

# INTERNATIONAL HANDBOOK OF FOODBORNE PATHOGENS

EDITED BY  
**MARIANNE D. MILIOTIS**  
*U.S. Food and Drug Administration  
College Park, Maryland, U.S.A.*

**JEFFREY W. BIER**  
*Food Safety Consultant  
Alexandria, Virginia, U.S.A.*



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# Foreword

In every part of the world, people wage a constant battle against food contamination, foodborne diseases, and food wastage. Efforts to reduce the devastating consequences of food contamination started long before written records. Cooking, smoking, and simple sun drying were probably the first methods ever used. Despite considerable advances in food science and technology, the safety of our food supply is even today a cause for considerable concern.

In 1983, an Expert Committee on Food Safety concluded that “illness due to contaminated food was perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity” (WHO, 1984). In 1992, the International Conference on Nutrition stated that hundreds of millions of people suffer from communicable diseases caused by contaminated food and drinking water. This conference declared that “access to nutritionally adequate and safe food is a right of each individual” (WHO, 1996).

Not only has epidemiological surveillance during the past two to three decades shown an increase in the prevalence of foodborne illness, there have also been devastating outbreaks of diseases such as salmonellosis, cholera, enterohemorrhagic *Escherichia coli* (EHEC) infections, and hepatitis A in both developed and developing countries. Furthermore, cholera and other diarrheal diseases, particularly infant diarrhea, which were traditionally considered to be spread by water or through person-to-person contact, were shown to be largely foodborne. In industrialized countries, sentinel studies showed an unexpectedly high annual prevalence of foodborne disease, i.e., 10 to 15% of the population. In the United States, this figure may be as high as 25 to 30% (Mead et al. 1999). One can safely assume this figure to be higher in developing countries, and the health consequences more severe.

Regarding chemical aspects, surveys made in industrialized countries suggest that the food supply is largely safe thanks to regulatory efforts and the general level of responsibility of the food industry. However, even in those countries accidental contamination or adulteration does occur, with potentially grave consequences. The situation in developing countries is virtually unknown due to lack of monitoring and surveillance programs. But reports of accidental or deliberate food contamination are brought time and again to the attention of health authorities.

It is certain that the problems of food safety will plague mankind in the 21st century, especially as several global changes continue to negatively influence the safety of food and drinking water. Such changes include population growth, urbanization, poverty, international trade in food and animal feed, and international tourism.

The World Health Organization has, for the past 20 years, urged governmental public health agencies, the entire food industry, and consumers to assume greater responsibility for food safety. As a consequence of this advocacy, but also because the health and economic consequences related to contaminated food became more noticeable, the World Health Assembly adopted in May 2000 a resolution on food safety (WHA 53.15, May 20, 2000). This resolution calls upon countries, but



also upon WHO itself, to integrate food safety as one of their essential public health functions. This is truly a milestone in the history of public health since, for the first time in WHO's 52 years of existence, food safety has been recognized not only as a public health responsibility but as an essential function of the public health community. It remains to be seen if and when WHO and its Member States adopt the necessary consequences as a result of this resolution, in the form of the provision of adequate resources and strengthening of national and international food safety programs.

In the past decade or so, more books on food safety have been published than ever before. This is a very desirable development, indicative of the greater interest in this branch of public health. The present book is a laudable addition to the already available arsenal of food safety books, providing up-to-date information on foodborne pathogens, on the incidence of foodborne diseases in various parts of the world, and on international food regulatory developments. The publisher of this book as well as its two editors, Drs. Miliotis and Bier, deserve praise for taking this initiative in this truly timely manner. I hope the book has many interested readers.

*Fritz Käferstein*  
*International Food Safety Consultant*  
*Nyon, Switzerland*  
*Former Director of WHO's Food Safety and Food Aid Programme*  
*Geneva, Switzerland*  
*Founding Fellow*  
*International Academy of Food Science and Technology*

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# Preface

Over the past 20 years, there has been a major change in the epidemiology of foodborne illness. Many factors have contributed to the change, including genetic factors, host susceptibility, new foodborne zoonoses, antimicrobial resistance, and a substantial increase in international travel and in globalization of food trade. In fact, international food trade increased 300% over the two decades and the dollar level reached over \$400 billion in 1998. This has led to foodborne illness episodes and epidemics that are no longer confined to local or regional geographic areas. The recently emerged protozoan *Cyclospora cayetanensis* outbreaks in North America, which are thought to be associated with consumption of contaminated imported produce, illustrate this transnational challenge of illness spread through international food trade.

The purpose of this book is to serve as a reference—a problem-solving compendium for food microbiologists, public health professionals, prudent processors (especially those engaged in international trade), food scientists, and biological science students with an interest in food safety. Our intent is to provide current information from an international perspective on the identification and characterization of the microbes, geographic incidence, and the challenge of control and prevention. We hope that practitioners, researchers, and students will find this a useful resource for topics related to foodborne disease of microbial origin.

This handbook is divided into three major parts. Part I characterizes the microbes. The same general topic format is presented for each organism or group of organisms. (For some organisms, the format varies because of lack of knowledge or research in certain topics.) Academic, government, and industry professionals who are actively pursuing research on the microbes were chosen as the authors of these chapters. Besides the conventional microbial foodborne pathogens described in this handbook—viruses, bacteria (including emerging bacterial pathogens, such as *Mycobacterium avium paratuberculosis* and *Enterobacter sakazakii*), fungi, protozoa, helminths, and marine organisms—a new infective form, the prion, has recently been described. Prion proteins have been implicated in transmissible spongiform encephalopathies (TSE). Recently, a variant form of Creutzfeldt-Jakob Disease (CJD), the spongiform encephalopathy of humans, has been associated with consumption of beef from cows with mad cow disease (the bovine form of TSE, BSE). A cluster of CJD cases was recently reported in the United Kingdom during a widespread epizootic of BSE. Since many aspects of this syndrome are poorly understood, numerous opposing theories have been presented to explain this disease. One of the many objectives of this manual is to provide a better understanding of this complex syndrome.

Part II is a geographic summary, which calls on various public health officials to assess the incidence, epidemiology, and risk assessment of foodborne illness in their respective regions of the world. Part III describes microbial risk assessment, the hazard analysis and critical point (HACCP) approach to providing a safe food supply, and the role of international bodies such as ISO, CODEX Alimentarius, and OIE in attaining this goal.

We are indebted to all the authors for their immense efforts in preparing their informative and innovative chapters. We also greatly appreciate the efforts of Dr. Fritz Käferstein for his guidance and assistance in this endeavor.

*Marianne D. Miliotis  
Jeffrey W. Bier*

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## Caliciviruses

**David H. Kingsley and Gary P. Richards**

*U.S. Department of Agriculture, and W.W. Baker Center, Delaware State University,  
Dover, Delaware, U.S.A.*

### I. BACKGROUND

Caliciviruses are a group of enteric viruses that infect a broad variety of terrestrial and marine animals (1–3). Although human caliciviruses are currently classified in three genogroups, the human viruses are of two basic morphological and biological types: the classic human caliciviruses and the Norwalk-like viruses (NLVs). The latter are also referred to in the literature as small round structured viruses (SRSVs). Although examples of classic calicivirus strains causing gastroenteritis in adults have been reported (4), classic caliciviruses are normally associated with self-limiting gastroenteritis in infants and children. Overall, the classic caliciviruses only account for a small portion of infantile gastroenteritis cases (5). Consequently, classic caliciviruses are generally considered of less medical and foodborne significance than the NLVs.

The more common NLVs are associated with gastrointestinal illness in persons of all ages. The prototypical Norwalk virus was identified by Kapikian and coworkers from an outbreak of gastroenteritis among students and staff at an elementary school in Norwalk, Ohio, in 1968 (6). Since then, numerous NLV strains have been implicated in gastroenteritis outbreaks worldwide. Most of these strains have been named for the locations in which the outbreaks occurred, such as the Hawaii virus, Snow Mountain virus, Mexico virus, Desert Shield virus, etc. (7,8). While there are well over 100 different human enteric viruses capable of causing gastroenteritis, molecular epidemiological studies indicate that most of the recent nonbacterial gastroenteritis cases are caused by NLVs. In the United States, a recent study by the Centers for Disease Control and Prevention (CDC) implicated NLVs in 86 of 90 (96%) of the outbreaks of nonbacterial gastroenteritis reported to 33 state health departments between January 1996 and June 1997, with the consumption of food being the most commonly identified mode of transmission (9). Perhaps of even greater significance, Norwalk-like (NL) illness is common among the general population worldwide. The CDC estimates that approximately 23 million cases of NL illness occur annually in the United States, with 40% of these (9.2 million cases) being associated with the consumption of contaminated food (10). Overall, the CDC estimates that among foodborne illness mediated by known agents, NLVs account for 76% of all cases, 33% of the hospitalizations, and 7% of the deaths (10).

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## II. CHARACTERISTICS

The classic caliciviruses and the NLVs are small (27–40 nm diameter), nonenveloped particles which are readily distinguishable from each other by their differing morphologies when examined by electron microscopy. They have a buoyant density of 1.33–1.41 g/cm<sup>3</sup> (7,11). Classic caliciviruses or Sapporo-like viruses were originally named for the 32 calyces or cup-shaped depressions on the virion surface giving a Star of David–like appearance (12). Norwalk-like viruses have a less clearly defined or more ragged morphology. Although morphological differences are readily apparent, classic calicivirus and NLVs apparently have some common antigenic epitopes as well as similar genomic structures (1,13,14). The virion of the NLV is composed of a highly variable outer coat (capsid), a small basic protein, and a 7.7-kb positive-polarity, single-stranded RNA genome (13). X-ray crystallography performed on recombinant capsid-like particles demonstrated that the capsid has a  $t = 3$  symmetry and is assembled from 180 monomeric units of a single 56.6 kDa protein (15–17). Several NLV genomes have been cloned and sequenced. All genomes encode three open reading frames (orf) (18–22). The first orf encodes a large polyprotein, which is proteolytically processed into a number of different proteins including a helicase, a cysteine proteinase, and an RNA-dependent RNA polymerase. The second and third orfs encode the capsid protein and small basic proteins, respectively. The function of the small basic protein has not formally been ascribed; however, its basic amino acid composition coupled with its presence in the virion suggests it may be an RNA-binding protein (3). A long poly A tail is located at the 3' end of the genome.

Human caliciviruses are presently divided into three genogroups based on antigenic and genome sequence differences, as well as slight differences in length and alignment of the open reading frames. Genogroup I includes the prototypical Norwalk virus, as well as Desert Shield, Cruise Ship, Montgomery County, and Southampton viruses. Genogroup II, of which Snow Mountain is the prototype, includes the Gwynedd, Toronto, Hawaii, Lordsdale, Mexico, Camberwell, Melksham, and White River viruses. Collectively genogroups I and II comprise the NLVs. The classic human caliciviruses are classified as genogroup III. These include the prototypical Sapporo virus, as well as the Houston, London, and Parkville virus strains (4,23). Despite genomic similarities, these three genogroups are quite divergent and a considerable degree of genetic variation is readily observed among individual strains within these three genogroups (4,23–29). For example, when 16 orf 1 PCR amplicons from genogroup I viruses were sequenced, the nucleotide identity was as low as 64% (30). The comparison of 20 genotype II strains revealed as little as 61% nucleotide sequence identity in orf 1, while analyses of orfs 2 and 3 for genogroups I and II also demonstrated a high degree of diversity (30). Although genogroup I viruses are common in the United States, genogroup II viruses may predominate as the leading cause of infections within the United States (26,29). Genogroup III caliciviruses cause gastroenteritis, principally in infants, and are less commonly associated with foodborne transmission than viruses in genogroups I and II (12).

## III. CLINICAL MANIFESTATIONS AND PATHOGENESIS

Clinical features of human calicivirus infections are variable, ranging from severe gastroenteritis to subclinical infections. Characteristic symptoms of NL illness include vomiting (69%), nonbloody diarrhea (66%), nausea (79%), abdominal cramps (30%), fever (37%), chills (32%), myalgia (26%), and sore throat (18%) (11). Norwalk virus has an incubation period of approximately 48 hours, with illness typically lasting 24–48 hours after onset (31). Experimentally-induced Norwalk illness causes blunting (shortening) and broadening of the villi, crypt hypertrophy, increased cellularity of the lamina propria, and increased mitosis in the crypts as early as 12 hours after virus ingestion and persisting for at least 5–6 days, usually 2–4 days after clearance of clinical symptoms (32,33). Changes in the absorptive cells lining the villi include decreased height, extensive vacuolization within the cytoplasm, and disordered nuclear polarity of the epithelial cells. Mononuclear cells increase in number within intracellular spaces of the epithelial cells. The lamina propria undergoes increased cellularity and infiltration by polymorphonuclear leukocytes.

Inflammation of the mucosa of the small bowel reduces the absorptive capacity of the villi contributing to diarrhea. Both crypt hypertrophy and epithelial cell proliferation may represent the body's response for replacing virus-damaged absorptive cells (33). The colonic mucosa and stomach remain relatively unaffected during Norwalk virus infection; however, the involvement of the distal portion of the small intestine is uncertain. Although the propagation of Norwalk virus reportedly occurs in the jejunum, the presence of virions within these cells was not observed (33,34).

#### IV. REPLICATION AND HOST RANGE

Humans are the only known reservoir of the NLV pathogens. Chimpanzees can be experimentally infected with NLVs. They can shed virus in their stools and seroconvert, but are refractory to onset of clinical symptoms and disease (25,31). Although some animal caliciviruses have genetic and morphological similarities to human NLVs, foodborne zoonotic infection of humans by animal caliciviruses has not been demonstrated to date (35–39). However, zoonotic transmission of animal caliciviruses is not unprecedented, since nonfoodborne transmission of the San Miguel sea lion virus to a laboratory worker has been reported (40). Efforts to propagate human caliciviruses *in vitro* have been unsuccessful, and there is no practical laboratory animal which can be used to culture the virus *in vivo*. Consequently, the only source of NLVs are the stools of infected individuals. Virus shedding occurs as early as 15 hours postinfection and peaks between 25 and 72 hours (41). However, virus shedding for as long as 2 weeks postinfection has been demonstrated (42).

Unlike enteric bacterial pathogens in foods, NLVs do not replicate within foods. Therefore, NL illness does not result from temperature abuse of food products. Exactly how much virus constitutes an infectious dose of NLV is not known; however, the amount of virus required to elicit illness is likely to be only 10–100 virus particles (35).

#### V. PROBLEMS

Today, NL illness is the number one cause of foodborne illness in the United States (10) and perhaps throughout much of the world. As an enteric virus, NLVs are transmitted by the fecal-to-oral route. The principal means of transmission are through contaminated food and water and by person-to-person spread. Norwalk-like viruses can be introduced into foods by improper hygiene of food handlers, through irrigation or fertilization of crops with contaminated water or sewage sludge, and from the use of contaminated water during food processing, sanitation, or ice preparation (43). Field workers can contaminate fruits and vegetables during harvest as a result of inadequate bathroom facilities or poor hygiene while working in the fields. Among the foods most commonly cited as contributing to viral illness from handling by infected workers are salads, raw fruits and vegetables (44–48), and bakery products (49,50)—foods that are generally served raw or that may be adulterated after cooking.

Consumption of raw or undercooked shellfish harvested from contaminated estuaries is a common cause of NLV outbreaks (51,52). Bivalve mollusks such as oysters, clams, and mussels are especially prone to transmit NLVs when raised in water that is subject to fecal contamination. Shellfish effectively bioconcentrate these viruses from the water through their normal filter feeding activities (53).

As a group, the nonenveloped enteric viruses are relatively resistant to physical and chemical inactivation and consequently can persist for extended periods in aquatic environments. It has been suggested that enteric viruses adsorbed to colloidal clays and debris have enhanced stability in water (54–56). Using feline calicivirus (FCV) as a surrogate for Norwalk virus in long-term stability studies, a  $10^{9.5}$ -50% tissue culture infectious dose (TCID<sub>50</sub>) of FCV was reduced by one order of magnitude over 60, 20, and 10 days at 4, 25, and 37°C, respectively (57). However, when dried, the virus was unstable at 37°C and did not survive more than 24 hours (57). At 60°C, the virus remained viable for as long as 30 minutes.

Norwalk virus may be especially thermally stable in the context of contaminated oysters. Inadequately cooked shellfish can readily transmit NLVs, since the shells and the high protein content of oysters afford some degree of thermal protection. Even if “properly cooked,” shellfish may not be absolutely free of infectious Norwalk virus, since some recent outbreaks have been associated with cooked oysters (58–60). Product contamination after cooking may also attribute to some cases of NL illness.

Early evidence suggested that Norwalk virus may be resistant to chlorination (61); however, further studies are needed. It is unclear whether properly functioning sewage treatment plants can discharge viable Norwalk virus. Less than optimal chlorination of drinking water has clearly been implicated in Norwalk virus outbreaks (62). The use of contaminated water in food processing or sanitation has led to food contamination (63–65).

## VI. EPIDEMIOLOGY

Norwalk-like illness was originally described in 1929 as “epidemic winter vomiting disease” syndrome (66). Although it occurs with some increased frequency in the winter months, NL infections readily occur year-round (67). Identification of Norwalk virus as the etiological agent for acute gastroenteritis was demonstrated after administration of bacteria-free stool filtrates from a previous outbreak to healthy volunteers. Among these volunteers, rising antibody titers against Norwalk virus correlated with illness, while persons who remained healthy did not show a substantial rise in antibody titers (68).

While the reporting of NL gastroenteritis has increased in recent years, NL infections have been grossly underreported. There are several reasons for the underreporting. Norwalk-like illness may be very mild and not of sufficient consequence to warrant a visit to the doctor. Only in severe cases, especially when involving young children, the elderly, or persons with various other medical problems, is medical care generally sought. Reporting of NL illness is not required in most countries. In the United States, there are no national reporting requirements for NL illness by health care providers. In most instances, laboratory diagnostic tests are not routinely performed to confirm the etiological agent involved in the illness.

Consistent with a large number of estimated annual NL illnesses, serological evidence suggests that many adults secrete NLV-specific antibodies (69). This confirms that infection with genogroup I or II viruses is quite common and that antibodies readily circulate among different worldwide communities (69,70). In fact, multiple genotypes are occasionally identified during investigations of outbreak sources (62,71,72). This background level of community infection is probably unnoticed until an infected individual contaminates a common food or water source or facilitates rapid person-to-person spread via close contact with individuals in a closed or institutional-type setting. In these settings, direct person-to-person transmission can occur via contact with soiled linens, vomitus, feces, aerosols, or fomites (48,73). Outbreaks of NL illness have occurred from exposure at banquets, geriatric facilities, psychiatric wards, emergency rooms, cafeterias, recreational lakes, swimming pools, dormitories, campgrounds, hotels, schools, restaurants, and cruise and military ships (9,73–75).

Two recent outbreaks that have been extensively studied reveal how readily Norwalk virus is transmitted and how long it may persist within aquatic settings. In a Finnish town, more than 1600 cases of NL gastroenteritis resulted from the ingestion of improperly chlorinated tap water from a lake (62). Based on reverse transcription–polymerase chain reaction (RT-PCR) and DNA sequence analyses, it was determined that this virus was identical to a Norwalk-like strain causing a foodborne outbreak four months previously in a town 70 km upstream. The clear implication is that this virus was discharged upstream and remained viable for 4 months in ice-covered lakes and streams while eventually drifting downstream to the town’s water intake.

Another well-documented outbreak of NL illness involved Louisiana oysters from Mississippi Sound. This outbreak, which affected 70 people within Louisiana and 120 from five other states, illustrates the manner in which Norwalk viruses can be spread via shellfish consumption (76,77).

In November 1993, the Louisiana public health office received reports of persons becoming ill after eating raw oysters. The outbreak was subsequently tracked to a particular harvesting area. Daily harvest from this area was 1 million oysters per day, with approximately 60% of this harvest subsequently shipped beyond Louisiana borders. Fecal coliform testing was minimal in this area because the shellfish bed was believed to be quite distant from potential sources of sewage contamination; the reef was approximately 10 miles from the nearest sewage outlet and was separated from shore by open water. Results of subsequent coliform testing of water from a monitoring station closest to the shellfish bed one week prior to shellfish harvest and from the shellfish bed 2 days after harvest were within acceptable limits. The investigation of potential viral contamination sources revealed that boats routinely discharged sewage into harvest waters, despite regulations against the practice. Six harvesters reported diarrhea or vomiting during November 1993; two between November 7 and 10, when contamination was believed to have occurred. Both men reported discarding vomitus and feces overboard.

Although shellfish are well known for their ability to transmit NL illness, other foods have contributed substantially to NL infection. Raw fruits and salads are commonly seen as vehicles of virus transmission. Contamination can occur as a result of surface contamination of fruits and vegetables from irrigation or fertilization practices, from the harvester or transporter, or from contamination during food preparation. Sick food handlers have contributed greatly to the incidence of NL illness, either by contaminating foods with unsanitized hands or exposing products to unclean surfaces. One outbreak of NL illness was attributed to the contamination of potato salad by mixing it in a sink that a restaurant worker had vomited in a day earlier (48). Epidemiological investigations demonstrate that many outbreaks of NL illness result from a general lack of knowledge among some food handlers due to inadequate training in sanitation and hygiene.

## VII. ISOLATION AND IDENTIFICATION

The identification of Norwalk virus was first accomplished by immunoelectron microscopy in 1972 (6). This method is not considered a practical method of identification. Currently there are several methods of diagnosing NL infection. Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay can be employed to detect IgG, IgM, and viral antigen (70,78,79). The presence of IgM specific for NLVs is considered evidence for recent Norwalk infection. At least a fourfold increase in ELISA antibody titer from paired sera, before and after illness, is considered evidence of recent exposure. Normal virus isolation methods, such as propagation in tissue culture, are ineffective since NLVs have not been successfully replicated *in vitro*. Consequently, virus must be isolated from the feces of infected individuals. Currently, the most common method of detection is RT-PCR from stool samples (26,80–84). A plethora of papers describing molecular biological methods for identification of Norwalk virus have appeared in the literature in recent years. Methods include tests for NLVs in feces (85–87), water (88,89), shellfish (24,90–93), and other foods (94). Unfortunately, direct testing of shellfish is not yet a practical means of preventing foodborne NL illness. While RT-PCR can be a very sensitive detection method, it is plagued by inhibitors in food and environmental extracts (95–97). Current shellfish testing methods rely on the use of long and cumbersome extraction methods, often utilizing many steps including PEG precipitation, freon extraction, flocculents, and multiple changes in pH (98). While potentially very sensitive, RT-PCR based techniques have a major limitation; RT-PCR detects the presence of viral nucleic acid and cannot be used to discriminate between viable infectious virus and inactivated noninfectious virus (96). Consequently, beyond administration to healthy volunteers, there is presently no way to determine the infectivity of NLVs.

## VIII. CONTROL MEASURES

The reduction of foodborne NL infections relies on a broad range of strategies encompassing all aspects of (a) food production, harvesting, transport, processing, and distribution, (b) water treatment

and distribution, and (c) person-to-person spread. Foods must be protected from contamination both preharvest and postharvest. The use of wastewater for crop irrigation and sewage sludge for fertilization should be banned or carefully controlled in all countries. Field workers harvesting fruits and vegetables should be provided with adequate bathroom and wash facilities to reduce the potential of fecal contamination. All water from which shellfish are harvested should be carefully monitored for quality. Although the current method of monitoring water quality using coliform standards may be less than ideal, outbreaks of Norwalk and other enteric virus illnesses are relatively infrequent when shellfish are obtained from approved waters. Consequently, vigilant monitoring and protection of these waters by various environmental agencies is currently the best means of reducing viral contamination of shellfish and subsequent NLV outbreaks. Laws regarding the discharge of sewage from water craft should be strictly enforced, and boat owners should be educated about the health hazards of releasing raw sewage into oceans, lakes, rivers, and estuaries. Wastewater should be subjected to at least primary and secondary treatment followed by chlorination or ultraviolet light disinfection to reduce virus levels prior to environmental discharge. Harvested shellfish should be from approved waters and wholesale and retail tagging requirements for shellfish should be enforced. Laws against "bootleg" harvesting of shellfish (i.e., illegal harvesting from non-approved waters) should be strictly enforced, and penalties should be substantial to provide a strong deterrent. Depuration, a commercial processing technology by which shellfish are allowed to purge contaminants in tanks of clean seawater for 48–72 hrs, does not eliminate viruses in shellfish (99–102); therefore, the use of depuration to remove viral contamination from shellfish is of limited value. The practice of relaying, or transferring oysters from non-approved waters to approved shellfish harvesting waters, can be effective in reducing many viruses because relaying is performed for periods sufficient to allow the inactivation of viruses by physical, chemical and biochemical processes (100,101). Relay periods of up to one month are commonly employed.

One of the primary sources of foodborne contamination after harvest is by the hands of sick food preparers; therefore, careful adherence to sound hygienic practices during food preparation is critical. Personnel should thoroughly wash hands before handling foods. Clean gloves may be worn to prevent the possibility of fecal contamination of prepared food items. Ill kitchen staff should be sent home. Since NLVs can be shed for several days after recovery (41,42), convalescing kitchen staff should be given alternate duties immediately following possible NL infection. Employers should provide sick leave for ill kitchen staff to remove the economic incentive to conceal illness. Fruits and vegetables should be thoroughly washed with potable water prior to serving.

Some processing technologies are effective in eliminating enteric viruses in foods. Unfortunately, specific studies have not been conducted on the inactivation of NLVs because the only method to test for virus inactivation is through costly volunteer studies. Among food-processing methods likely to be effective in eliminating NLV are cooking and ultraviolet, ionizing, and microwave irradiation. Cooking can totally inactivate enteric viruses; however, cooking times and temperatures required for inactivation will depend on the composition of the food (43,103,104). High protein and fat contents of food products may afford enteric viruses some protection from thermal inactivation. Also, the method of cooking (i.e., frying, boiling, steaming or baking) can affect the efficiency of virus inactivation (105).

Enteric virus reductions may be accomplished using ionizing radiation, ultraviolet light, and microwave energy. Ionizing radiation fragments viral nucleic acids and is becoming more generally accepted for food processing (106,107). Depending on the product, irradiation levels as high as 10 kGy have been proposed (43,108). Ionizing radiation penetrates foods to kill the microorganisms within; however, ultraviolet irradiation only inactivates viruses in direct contact with the rays. Germicidal lamps are effective in eliminating viruses on the surface of foods. Rotavirus and poliovirus demonstrated similar rates of UV inactivation but required three to four times the dose of UV necessary to inactivate *E. coli* (109). No specific data are available on the effectiveness of UV irradiation on NL caliciviruses.

Microwave energy inactivates viruses primarily as a function of the heat produced. Hepatitis A and poliovirus were inactivated in foods by microwaving (110,111). Microwave processing heats

foods unevenly, often leaving cold spots where viruses may survive. Foods should be thoroughly stirred or frequently turned during microwave processing to reduce the effects of unbalanced heating.

Another potential source of NLVs is contaminated water used for drinking or for the manufacture of ice, for plant sanitation, and for food preparation. Municipal water supplies should use levels of chlorine and retention times sufficient to ensure inactivation of NLVs. Only potable water should be used for ice and food preparation.

The NLVs are readily transmitted in institutional settings. Procedures such as minimizing contact between sick and healthy persons and thorough decontamination of surfaces in contact with vomitus and feces may prevent further spread. Caregivers should wear gloves and gowns if contamination with fecal material is possible. Gloves should be replaced or disinfected frequently. Soiled laundry must be handled carefully. Care should be taken not to have chambermaid, laundry, patient care, or janitorial staff assigned kitchen duties within these institutions.

## **IX. VACCINE PROSPECTS**

Whether or not an efficacious vaccine against NLVs will be developed is unclear. Unfortunately, immunity following NL infection is not longlasting. Immunity against challenge has been demonstrated 2 months postinfection, but immunity to homologous virus declines after 2 years (112,113). This transient immunity induced by NLV infection is strain specific, since protection induced from one NLV strain does not afford substantial protection from different NLV strains (68,112). Curiously, persons with the highest preexisting levels of Norwalk antibodies are at the highest risk of developing symptomatic infection, suggesting that antibodies are not protective against NLV infection (7,41,113).

Currently, recombinant NLV capsids are being evaluated as prospective vaccines (114). Expression of the Norwalk capsid genes in plant and insect cells (115–120) results in self-assembly of capsid proteins into virus-like particles. It was subsequently shown that Norwalk capsid proteins expressed in tobacco leaves and potatoes are immunogenic when fed to mice (120). Recombinant NLV elicits IgG and IgA responses when administered orally without requiring adjuvant or a specific delivery system (121). Recently, published results of phase 1 clinical trials in human volunteers indicate that ingestion of recombinant NLV capsid proteins does not result in adverse medical effects (122,123). Whether these recombinant NLV capsids will be efficacious for the prevention of Norwalk-mediated illness or useful as an adjuvant for vaccination against other intestinal pathogens remains to be determined.

## **X. FUTURE DIRECTIONS**

Human caliciviruses will continue to intrigue the research and public health sectors as new viruses continue to be added to the already long list of members. The extraction and assay of these viruses will remain a challenge. To date, the human caliciviruses have defied cell culture propagation, and novel methods must be evaluated to develop a system for detecting infectious virus particles. Processing methods must be evaluated to delineate the most effective means of eliminating these viruses from foods. Potential reservoirs of NLVs may be found and could lead to new areas of research and epidemiological investigations. In the nearly 30 years since Norwalk virus was first discovered (6), progress has been made in isolating, cloning, and sequencing the genome (124). The next decade will likely provide exciting new revelations to help combat this pervasive enteric pathogen.

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# 2

## Hepatitis

**Barry D. Schoub**

*University of the Witwatersrand, Johannesburg, South Africa*

### I. GENERAL BACKGROUND

Over the past 3 decades an ever-increasing number of hepatitis viruses have been described, designated by letters of the alphabet: A, B, C etc. The most important of these hepatitis viruses, A, B, and C, are major causes of morbidity and mortality worldwide. Hepatitis viruses D and E are also significant contributors to illness burden in certain parts of the world. Hepatitis F virus, obtained from the stools of a hepatitis patient, has been transmitted to primates but is probably of doubtful significance as a human pathogen (1). Hepatitis G virus, also known as hepatitis GB virus C (GB being the initials of the surgeon from whom the virus was initially isolated), is widely distributed and, in developing countries, is found in up to 18% of random outpatient blood samples (2); however its disease potential is still uncertain. Similarly, the recently described TT virus (also named after a patient, TT, from whom it was recovered) is found in 1.9% of blood donors, but its disease association is unclear (3).

The only hepatitis viruses spread by the fecal-oral route are hepatitis A and E viruses; the others are exclusively spread parenterally. Hepatitis A virus (HAV) is mainly spread enterically, i.e., by the fecal-oral route, through direct person-to-person contact or via contaminated food and water. Parenteral spread of HAV, for example, sexual transmission, particularly among homosexuals, does occur, and spread via blood products, particularly factor VIII, has been reported on a number of occasions. Hepatitis E virus is known to be spread by contaminated water and possibly also by contaminated food; parenteral transmission of hepatitis E virus is not known to occur, with the possible exception of vertical transmission (4).

### A. Hepatitis A Virus

The clinical disease entity due to hepatitis A virus (HAV) infection has been known since antiquity. In 1912 its infectiousness was recognized, and in 1947 infectious jaundice was divided into two main groups: hepatitis A, spread by the fecal-oral route with a shorter incubation period, and hepatitis B, spread parenterally with a long incubation period. The distinction between these two forms of hepatitis was further refined in the famous studies in the 1950s and 1960s at the Willowbrook State School for the mentally retarded in New York (5). The development of the marmoset monkey as an animal model for hepatitis A allowed for the propagation of the virus (6), and in 1978 the protective immunogenicity of a formalin-treated preparation of hepatitis A derived from the liver of an infected animal was demonstrated, by intravenous challenge, to protect vaccinated marmosets (7). In 1979, hepatitis A virus was propagated in cell culture including human fibroblast cell cultures, and this ultimately led to the development of a vaccine against the virus suitable for human use (8,9).

HAV infection is widespread throughout the world. Clinical hepatitis, usually with jaundice, occurs as an acute and usually self-limiting illness with a very low mortality, although the disease

is often debilitating and may be prolonged. It is a significant cause of illness burden in countries with intermediate endemicity of infection. It is estimated that approximately 1.5 million clinical cases of hepatitis A occur annually worldwide (10). The illness burden due to the virus is, however, markedly different in various regions of the world and is directly dependent on socioeconomic development and levels of sanitation. The disease is lowest in countries with both low and high endemicity; in the former case, infection and disease is uncommon because circulation of the virus in the population is low due to good hygiene and adequate sanitation, while in countries with high endemicity, virus circulation is high and thus infection is common in young children, where it is usually asymptomatic and unrecognized and results in life-long protection. It is in the regions of intermediate levels of endemicity, or countries with both developed and developing populations living side by side, where the highest levels of disease are seen.

## **B. Hepatitis E Virus**

The development of serological tests for hepatitis A and hepatitis B viruses demonstrated that a significant proportion of cases of hepatitis were due to neither of these viruses (36). This led to the coining of the term non-A, non-B hepatitis, of which two forms were epidemiologically apparent: a parenteral form with routes of transmission, which appeared similar to parenterally spread hepatitis B virus infection, and an enterically spread non-A, non-B hepatitis with routes of transmission similar to enterically spread hepatitis A virus.

Enterically spread non-A, non-B hepatitis was apparently responsible for vast epidemics of waterborne hepatitis on the Indian subcontinent, Southeast Asia, Asian republics of the former USSR, Africa, and Central America. The development of serological tests confirmed that the etiological agent was a novel virus, unrelated to hepatitis A, and that infection was considerably more widespread than suggested by the geographic location of the large waterborne outbreaks. In addition to outbreaks, the virus, now termed hepatitis E virus (HEV), was also responsible for sporadic cases of hepatitis both in developing and in developed countries, among indigenous populations as well as imported cases from travelers.

## **II. CHARACTERISTICS**

### **A. Hepatitis A Virus**

HAV is a member of the large Picornaviridae family of viruses. The family consists of small (25–28 nm), round virus particles morphologically indistinguishable from each other. The viruses are nonenveloped (naked) and are therefore generally robust. HAV has a number of characteristic biochemical and biophysical features, which distinguish it from other members of the Picornaviridae family, such as poliovirus and coxsackievirus, and it is therefore classified in a genus of its own called *Hepatovirus* (11). One of the characteristic features of HAV is that it is a particularly stable virus, able to survive for months in the environment. It is also resistant to lipid solvents such as ether and is stable at a low pH of 3.0. It is also relatively heat resistant, surviving exposure to 60°C for 10 hours, but it is rapidly inactivated within 5 minutes by boiling. It is also inactivated by ultraviolet irradiation, formaldehyde at a 1:4000 concentration for 72 hours at 37°C, and chlorine at a concentration of 1 mg/L for 30 minutes (12).

The small (7.5 kb) genome consists of a single strand of RNA, which codes for four polypeptides, the outer two of which are important for immunity to infection. The virus is exquisitely sensitive to neutralization by specific antibodies directed to these polypeptides. HAV cannot be readily isolated in tissue culture, although a small number of strains have been adapted for propagation in tissue culture, and these are used for the manufacture of vaccine.

### **B. Hepatitis E Virus**

Biological properties of HEV suggest that the virus is distinct from other members of the Picornaviridae family. For example, the virus is somewhat larger, 32–34 nm in diameter, and is less stable

than HAV. It shares many characteristics of the Caliciviridae family. At present the virus is not cultivable.

### **III. DISEASE**

#### **A. Hepatitis A Virus**

Clinical disease due to HAV resembles other types of acute hepatitis, and diagnosis is generally dependent on laboratory confirmation of infection by the virus. The incubation period is approximately 28 days but can vary from 15 to 50 days. Initial symptoms include pyrexia, malaise, anorexia, which is often quite prominent, nausea, vomiting, and abdominal discomfort or pain. These initial symptoms are followed a few days later by the development of jaundice with a typical yellow discoloration of the skin, especially the sclera of eye, and darkening of the urine. The acute phase usually lasts between 1 and 3 weeks and is followed by a varying period of convalescence characterized by fatigue, malaise, anorexia, and persisting nausea.

Complications are rare. Approximately 5% of patients develop cholestatic hepatitis with pruritis and steatorrhea. Fulminant hepatitis, characterized by precipitous loss of liver function rapidly leading to liver failure and death, occurs in approximately 0.01% of cases. Chronic disease and chronic excretion of virus does not occur. However, up to 10% of patients will relapse, that is, will suffer a recurrence within 1–4 months after acute disease, and these recurrences may persist for several months.

The severity of hepatitis A disease is directly dependent on a number of factors: age, underlying liver disease, immunosuppression, and pregnancy (13). Thus, jaundice occurs in less than 10% of children under 6 years of age, 40–50% of older children, and 70–80% of adults. The overall mortality in the United States is less than 0.1%, but mortality is somewhat higher in children less than 5 years of age (0.15%) and even higher in older persons over 50 years of age (2.7%). Similarly, increased mortality is seen in immunosuppression and in patients with underlying liver disease such as cirrhosis. In most older children and adults the disease is, at best, an uncomfortable to debilitating illness, which runs a course of 1–2 months. However, some 11–22% of cases are hospitalized (14), and studies of work absenteeism have shown that sick leave for hepatitis A can vary from 27 to 60 days (60 days was found at the Willowbrook State School to be the average duration of sick leave among staff working at the institution) at an estimated annual direct and indirect cost in the United States of over \$300 million (15).

#### **B. Hepatitis E Virus**

Hepatitis E shares many of the characteristics of hepatitis A. The incubation period is somewhat longer—an average of about 6 weeks. As with hepatitis A, the disease is generally mild and self-limiting, with a very low mortality of 0.5–3% even in deprived populations. A striking exception to this is HEV disease in pregnant women, particularly in the third trimester, where mortality rates of up to 20% have been reported in outbreaks. The reason for this uniquely high mortality is still unknown.

### **IV. EPIDEMIOLOGY**

#### **A. Hepatitis A Virus**

The only reservoir of HAV is infected humans, and the major source of infectious virus is human feces. Virus is detectable in feces as well as blood 10–12 days after infection. Generally the most infective period is 14–21 days before the onset of clinical symptoms until 1 week after the onset of symptoms (16). Infectivity, thus, rapidly wanes with the onset of jaundice. During acute infection, up to  $10^8$  infectious virus particles per mL may be found in fecal specimens. Fecal excretion of

virus occurs during most of the incubation period, and individuals are contagious for a duration of at least 3 weeks or more, mostly when they are asymptomatic. Virus shedding in the feces drops rapidly after the onset of jaundice and continues for less than a week after jaundice begins, although, with relapses, it has been detected for up to a further 2 months.

Considerably less virus, up to  $10^5$  particles per mL, is found in the blood, and the viremic stage coincides largely with the period that the virus is excreted into the feces. This may now explain the increasing finding of bloodborne hepatitis A infections (17). Virus is also excreted into the saliva, although it has not been established whether saliva plays any role in hepatitis A virus transmission (18). There is, for example, no evidence that sharing eating utensils, cigarettes, or kissing can transmit hepatitis A infection.

By far the most common route of transmission of hepatitis A virus is the fecal-oral route, with person-to-person being the most important mode of transfer of the virus. This has been clearly demonstrated by observations of the rates of infection among household contacts of patients and among children in the setting of daycare centers (19). The virus is robust and is able to survive in water and food for 12 weeks to 10 months. As a result, foodborne outbreaks (common-source outbreaks) are also common (20). Foodborne outbreaks may be due to contamination of food by food handlers—typically a food handler in the asymptomatic preicteric phase of the illness. This would occur especially in situations where there are poor hand-washing practices and uncooked foods such as salads, sandwiches, cold meats, etc. are touched by hand. In addition, foods themselves may be contaminated, especially shellfish harvested from waters close to sewage outlets or vegetables fertilized with untreated human nightsoil (21). Large water-borne epidemics due to drinking or swimming in fecally contaminated water also occur from time to time (22).

Although still rare, parenteral spread of HAV is being increasingly recognized, for example, following receipt of contaminated blood (i.e., blood from donors in the asymptomatic viremic stage of the incubation period) and blood products, especially factor VIII concentrates (17). Hepatitis A virus transmission via contaminated syringes and needles is being increasingly seen among intravenous drug abusers (23). The role of sexual transmission of hepatitis A virus has not been clearly defined, although the prevalence of infection has been shown to be higher among attendees of sexually transmitted disease (STD) clinics than among blood donors, and especially among male homosexuals (where oral-anal transmission may be important) (24).

The epidemiology of hepatitis A differs markedly in various parts of the world and in different populations. Classically, three epidemiological patterns of HAV endemicity have been described depending on socioeconomic circumstances such as levels of hygiene and sanitation and crowding (10). Countries with high endemicity in the developing world would experience relatively low levels of disease, although the virus circulation is high; this is due to widespread inapparent infection in childhood resulting in almost all older children and young adults having protective antibodies by the time they reach the age when infection usually causes disease. The second pattern, intermediate endemicity, associated with the majority of disease, is seen because circulation of virus is still at a fairly high level but there is still a significant immunity gap at the vulnerable age group of older children and young adults. With the third epidemiological pattern, countries with low endemicity of infection, disease is infrequent because circulation of virus is low and cases are usually linked to importation or are found in travelers to developing countries.

There is little doubt that the global epidemiology of hepatitis A infection has undergone marked changes as a direct result of improvements in the provision of clean water and sanitation and in personal and public hygiene. In some highly industrialized countries such as Scandinavia, Germany, Switzerland, and Japan, endemic transmission has all but ceased, and the major proportion of infection in these countries is due to importation or occurs in travelers returning from developing countries (25). The prevalence of antibodies to HAV in blood donors from these countries is less than 10%. In most other developed countries, endemic transmission of virus is also declining, and this has resulted in an increasing susceptibility in adults with its attendant greater morbidity and mortality (26). Thus, the declining transmission of HAV has created a large pool of susceptibles among older children and adults who are vulnerable to outbreaks of infection. These outbreaks may either be



community outbreaks (often centered around, e.g., daycare centers, groups of individuals living in closed living conditions, especially the military, prisons, boarding schools, or residents of institutions such as those for the mentally handicapped), or common source outbreaks (food- or waterborne outbreaks). There is also an increasing number of adults who, while they are susceptible to infection, are nevertheless coming into increasingly greater contact with HAV, either because of travel (business or recreational) or occupational exposure, e.g., staff at daycare centers for children or institutional personnel, health care workers, personnel working in sewage plants, etc. A further consequence of the decline in HAV transmission and increasing infection in adulthood is the relative rise in the importance of bloodborne transmission as more and more individuals in the asymptomatic viremic period may be donating blood.

In the United States communities have been divided into two categories communities with high rates and those with intermediate rates of hepatitis A (13):

1. Communities with high rates of hepatitis A: These communities are characterized by high rates of infection, high rates of disease, but few cases in individuals over 15 years of age and epidemics every 5–10 years. Seroprevalence studies have shown that 30–40% of children are seropositive before 5 years of age, and virtually all individuals are positive by the time they reach young adulthood. In the United States this pattern is found among Native American populations and some Hispanic and religious communities.
2. Communities with intermediate rates of hepatitis A: These communities are characterized by the majority of disease occurring in children, adolescents, and young adults; disease rates are some 5–10 times lower with less clearly defined risk activities. The seroprevalence of antibodies to hepatitis A virus varies from 10–25% in children less than 5 years of age to about 50% in individuals over 15 years of age.

## **B. Hepatitis E Virus**

As with HAV, transmission of HEV is by the fecal-oral route. However, it is only the waterborne route of transmission, which has been shown to be of importance in the large outbreaks. Presumably food also plays a role in transmission of infection, as does direct person-to-person contact, although these latter two routes have not been as well established as in the case of HAV. Another exceptional characteristic of HAV is the virtual absence of secondary cases following on importation of infection—this may well be due to the relative lability of the virus. Lastly, a particularly striking feature of the epidemiology of HEV has been the extent of the waterborne epidemics. For example, 29,000 cases in New Delhi in 1955 (37), 10,800 cases in the Kirgiz Republic of USSR between 1955 and 1956, over 20,000 cases in Myanmar between 1976 and 1977 (36).

Sporadic cases outside of the regions where outbreaks have been reported are uncommon. However, seroprevalence studies in different populations have shown that up to 5% of random population samples may be positive for HEV, while 6–19% of some developing population samples were seropositive (38).

The discovery of viruses resembling HEV in herds of pigs in the United States has led to speculation that the pig may act as a reservoir for the virus. However, whether animal-to-human transmission takes place still remains to be established (39).

## **V. ISOLATION AND IDENTIFICATION**

### **A. Hepatitis A Virus**

HAV isolation is not carried out for routine clinical or epidemiological diagnosis, and culture of the virus is limited to few research centers. The virus is also not readily visualized by electron microscopy. Viral genome is detectable by reverse transcriptase–polymerase chain reaction (RT-PCR) 10–12 days after infection. With sensitive techniques such as nested RT-PCR, HAV RNA

may be detected 17 days before the alanine aminotransferase peak, and the viremia can then be detected for an average of 79 days after the peak of liver enzymes (27). Detection of virus in stools by RT-PCR may also persist for longer.

Generally speaking, however, detection of HAV infection for clinical diagnostic purposes is dependent on serology—radioimmunoassay or more commonly enzyme immunoassay. Antibodies develop early in the course of infection and are almost always present at the onset of clinical symptoms. HAV IgM antibodies remain detectable for 45–60 days after the onset of symptoms. HAV IgG antibodies increase with convalescence and persist for many years, if not for a lifetime. Liver biopsy is seldom required for diagnosis.

## B. Hepatitis E Virus

The virus cannot be isolated in cell culture. Detection of virus is usually carried out by RT-PCR as virus particles are difficult to visualize under electron microscopy and the level of antigen excreted in the stools is usually too low to allow for reliable detection by serological tests. Diagnosis of infection is usually carried out by demonstration of specific IgM and IgG antibodies by enzyme immunoassay.

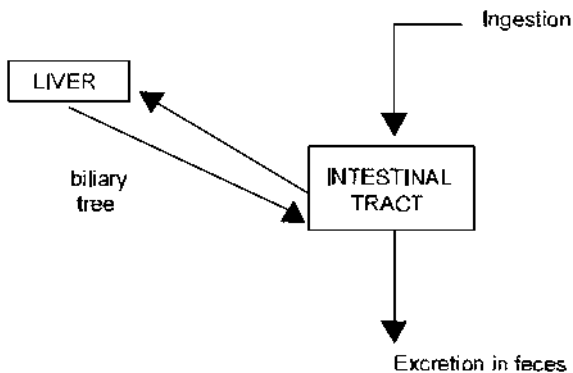
## VI. PATHOGENESIS

### A. Hepatitis A Virus

The virus is ingested with contaminated food or water or from oral contact with contaminated hands. It infects the intestinal tract and is then transported to the liver (Fig. 1). The exact area of infection in the gastrointestinal tract is not known. Infectious particles appear in the blood for only about a week at most, although, as mentioned above, investigations by nested RT-PCR have shown that the duration of viremia may be considerably longer than initially thought. The liver is essentially the only target organ of the virus. This means that the hepatocyte is the only cell in which the virus replicates. HAV is then transported back to the gastrointestinal tract via the biliary tree.

The typical HAV pathology of the liver includes necro-inflammatory lesions in the hepatic parenchyma. The pathogenesis of hepatitis A infection does not involve direct viral cytopathology but is rather due to the host immune response. It is also not due to antibody responses to the virus.

The pathogenesis is essentially a cell-mediated immune injury—specifically the CD8 T-lymphocyte response and various inflammatory cytokines. Apoptosis or programmed cell death also plays a major role.



**FIGURE 1** Pathogenesis of Hepatitis A virus.

## B. Hepatitis E Virus

Relatively little is known of the pathogenicity of HEV. It is still not clear whether the pathological changes seen in the liver—hepatic necrosis with lymphocytic and polymorphonuclear leukocyte infiltration—are a reflection of direct cytopathic changes or the result of immunopathological damage.

## VII. CONTROL MEASURES

### A. Hepatitis A Virus

#### 1. *General Measures*

The measures generally recommended for the prevention of fecal-oral transmission of enteric pathogens are equally applicable to hepatitis A virus infection (20). These are particularly pertinent to travelers journeying to developing countries where standards of hygiene and sanitation may be poor. Water used for drinking, washing of vegetables or fruit, or brushing of teeth is a major hazard to travelers. If in doubt, water should be boiled for at least 1 minute or, at high altitudes, for a few minutes. Alternatively, proprietary chlorine tablets can be used to disinfect water (provided that the water is clear and free of contaminating organic material). Ideally, bottled water should be used for drinking. (Portable filters have not been shown to be reliable and are currently not recommended.) Ice is a common source of contamination and should not be used for drinks unless the safety of the source of the water is assured. Similar precautions would hold for iced lollipops and frozen flavored ices. Unpasteurized milk and products made from raw milk must be avoided; unpasteurized milk should be boiled before being consumed. Food must be thoroughly cooked and should be eaten while still hot and steaming; this is especially important with minced meat dishes. Street vendors should be avoided if possible, as they are a frequent source of foodborne illness among travelers. Raw fruits and vegetables that cannot be peeled should be avoided. Some vegetables, such as lettuce, are particularly difficult to clean thoroughly and are often contaminated. Hands should be washed thoroughly and frequently using soap. The old adage for travelers still remains pertinent today: “Boil it, cook it, peel it, or forget it!” Staff caring for young infants in diapers in daycare centers or institutions for the mentally handicapped should be instructed regarding appropriate hygienic precautions, particularly adequate hand washing, if there is a possibility of contact with human feces.

#### 2. *Use of Immunoglobulin for Prophylaxis—Passive Immunization*

Human immunoglobulin preparations have been successfully used for some 50 years to provide passive protection against hepatitis A virus infection (28). The immunoglobulin fraction is prepared from large numbers of plasma units from blood donors (up to 1000 units) by alcohol precipitation to produce a 16% protein preparation. Immunoglobulin is administered intramuscularly into the gluteal or deltoid muscle (in infants it can be given in the anterolateral muscle mass of the thigh). The dosage varies from 0.02 to 0.06 mL/kg body weight (the latter dose giving longer-lasting protection). The injection is painful but other side effects are very uncommon. The only contraindication is selective IgA deficiency (because of the risk of development of autoantibodies). Immunoglobulin is rapidly absorbed into the bloodstream to give almost immediate protection. Protection is, however, only temporary—usually up to 3 months, as the administered immunoglobulin is degraded.

Immunoglobulin preparations can be given simultaneously with other vaccines (at different sites of the body) (13). However, following administration of immunoglobulin, there should be a delay of at least 3 months before MMR (measles, mumps, rubella) vaccine is administered and 5 months before varicella vaccine is administered because of the potential of interference with these live vaccines by antibody in immunoglobulin preparations.

Immunoglobulin can be given as preexposure or postexposure prophylaxis. Preexposure prophylaxis applies generally to travelers who urgently require protection because of imminent departure to an endemic area. Protection will be afforded for 1–2 months if a dose of 0.02 mL/kg is

administered and for up to 3–6 months if a higher dose of 0.06 mL/kg is given. Immunoglobulin may be given together with vaccine to combine the advantage of immediate protection by immunoglobulin with long-term protection by vaccine. Postexposure prophylaxis immunoglobulin is maximally effective if administered as soon as possible after exposure and rapidly loses its efficacy if there is a delay in administration. If there is a lapse of greater than 2 weeks after exposure, there is no point in giving immunoglobulin. The following are the most commonly recommended indications for postexposure prophylaxis:

- Close personal contact with a patient with hepatitis A virus infection, e.g., all household and sexual contacts
- Staff and children of daycare centers where a case of hepatitis A occurs, especially if there are children in diapers
- Hepatitis A occurring in an institutional setting including barracks, prisons, and similar living conditions

The extent of passive immunization of contacts would need to be carefully evaluated depending on epidemiological considerations. The following circumstances generally do not warrant administration of immunoglobulin:

- A case of hepatitis in a day school or office environment where there is only casual contact.
- Healthcare workers exposed to a case of hepatitis in a hospital setting.
- In most situations of common source outbreaks—by the time the first patient who has ingested the offending food displays symptoms of hepatitis, it is usually too late for passive immunization to be effective.

### 3. *Active Immunization—HAV Vaccine*

A number of HAV vaccines have been developed following on the successful propagation of HAV virus in cell culture. Currently four vaccines are available and in widespread use internationally (10). Three of these vaccines are produced by growing cell culture–adapted strains of HAV in human fibroblasts followed by formalin inactivation and adsorption onto an aluminum hydroxide adjuvant. A fourth vaccine has been produced using a virosome (virus-like particle) design (29). The inactivated fibroblast-grown virus particles are adsorbed onto phospholipid vesicles into which have been incorporated the hemagglutinin and neuraminidase proteins of influenza virus. The virosome is designed to target antibody-producing cells, which have been primed by previous exposure to influenza virus, and also macrophages in order to produce more rapid and effective T- and B-cell proliferative responses.

HAV vaccine is administered as a two-dose schedule by intramuscular injection. The immune response is highly satisfactory even after one dose, but a second dose given 6–12 months later is recommended for long-term immunity. Side effects are rare and usually trivial and consist mainly of allergies to one of the vaccine components. The vaccine is presently contraindicated in children less than one year of age because of doubtful efficacy due to possible neutralization by passively acquired maternal antibodies.

Specific neutralizing antibodies are produced in 95% of adults within 15 days and 100% of adults within 2 months. In children antibodies are formed in 97–100% of recipients after the first dose. Efficacy studies in Thailand and New York demonstrated protection of 94% and 100% of cases, respectively (30,31). The long-term duration of protection is not yet known, but kinetic models predict durability of protection for at least 20 years (10).

The following individuals have been targeted for routine immunization with HAV:

- People who may be exposed to the virus in their occupation (doctors, nurses, daycare center personnel)
- People with liver disease

Travelers (over the age of 2 years)  
Individuals with clotting factor dysfunction  
Homosexual men  
Illegal drug users

HAV vaccine can also be given as postexposure prophylaxis, usually together with immunoglobulin (at different sites of the body); as with immunoglobulin, it needs to be given as soon as possible after exposure.

Selective administration of vaccine to at-risk individuals is highly effective for personal protection but has little or no effect on the circulation of the virus in the population. Studies of the source of infection in cases of HAV disease have shown that 22–26% of cases of transmission occur as a result of household or sexual contact, 14–16% in daycare centers, 4–6% in international travelers, and 2–3% as a result of food- or waterborne outbreaks (32). Thus, in approximately 50% of cases there is no identified source of infection. It is therefore clear that directing immunization to the target populations “at risk” may be highly effective in protecting those individuals from infection but would have no effect on the circulation of the virus in the population. This has recently given rise to considerations for the need to institute HAV vaccination in the routine immunization program (33–35). This has been strengthened by comparisons with polio, with which HAV has much in common. For example, both viruses share similar fecal-oral transmission routes and epidemiology. Second, both are exquisitely sensitive to neutralizing antibodies. Third, in both cases immunity is directed to their outermost VP1 protein. Fourth, neither virus is shed chronically. For universal immunization to be successful, however, high routine coverage would need to be achieved, otherwise the situation may be aggravated by coverage that was only partial, causing an upward age shift of infection, resulting in the widening of the immunity gap in the vulnerable older children and young adult population. In addition, the high cost of HAV vaccine mitigates against the introduction of universal immunization at present (10).

## B. Hepatitis E Virus

Prevention of waterborne epidemics involves public health measures such as ensuring the provision of clean, safe drinking water and preventing fecal contamination of water supplies. Travelers to endemic countries need to take the same precautions against HEV as were enumerated above under HAV. At present, no passive or active immunization options are available, although preliminary phase I and II trials of experimental vaccines are being carried out.

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# 3

## ***Bacillus cereus***

**Kathleen T. Rajkowski**

*U.S. Department of Agriculture, Wyndmoor, Pennsylvania, U.S.A.*

**Reginald W. Bennett**

*U.S. Food and Drug Administration, College Park, Maryland, U.S.A.*

### **I. BACKGROUND**

*Bacillus cereus* has a ubiquitous distribution in nature, and this aerobic endospore-forming rod is normally present in soil, dust, and water. Because of its wide and generalized presence in the environment, it can be isolated from a variety of processed and raw foods—particularly in vegetation, which comes directly in contact with the soil. Its presence in foods, however, is not a significant health hazard unless the organism is able to grow (1) and produce toxins giving rise to foodborne illness. However, the isolation of high levels of *B. cereus* is suggestive of the organism's involvement in food poisoning (2). The consumption of food containing more than  $10^5$  viable toxigenic *B. cereus* organisms per gram of food has resulted in outbreaks of food poisoning (3–5). Although *B. cereus* was long considered a harmless saprophyte, its role as a potential foodborne pathogen associated with food poisoning was recognized in Europe as early as 1906 (6). Its role as a significant food-poisoning organism has been recognized since the 1950s (4,7). The first clearly documented outbreak occurred in Great Britain in 1971 (8). All populations appear to be susceptible to food poisoning caused by this organism. Although *B. cereus* is the most common organism associated with food poisoning, other *Bacillus* species such as *B. licheniformis*, *B. subtilis*, *B. pumilus*, and *B. brevis* have also been linked to food-poisoning outbreaks and have been documented elsewhere (5,9,10). Other enterotoxin-producing bacilli, including psychrotrophic *B. circulans*, *B. lentus*, *B. thuringiensis*, *B. polymyxa*, *B. carotarum*, and *B. pasteurii*, have been isolated from foods (11). Moreover, in recent years, *B. cereus* and other species proven to be sometimes psychrotrophic and isolated from foods stored at refrigeration temperatures have been, in fact, enterotoxigenic and are of increasing concern to the food industries (12–14).

### **II. CHARACTERISTICS**

#### **A. Organism**

The organism *Bacillus cereus* is a gram-positive rod primarily characterized by spore formation. The organism has two morphological forms, that of an endospore and a vegetative cell (15). The organism has a minimal growth temperature range of approximately 10–12°C and demonstrates a maximum range of about 48–50°C (8) with optimal growth temperatures of 28–35°C (16). The generation time of the organism occurs between 18 and 27 minutes. Growth has been demonstrated over a rather wide range of pH—4.9–9.3—and the organism tolerates salt concentrations up to 7.5%. The spores possess a resistance to heat that is typical of other mesophiles (6), with a germination frequency of up to 100%. The spore is characterized by nonswelling of the sporangium. Exposure to air does not repress sporulation, and the endospore is resistant to numerous environmental



and adverse conditions. The vegetative cells of *B. cereus* are facultative aerobic rods, which vary in width from 1.0 to 1.2  $\mu\text{m}$  and in length from 3.0 to 5.0  $\mu\text{m}$ . The rods tend to grow in long chains. During early cell growth, they are gram-positive, but they can be gram-negative in late growth. The motile *B. cereus* possess peritrichous flagellae, although nonmotile strains have been observed.

## B. Toxins

Food-poisoning strains of *B. cereus* produce a wide variety of extracellular toxins and enzymes, including lecithinase, proteases,  $\beta$ -lactamase, sphingomyelinase, cereolysin, and hemolysin BL (6). The diarrheagenic toxin is an extracellular protein complex that elicits diarrhea in monkeys and can be identified in culture fluids by serological assay (17–19). This mature protein complex induces vascular permeability in the skin of rabbits and elicits fluid accumulation in the rabbit ileal loop. The component is reported to have a molecular weight of 50,000–60,000 (20). This component is heat labile (inactivated in 5 min at 56°C) and is sensitive to trypsin and pronase (21). A number of the properties of the diarrheal and emetic toxins produced by *B. cereus* have been tabulated by Johnson (2). The diarrheagenic illness appears to be produced by a tripartite complex, which consists of components B, L, and L<sub>2</sub> and the hemolysin designated as BL (HBL). This complex exhibits a number of phenomena, including hemolysis, cytolysis, demonecrosis, vascular permeability, and enterotoxic activity (22). While no single enterotoxin has been demonstrated, the HBL component appears to be responsible for the diarrheagenic syndrome (23). For use in diagnostic tests, specific antibodies can be raised against the protein (17,19). The emetic toxin (vomiting type) is different from the diarrheal-type toxin in function and has a molecular weight of about 1.2 kDa. This toxin has been determined to be a cereulide, an ionosphoric water-soluble peptide that is closely related to the peptide antibiotic valinomycin (24). It induces the formation of vacuoles in Hep-2 cells and is highly resistant to heat: its activity is not lost after heating at 120°C for >1 hour. Additionally, this cereulide is stable from pH 2.0–11.0, is not hemolytic, and does not induce vascular permeability in the skin of the rabbit. The structural details and other functional characteristics of this toxin have been described in more detail (25).

A similar response in cats is also produced by the injection of heated (100°C for 90 min) culture fluids from *B. subtilis* and *B. licheniformis* cultures (18) that have been implicated epidemiologically in food poisonings (26,27). Previous investigations have shown that *B. thuringiensis* is capable of producing the diarrheal or emetic toxins and has been involved in food-poisoning outbreaks. In 1987, honey produced in Maine was contaminated with *B. thuringiensis* that caused a food-poisoning outbreak in which 3 of 5 family members became ill. The primary symptoms were vomiting and diarrhea. The incubation period was 5–10 hours, with a duration of 18 hours.

## III. NATURE OF ILLNESS

### A. Diarrheal Syndrome

Consumption of foods containing millions of enterotoxigenic *B. cereus* per gram frequently results in food poisoning. Two distinct types of illness have been attributed to the consumption of foods contaminated with *B. cereus* and other species of the genus. The first and best-known type of illness is characterized by nausea (rare vomiting), abdominal distress, rectal tenesmus, and watery stools, not unlike the symptoms produced by the consumption of food containing large numbers of *Clostridium perfringens* (28,29) (Table 1). The diarrheal illness caused by enterotoxigenic *B. cereus* has an incubation period of 8–19 hours. Symptoms usually last for 12–24 hours. Fever is generally absent (6).

### B. Emetic Syndrome

This form of *B. cereus* food poisoning is significantly more severe and acute than the diarrheal syndrome (6). The incubation period for this syndrome ranges from 1 to 6 hours, with a mean time

**TABLE 1** Clinical and Epidemiological Similarities Between Illnesses Caused by *B. cereus*, *C. perfringens*, and *S. aureus*

	<i>C. perfringens</i> enterotoxin	<i>B. cereus</i> toxins		
		Diarrheal syndrome	Emetic syndrome	<i>S. aureus</i> enterotoxin
Onset of symptoms, h	8–22	8–16	1–5	2–6
Duration of illness, h	12–24	12–14	6–24	6–24
Diarrhea, abdominal cramps	Predominant	Predominant	Fairly common	Common
Nausea, vomiting	Rare	Occasional	Predominant	Predominant
Pathogenesis	Toxin mediated <sup>a</sup>	Toxin mediated <sup>b</sup>	Toxin mediated <sup>c</sup>	Toxin mediated <sup>c</sup>
Principal food vehicles	Cooked meat and poultry sources	Meat products, soups, vegetables, puddings, poultry, dairy products	Cooked rice and pasta	Cold cooked meat and poultry

<sup>a</sup> Sporulation-associated toxin released in the small intestine.

<sup>b</sup> Toxin may be preformed in food or produced in the small intestine.

<sup>c</sup> Toxin preformed in food.

Source: Modified from Ref. 9.

of approximately 3 hours. This syndrome is characterized by an acute attack of vomiting after ingestion of food contaminated with the emetic toxin. Diarrhea is generally not associated consistently with this type of illness, although it has been known to occur (30). The symptoms of *B. cereus* emetic-type food poisoning mimics staphylococcal food poisoning (Table 1). A number of typical food-poisoning outbreaks have been described in relative detail by other investigators (5,25,31).

### C. Dose

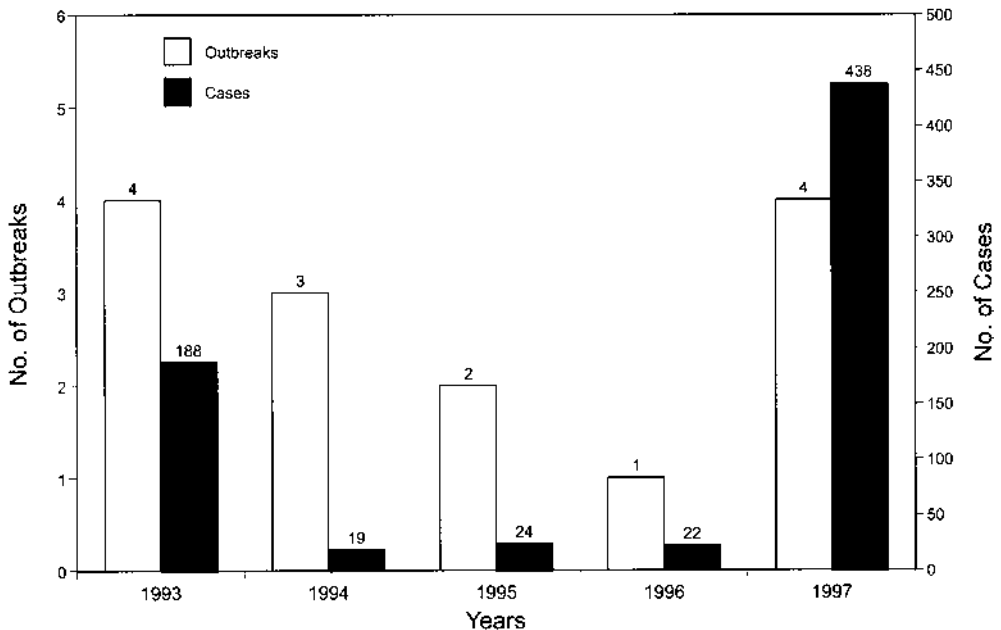
Outbreaks caused by *B. cereus* and other *Bacillus* spp. between 1950 and 1978 have been summarized by Gilbert (3). Plate counts enumerating the levels of contamination on leftover foods ranged from  $10^5$  to  $9.5 \times 10^5$  organisms/g with many foods incriminated in outbreaks in the range of  $10^7$ – $10^8$  organisms/g (6). The first and best-documented outbreaks were reported by Hauge (7), in which counts ranged from  $2.5 \times 10^7$  to  $1 \times 10^8$  organisms/g in contaminated vanilla sauce (6). Other *B. cereus* foodborne incidents reported from 1992 to 1997 were documented by Rajkowski and Smith (25).

## IV. EPIDEMIOLOGY

Many thousands of food-poisoning outbreaks in all countries go unreported to health agencies responsible for the collection of epidemiological data for tracking cases and outbreaks of foodborne illnesses. Proper documentation of specific outbreaks is imperative to construct epidemiological profiles. Such documentation should include brief histories of the illness (symptoms, incubation, and duration), details on those affected (type of population involved and attack rate), and information concerning the incriminated food (nature, where obtained, preparation, storage, and handling). This documentation is helpful in the laboratory investigation of such illnesses. In spite of improved harvesting techniques, processing approaches, packaging, and storage improvements, outbreaks caused by *Bacillus* continue to occur.

### A. Frequency of Illness

Among the outbreaks for which the etiology was determined, bacterial pathogens caused the largest percentage of outbreaks (75%) and the largest percentage of cases (86%) in the United States from



**FIGURE 1** Comparative numbers of reported outbreaks and cases caused by *Bacillus cereus* in the United States, 1993–1997.

1993 to 1997. Of the outbreaks that occurred during this period, 14 (0.5%) involving 691 (0.8%) cases were attributed to the contamination of foods by *B. cereus* (32). The number of documented outbreaks caused by *B. cereus* and related species occurring in the United States from 1993 to 1997 is presented in Figure 1. One of the difficulties in the investigation of foodborne illness is that symptoms of those afflicted might be similar to symptoms caused by other etiological agents. The *B. cereus* diarrheal syndrome symptoms mimic the clinical features of *C. perfringens*, while symptoms elicited by the emetic toxin are not unlike those of staphylococcal intoxication. *Bacillus cereus*–*Staphylococcus aureus* food-poisoning symptoms are given in Table 1. As a consequence of these symptomatic parallels, it is imperative that laboratory investigations be carried out completely. Laboratory analysis should include enterotoxigenicity data of the recovered organism and demonstration of preformed toxin in the incriminated food.

## B. Foods Incriminated

The presence of the diarrheal factor is usually associated with proteinaceous foods, vegetables, sauces, and puddings. In contrast, the emetic form of the illness is associated with farinaceous foods, particularly cooked rice and other starchy foods. A vast number of foods previously involved in food-poisoning outbreaks have been documented in other investigations (5,25,31), including vanilla puddings, cooked meat and vegetable dishes, boiled and fried rice, dairy products, and vegetable sprouts (17). During 1993–1997 in the United States, the greatest number of outbreaks involved Chinese food, followed by salads (fish, poultry, egg), Mexican food, nondairy beverages, pork, chicken, and multiple vehicles caused by cross-contamination.

In most food-poisoning outbreaks of this type, the causative strain was found to be present in large numbers. Occasionally, one or both symptom categories (i.e., vomiting and diarrhea) is experienced in the same outbreak, which might indicate that both toxins are produced by the same strain.

Since the symptoms of these foodborne illnesses mimic those caused by other bacteria, laboratory confirmation becomes essential before a definite diagnosis can be made (17).

In order to improve more significant epidemiological data collection in the surveillance of food-poisoning outbreaks, a new technology has been applied to help and more effectively identify episode clusters or outbreaks. PulseNet is a National Network of public health laboratories involving state and federal agencies that perform pulsed-field gel electrophoresis (PFGE) on bacteria that might be foodborne (33,34). This network permits rapid comparison through an electronic database at the Centers for the Disease Control and Prevention (Atlanta, GA). Closely related PFGE patterns in bacterial isolates from foods, outbreak victims, and/or contact environments involved in outbreaks suggest a common source, thus becoming helpful in resolving epidemiological investigations (32).

## V. ISOLATION AND IDENTIFICATION

Although *B. cereus* and other *Bacillus* species have a ubiquitous distribution in the environment and can be isolated from a wide variety of processed and raw foods, its presence in foods is not a significant hazard to consumer health unless it is able to proliferate and produce toxins (18). However, consumption of contaminated foods containing  $\geq 10^5$  viable enterotoxigenic *B. cereus* cells per gram of food has resulted in outbreaks of food poisoning.

### A. Enumeration and Isolation

Surface plating procedures are generally employed to determine viable *Bacillus* populations in foods incriminated in food-poisoning outbreaks or in surveillance and survey samples. In the United States and many parts of Europe, identification agars make use of (a) capability of *B. cereus* organisms to produce turbidity surrounding colonies growing on agar containing egg yolk and (b) the resistance of *B. cereus* to the antibiotic polymyxin B to create a selective and differential initial plating medium (17). Mannitol–egg yolk agar specified for use in Official Methods of Analysis, published by the Association of Official Analytical Chemists International (AOAC International), for the enumeration of *B. cereus* in foods (35) is preferred. However, other plating media of similar efficiency have been described (5). In cases where the number of viable *B. cereus* is  $<1000$  organisms/g, the alternative AOAC International Most Probable Number (MPN) method using tryptic soy polymyxin broth is recommended (35) and described in detail (1). Since it is not always possible to predict the levels of *B. cereus* in a suspect food, both direct plating and MPN procedures should be conducted simultaneously.

Lecithinase-positive, mannitol-negative colonies on MYP agar are highly indicative of *B. cereus*; however, because of the similarity of reactions by other members of the *B. cereus* group, including *B. anthracis*, *B. thuringiensis*, and rhizoid strains classified as *B. cereus* var. *mycoides*, isolates must be further tested to differentiate them from other *Bacillus* species before definite identification can be made (17,19). The most important features of the four biotypes of the *B. cereus* group are summarized in Table 2 (1). The procedures for differentiating members of the *B. cereus* group have been described in detail (15,17,19).

### B. Identification of *B. cereus*

Colonies from MYP and other comparable media (i.e., KG agar) that meet the criteria described earlier should be provisionally identified as *B. cereus*. Additionally, isolates that fulfill the criteria listed in Table 2 are large gram-positive bacilli that produce lecithinase, are negative for mannitol fermentation on MYP agar, grow and produce acid from glucose anaerobically, reduce nitrate to nitrite (a few strains are negative), produce acetylmethylcarbinol, decompose L-tyrosine, grow in the presence of 0.001% lysozyme, exhibit motility, are hemolytic, and do not produce endotoxin crystals or rhizoid growth. The plate count of such colonies, or the most probable number, times the dilution factor is the confirmed *B. cereus* count.

**TABLE 2** Identification Characteristics of *Bacillus cereus* and Culturally Similar Species

Feature	<i>B. cereus</i>			
	<i>B. cereus</i>	var. <i>mycoides</i>	<i>B. thuringiensis</i>	<i>B. anthracis</i> <sup>a</sup>
Gram stain	+	+	+	+
Catalase	+	+	+	+
Egg yolk reaction	+	+	+	(+)
Motility	(+/-)	-	(+/-)	-
Acid from mannitol	-	-	-	-
Hemolysis (sheep RBC)	+	(+)	+	-
Rhizoid growth	-	+	-	-
Toxin crystals produced	-	-	+	-
Anaerobic utilization of glucose	+	+	+	+
Reduction of nitrate	+	+	+	+
VP reaction	+	+	+	+
Tyrosine decomposition	+	(+)	+	(+)
Resistance to lysozyme	+	+	+	+

<sup>a</sup> Additional tests are required to identify this species.

+, positive; (+/-), usually positive but occasionally may be negative; (+), often weakly positive; -, negative.

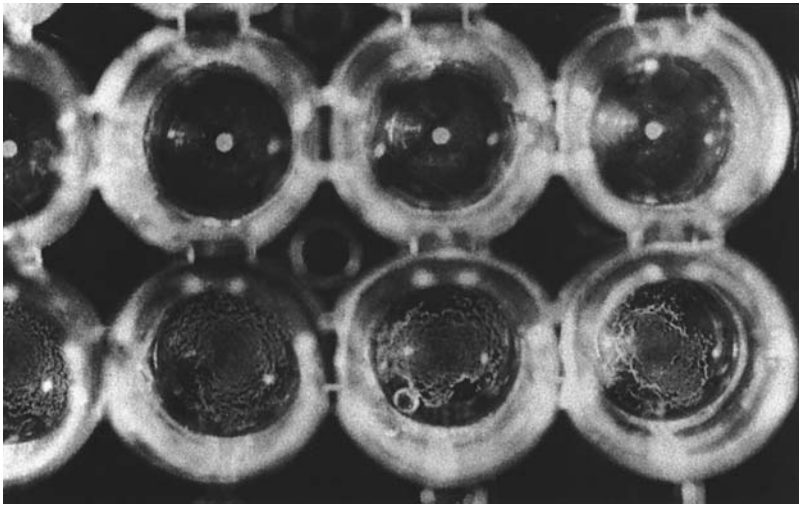
It has been suggested that the APT system (Analytal Products, Plainview, New York) be used to confirm isolates as *B. cereus* from selective media (38). More recently employed approaches such as PCR analysis (39,40), multiplex PCR (41), fluorogenic based PCR (42), and ribotyping (43) have been shown to be applicable in the identification of *B. cereus* and the differentiation of other species.

### C. Identification of Toxins

While the procedures for enumeration, isolation, and identification are effective in the taxonomic classification of the organism, they have little to do with whether the organism poses a potential or actual hazard to consumer health. This can only be accomplished, with the aid of epidemiological information, by determining whether the suspect *Bacillus* is enterotoxigenic, which provides circumstantial evidence of contamination of a suspect food. The ultimate assay is the demonstration of the actual presence of preformed toxin in foods incriminated in food poisoning outbreaks or in suspect foods.

#### 1. Diarrheal Toxin Identification

The toxic entities responsible for the diarrheal syndrome act in the small intestine of the host. These components, in recent studies, have been shown to be hemolysin BL (HBL), which is a tripartite entity consisting of three protein components and possessing hemolytic, cytotoxic, dermonecrotic, and vascular permeability activity (23,44). Additionally, this entity induces fluid accumulation in rabbit ileal loops. These three components, L<sub>2</sub> (46 kDa), L (38 kDa), and B (37 kDa), all appear to be necessary to achieve the diarrheal syndrome. Conversely, Lund and Granum (45) and Agata et al. (46) characterized a nonhemolytic toxin complex from *B. cereus* strains responsible for food-poisoning outbreaks. This nonhemolytic entity (NHE) also consisted of three protein moieties designated B (105 kDa), L<sub>2</sub> (54 kDa), and L (39 kDa), and all three are believed to be necessary to demonstrate maximal cytotoxicity. It is thought that enterotoxigenic (diarrheal type) strains of *B. cereus* may produce HBL or NHE or both protein complexes (45). The earliest adapted serological method for the diarrheal toxin was the microslide gel double diffusion test (17,47,48) followed by the development of more rapid methods. Presently, two antigen (toxin)-antibody methods have been

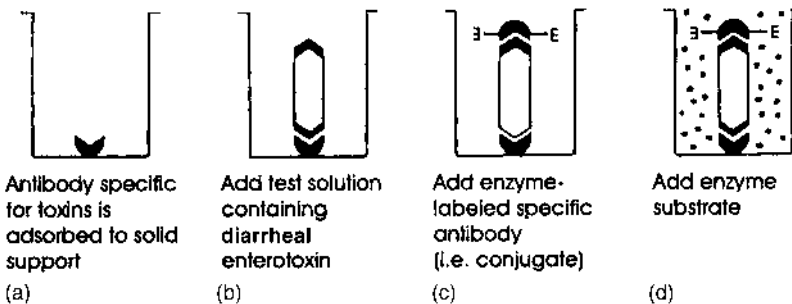


**FIGURE 2** RPLA test results showing smooth button negatives (top row) and “granular dispersed” positives (bottom row).

made commercially available for the detection of *B. cereus* diarrheal toxin(s): a reversed passive latex agglutination (Oxoid™, BCET-RPLA) (Fig. 2) and a double antibody sandwich” ELISA (TECRA™ International) (Fig. 3). The Oxoid RPLA commercial kit detects the L<sub>2</sub> of the HBL complex, while the ELISA kit measures the L<sub>2</sub> entity of the NHE complex (37). Beecher and Wong (44) have shown that a simple approach for differentiating HBL strains of *B. cereus* is their distinctive discontinuous hemolytic pattern on blood agar (25).

## 2. Emetic Toxin Identification

The emetic activity produced by *B. cereus* strains incriminated in emetic syndrome outbreaks has been demonstrated by monkey feeding (30) and in kittens by intravenous injection of heated culture fluids (17). The tissue culture assay using Hep-2 cells (49,50) and a recently described spermatozoa toxicity bioassay (51) may also be useful for the detection and quantitation of the emetic toxin. Other cell lines investigated for use in the detection of the emetic toxin, including



**FIGURE 3** *Bacillus cereus* diarrheal enterotoxin immunoassay. ELISA performed in a double antibody “sandwich” configuration. (a) Capture antibodies to diarrheal toxin adsorbed to plastic microtiter well; (b) addition of preparation containing suspect toxin; (c) addition of the enzyme-labeled specific antibodies to the diarrheal enterotoxin; (d) addition of enzyme substrate with enzyme action on the substrate if toxin is present in sample.

Int. 407 (embryonic intestine), CHO, Vero, HeLa, Y-I adrenal, and MA-104 (rhesus embryonic monkey kidney), have been documented by Schultz and Smith (31). Immunotechniques or other rapid and specific methods for the detection and quantitation of this toxin are yet to be developed.

## VI. PATHOGENICITY/TOXICITY

Most strains of *B. cereus* and other *Bacillus* species are capable of elaborating a wide range of extracellular metabolites, primarily during the exponential growth phase. These metabolites include a number of toxins including “virulence factors” demonstrated on the basis of their behavior in animal models and tissue culture or cell lines. The various activities of these have been summarized (5). Human feeding studies and results collected on experimental animals such as dogs and cats as well as rhesus monkeys using whole cell cultures or filtrates have shown that diarrhea can be induced and, therefore, have established *B. cereus* as a foodborne pathogen. Two of a variety of metabolites, the diarrheal and emetic toxins, have been established as separate toxic moieties, which present different clinical profiles. However, they show clinical manifestations, which are demonstrated by other bacterial toxins as shown in [Table 1](#).

It has long been recognized that *B. cereus* and other *Bacillus* species can be involved in clinical diseases other than those affecting the gastrointestinal tract. Some nongastrointestinal diseases induced by *B. cereus* include posttraumatic endophthalmitis (eye infections), primary cutaneous infections, postoperative and posttraumatic orthopedic wounds, pleuropulmonary infections, meningitis, bacteremia, mastitis (cattle and goats), and other types of infections. A number of nongastrointestinal clinical conditions have been described, in relative detail, by Schultz and Smith (31) and by other investigators (5).

## VII. GENETIC STUDIES

Despite the apparent close phenotypic relationship within the *B. cereus* group—*B. cereus*, *B. thuringiensis*, *B. anthracis*, and *B. mycoides*—these members are genotypically diverse, although several genes are common to all of these species (16). The basic criteria used to classify an organism as *B. cereus* or *B. thuringiensis* or *B. anthracis* is the inability to produce the insecticidal protoxin or anthrax toxin, respectively. Taxonomic classification of species or subspecies within this group becomes confusing because the various characteristics (i.e., flagellar H antigens, crystal toxin capability, shape, size, antigens, and/or plasmid profiles) yield conflicting results (52). A number of factors may be responsible for this taxonomic confusion; however, the major factor is probably the ability of the *Bacillus* group to exchange and rearrange genetic information (53). Heretofore, methods for genetic exchange of chromosomal and plasmid traits in this group of organisms have been restricted to general transduction, transformation of protoplasts and autoplasts, and a conjugation-like transfer process (54). In earlier studies on the genetics of the *Bacillus* group, the most prevalent methods for gene transfer were transformation and transduction (53). Additionally, a system that appears to involve cell-to-cell contact has been documented for the transfer of plasmids from selected *B. thuringiensis* subspecies to *B. cereus* (55), *B. anthracis* (56), and plasmid-cured variants of *B. thuringiensis* (55). The plasmids transferred most frequently by these and other investigators were those encoding protoxin genes or those involved in the regulation of protoxin synthesis (57). In addition to generalized transduction, transformation, and cell-cell contact processes, genetic analysis using multiple enzyme electrophoresis, carbohydrate profiles, phage, DNA-DNA homology, and or RNA suggest differences both within species as well as between species (16). A number of these genetic studies have been briefly described by Schultz and Smith (31), who reported on the development of a mating system involving the plasmid transfer of the ability to produce parasporal crystals from *B. thuringiensis* to *B. cereus* and *B. anthracis* by cell-cell contact (56). Aronson and Beckman (53) demonstrated a low frequency of chromosomal gene transfer from *B. thuringiensis* to *B. cereus* during cell mating experiments. Belliveau and Trevors (54) and Sabelnikov and Ulyashova (58)

demonstrated that DNA isolated from plasmids transformed *B. cereus* vegetative cells by use of electroporation and cellophane membranes, respectively. Kolsto et al. (59) constructed a physical map of the *B. cereus* chromosome that has enhanced the understanding of the genetic aspects of this organism. Yamada et al. (60) employed the gyrase B gene (*gyrB*) as a molecular diagnostic marker in the differentiation of *B. cereus* from closely related species by PCR amplification of the *B. cereus*-specific fragment. Daffonchio et al. (61) applied the randomly amplified polymorphic DNA (RAPD) fingerprinting technique in which the DNA marker was specific for the *B. cereus* group. However, restriction analysis of the fragment with the *AluI* gene distinguished *B. anthracis* from other species of the *B. cereus* group. The *B. cereus* group of bacteria demonstrate different phenotypical expressions and, more importantly, different pathological effects. *B. anthracis* causes acute fatal disease anthrax and is a potential human biological agent due to its high toxicity. *B. thuringiensis* produces an intracellular protein crystal toxic to a wide variety of insects and as a consequence used commonly as a biological pesticide. *B. cereus* is ubiquitous in the environment, particularly in soil, and is considered a significant pathogen causing at least two types of food poisoning. In contrast to their phenotypic differences, Helgason et al. (62) showed by multilocus enzyme electrophoresis and by sequence analysis of nine chromosomal genes that *B. anthracis* should be considered a lineage of *B. cereus*. These observations are not only a matter of taxonomy but may also have consequences with regard to virulence and pathogenicity. As the absolute requirements for virulence are better defined for *B. cereus*, new opportunities for functional identification schemes may be realized (16). Thompson et al. (63) suggested that the enterotoxic activity probably comprised more than one component while Beecher and Macmillan (64) reported that three components of hemolysin BL also composed the diarrheal toxin. However, other investigators (65,66) identified a single unstable protein of 43–53 kDa as an entity responsible for the diarrheal-type food poisoning. This confusion regarding whether the diarrheal toxin is a single protein or a multi-component has prompted studies at the genetic level to resolve these conflicting findings. A toxin gene (*bcet*) on a 2–9 kb DNA fragment of *B. cereus* B-4ac was cloned and expressed in *Escherichia coli* (24). The translated product in *E. coli* exhibited Vero cell cytotoxicity and was positive in a vascular permeability assay. Additionally, this product caused fluid accumulation in ligated mouse ileal loop and was lethal to mice upon injection. These biological characteristics are characteristic of diarrheal enterotoxin. Further genetic studies may need to be done to resolve this issue.

## VIII. PREVENTION/CONTROL MEASURES

Various parameters and factors for limiting and preventing the growth of *B. cereus* have been investigated. Low temperature is often used as a means of limiting or preventing the proliferation of *B. cereus*, although some studies have shown that some strains can grow and produce toxins at reasonably low temperature. Studies on growth temperature requirements with 50 strains showed some strain variation. In this study, all the strains grew at temperature ranges from 14 to 40°C, although only half of the strains grew at 45°C and 3 strains grew at 49°C. More than half the strains grew at 10°C, 6 strains grew at 8°C, and one strain grew at 6°C (67). Other studies have shown that *B. cereus* isolated from foods incriminated in food-poisoning outbreaks were able to grow and produce toxins at 4–7°C (31). Alkaline conditions lead to growth while pH 5.0 conditions have been shown to be more inhibitory to growth of clinical and veterinary strains of *B. cereus* than of food-poisoning strains. Methods involving the inhibition and inactivation of *B. cereus* spores include the determination of D-values. A study of *B. cereus* spores in heated custard shows D-values of 3.6, 2.8, and 2.2 minutes at 90, 95, and 100°C, respectively; however, when the product was adjusted to pH 6.2, the D-values were decreased. Conversely, increasing the pH (7.6) also increased the D-value. Studies have shown that *B. cereus* spores inoculated into uncooked rice were not inactivated completely during the cooking process (100°C for 30 min), although viable cells were destroyed in fried rice when subjected to 180–190°C for 5–7 minutes. Studies on the effect of various solutes on spore germination and growth of *B. cereus* show that spore germination is less sensitive to reduced water



activity ( $A_w$ ) than outgrowth or growth of vegetative cells of *B. cereus*. In a review on water activity, Troller (68) discussed the effect of various solutes on spore germination and growth of *B. cereus*.

Other methods investigating the inhibition and inactivation of *B. cereus* include microwave, treatment by irradiation, and chemicals (31). Studies have been conducted on the effects of NaCl, pH, and temperature combinations on the growth of *B. cereus*. Nitrite acts as a membrane-directed sulfhydryl agent against *B. cereus*, although nitrite inhibition is reversed when the exposure to nitrite is removed (69).

Nicotinamide-treated spores germinate poorly and lose their capability to germinate over extended storage. It has been suggested that the addition of nicotinamide to foods could be useful as an anti-*B. cereus* agent (31).

The ubiquitousness of *B. cereus* in the general environment, the stability and resistance of their spores, and their presence on raw agricultural products provides justifiable concern for *Bacillus* spp. as actual or opportunistic foodborne pathogens. Their presence on raw agricultural products ensures possible contamination of the food-processing environment and equipment. As a consequence, effective prevention and control measures would include (a) the control of *Bacillus* spore germination and (b) prevention of proliferation of the vegetative cells in foods. Effective heat or irradiation treatment may be necessary where complete destruction of the organism is desired. The creation of unfavorable conditions such as low temperatures, low  $A_w$ , or pH in foods may greatly reduce the spore germination of enterotoxigenic *Bacillus* spp., thus preventing toxin formation in foods. A number of cooking methods such as steaming under pressure, thorough roasting, frying, and grilling are likely to destroy both vegetative cells and spores, although cooking at temperatures below 100°C might allow survival of *Bacillus* spores. Of major concern to the consumer is the multiplication of the organism during inadequate cooling or the holding of moist foods in a nonrefrigerated state over periods that would allow for cell proliferation. Favorable conditions for enterotoxigenic *Bacillus* are sometimes provided by cooking procedures that activate spores and then by slow cooling and mass storage of foods at temperatures above 10°C and below 60°C. If food storage is necessary, it should be cooled rapidly to a temperature (8°C) that prevents growth of the organism. If a food must be held in a warm state, such as might be necessary in institutional settings, the temperature should be maintained above 60°C. The most frequent causes attributed to *B. cereus* food poisoning outbreaks in the United States from 1993 through 1997 are shown in Table 3. Of major concern to the food processor and retailer in the prevention of food-poisoning outbreaks should be the effective utilization of hazard analysis critical control point (HACCP) systems by all who are involved in the harvest, manufacture, distribution, storage, and serving of food.

**TABLE 3** Leading Causes of *Bacillus cereus* Food-Poisoning Outbreaks in the United States, 1993–1997

Cause	Number of outbreaks
Improper holding temperatures	10
Inadequate cooking	3
Contaminated equipment	2
Food from unsafe source	0
Poor personal hygiene	2
Other	1

Source: CDC Surveillance Summaries, 2000, Vol. 49, #SS1.

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# 4

## ***Staphylococcus aureus***

**Reginald W. Bennett and Steven R. Monday**

*U.S. Food and Drug Administration, College Park, Maryland, U.S.A.*

### **I. BACKGROUND**

The association of *Staphylococcus* with foodborne illness dates back as early as 1884, when spherical organisms in cheese caused a large food-poisoning outbreak in the United States. Other earlier outbreaks now attributed to the consumption of staphylococcal-contaminated foods occurred in France in 1894, Michigan in 1907, and the Philippines in 1914. In 1930 Dr. Gail Dack and his colleagues at the University of Chicago were able to demonstrate that the cause of food poisoning resulting from the consumption of contaminated sponge cake with cream filling was a toxin produced by isolated staphylococci (1).

The growth and proliferation of *Staphylococcus aureus* in foods presents a potential hazard to consumer health since many strains of *S. aureus* produce enterotoxins. The primary reasons for examining foods for *S. aureus* and/or their toxins are to confirm that this organism is the causative agent of a specific food-poisoning episode, determine whether a food or ingredient is a source of enterotoxigenic staphylococci, and demonstrate postprocessing contamination. The latter is usually due to human contact with processed food or exposure of food to inadequately sanitized food-processing surfaces. Foods subjected to postprocess contamination with enterotoxigenic staphylococci also represents a potential hazard because of the absence of competitive organisms that might otherwise restrict the growth of *S. aureus* and subsequent production of enterotoxins.

Of the various metabolites produced by the staphylococci, the enterotoxins pose the greatest risk to consumer health. Enterotoxins are proteins produced by some strains of staphylococci (2), which, if allowed to grow in foods, may produce enough enterotoxin to cause illness when the contaminated food is consumed. These structurally related, toxicologically similar proteins are produced primarily by *S. aureus*, although *Staphylococcus intermedius* and *Staphylococcus hyicus* have also been shown to be enterotoxigenic (3). Normally considered a veterinary pathogen (4,5), *S. intermedius* was isolated from butter blend and margarine in a food-poisoning outbreak (6,7). A coagulase-negative *S. epidermidis* was reported to have caused at least one outbreak (8). These incidents support testing staphylococci other than *S. aureus* for enterotoxigenicity if they are present in large numbers in a food suspected of causing a food-poisoning outbreak.

Foods commonly associated with staphylococcal food poisoning fall into general categories such as meat and meat products, salads, cream-filled bakery products, and dairy products. Many of these items are contaminated during preparation in homes or food service establishments and subsequently mishandled prior to consumption. In processed foods, contamination may result from human, animal, or environmental sources. Therefore, the potential for enterotoxin development is greater in foods exposed to temperatures that permit the growth of *S. aureus*. This is especially true for fermented meat and dairy products. Although the potential is there, it is only when incomplete fermentation (e.g., lactic acid failure) takes place that the development of staphylococcal enterotoxin occurs.

In processed foods in which *S. aureus* is destroyed by processing, its presence usually indicates contamination from the skin, mouth, or nose of food handlers. This contamination may be introduced directly into foods by process line workers with hand or arm lesions caused by *S. aureus* coming into contact with the food or by coughing and sneezing, which is common during respiratory infections. Contamination of processed foods may also occur when deposits of contaminated food collect on or adjacent to processing surfaces to which food products are exposed. When large numbers of *S. aureus* are encountered in processed food, it may be inferred that sanitation, temperature control, or both were inadequate.

In raw food, especially animal products, the presence of *S. aureus* is common and may not be related to human contamination. Staphylococcal contamination of animal hides, feathers, and skins is common and may or may not result from lesions or bruised tissue. Contamination of dressed animal carcasses by *S. aureus* is common and often unavoidable. Raw milk and unpasteurized dairy products may contain large numbers of *S. aureus*, usually a result of staphylococcal mastitis.

The significance of the presence of *S. aureus* in foods should be interpreted with caution. The presence of large numbers of the organism in food is not sufficient cause to incriminate a food as the vector of food poisoning. Not all *S. aureus* strains produce enterotoxins. The potential for staphylococcal intoxication cannot be ascertained without testing the enterotoxigenicity of the *S. aureus* isolate (circumstantial evidence) and, more importantly, demonstrating the presence of staphylococcal enterotoxin in food. Neither the absence of *S. aureus* nor the presence of small numbers of organisms can provide complete assurance that a food is safe. Conditions inimical to the survival of *S. aureus* may result in a diminished population or death of viable microbial cells, while sufficient toxin remains to elicit symptoms of staphylococcal food poisoning.

The method to be used for the detection and enumeration of *S. aureus* depends, to some extent, on the reason for conducting the test. Foods suspected to be vectors of staphylococcal food poisoning frequently contain a large population of *S. aureus*, in which case a highly sensitive method will not be required. A more sensitive method may be required to demonstrate an unsanitary process or postprocess contamination, since small populations of *S. aureus* may be expected. Usually, *S. aureus* may not be the predominant species present in the food, and, therefore, selective inhibitory media are generally employed for isolation and enumeration.

The methods for identifying enterotoxins involve the use of specific antibodies. The fact that there are several antigenically different enterotoxins complicates their identification because each one must be assayed for separately. Another problem is that unidentified enterotoxins exist for which antibodies are not available for in vitro serology. These unidentified toxins, however, appear to be responsible for only a very small percentage of food-poisoning outbreaks.

## II. CHARACTERISTICS

### A. Organism

*S. aureus* is a spherical gram-positive bacterium (coccus) that on microscopic examination appears in pairs, short chains, or bunched, grape-like clusters. Some strains are capable of producing a highly heat-stable protein toxin, which is capable of causing illness in humans. Other salient characteristics are that they are nonmotile and asporogenous; capsules may be present in young cultures but are generally absent in stationary phase cells (9). *Staphylococcus* species are aerobes or facultative anaerobes and have both respiratory and fermentative metabolism. They are catalase positive and utilize a wide variety of carbohydrates. Amino acids are required as nitrogen sources, and thiamine and nicotinic acid are also required. When grown anaerobically, they appear to require uracil (10).

Although the staphylococci are mesophilic, some strains of *S. aureus* grow at a temperature as low as 6–7°C. In general, growth of *S. aureus* ranges from 7 to 47.8°C, with an optimum temperature for growth of 35°C. The pH range for growth is between 4.5 and 9.3, with the optimum between pH 7.0 and 7.5 (9). As is true with other parameters, the minimum pH for growth is also dependent on the degree to which all other parameters are at optimal conditions (10). With regard to water

**TABLE 1** Characteristics of *Staphylococcus* Species

Property	<i>S. aureus</i>	<i>S. intermedius</i>	<i>S. hyicus</i>	<i>S. epidermidis</i>
Pigment	+ <sup>a</sup>	—	—	—
Coagulase	+	+	±	—
DNase	+	+	±	—
Hemolysis	+	+	—	±
Mannitol (an)	+	—	—	—
Acetoin	+	—	—	+
Clumping	+	+	±	—
Hyaluronidase	+	—	+	—
Lysostaphin	HS	HS	HS	SS

<sup>a</sup> >90%.

an, anaerobic conditions; HS, high sensitivity; SS, slight sensitivity.

activity ( $A_w$ ), the staphylococci are unique in being able to grow at lower levels than other nonhalophilic bacteria. Growth has been demonstrated at as low as 0.83  $A_w$  under ideal conditions. These low  $A_w$  conditions are too low for the growth of many competing organisms. Most strains of *S. aureus* are highly tolerant to the presence of salts and sugars and can grow over an  $A_w$  range of 0.83 to >0.99. *S. aureus* grows best at an  $A_w$  of >0.99, and growth at low  $A_w$  values depends on other growth conditions being optimal.

*S. aureus* is capable of producing a large number of extracellular enzymes, toxins, and other chemical components. It has been shown that *S. aureus* is capable of producing at least 34 different extracellular proteins, although no one strain of the organism is capable of producing all of these proteins (11). Some of these extracellular metabolites have been useful in the identification of *S. aureus* and differentiation from other commonly encountered staphylococcal species. The salient characteristics of *S. aureus* and some other staphylococcal species are presented in Table 1. The two most common metabolites that have been the most useful in the identification of *S. aureus* are coagulase, a soluble enzyme that coagulates plasma, and thermonuclease (TNase). TNase is a heat-stable phosphodiesterase that can cleave either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) to produce 3'-phosphomononucleosides. TNase is much more heat stable than ribonuclease and is useful in speciating staphylococci.

## B. Enterotoxins

The staphylococcal enterotoxins are single-chain proteins, which are antigenic with molecular weights of 26,000–29,000. They are neutral-basic proteins with isoelectric points of 7.0–8.6. They are resistant to proteolytic enzymes, such as trypsin and pepsin, which makes it possible for them to travel through the digestive tract to the site of action. The toxins are highly stable to heat, making them a potential health hazard when they present in canned foods. Their other general properties, such as amino acid composition and immunological characteristics, have been described elsewhere (1,12).

## III. NATURE OF ILLNESS

### A. Symptoms

The onset of symptoms in staphylococcal food poisoning is usually rapid (2–6 hours) and in many cases acute, depending on individual susceptibility to the toxin, the amount of contaminated food eaten, the amount of toxin in the food ingested, and the general health of the victim. The most

common symptoms are nausea, vomiting, retching, abdominal cramping, and prostration. Some individuals do not demonstrate all the symptoms associated with the illness. In more severe cases, headache, muscle cramping, and transient changes in blood pressure and pulse rate may occur. Recovery generally takes 2 days, but it is not unusual for complete recovery to take 3 days or longer. Death from staphylococcal food poisoning is very rare, although such cases have occurred among the elderly, infants, and severely debilitated persons.

## **B. Dose**

A toxin dose of less than 1.0  $\mu\text{g}$  in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when *S. aureus* populations exceed  $10^6$  cfu. However, in highly sensitive people a dose of 100–200 ng is sufficient to cause illness (1).

## **IV. EPIDEMIOLOGY**

The epidemiology of foodborne disease is evolving to better trace established organisms as well as newly recognized or emerging pathogens as etiological agents of foodborne illnesses. Many of the pathogens have reservoirs in healthy food animals, from which they spread to a wide variety of foods (13). These pathogens, including staphylococcal species other than *S. aureus*, cause millions of sporadic illnesses and chronic complications as well as massive and challenging outbreaks around the world. Recently, developed technologies and commercially available rapid methods have allowed for improved surveillance of such outbreaks. An outbreak investigation or epidemiological study should go beyond identifying a suspected food and removing it from the shelf to include determining the chain of events that allowed contamination with an organism in large enough numbers to cause illness (13). This approach would facilitate the design of strategies for preventing similar occurrences in the future.

### **A. Frequency of Illness**

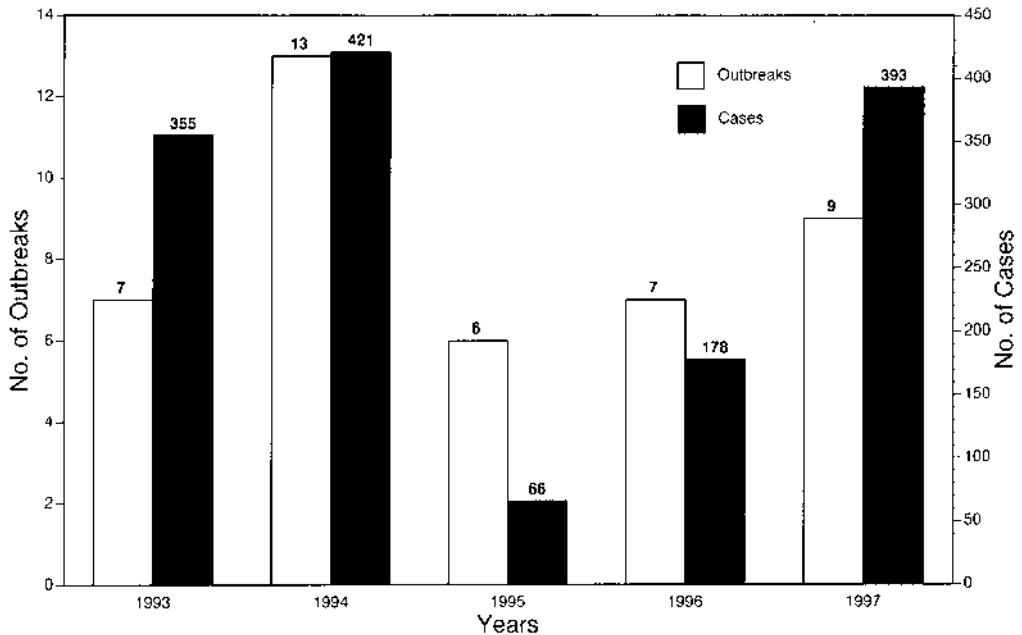
The true incidence of staphylococcal food poisoning is unknown for a number of reasons, including (a) poor responses from victims during interviews with health officials, (b) misdiagnosis of the illness, which may be symptomatically similar to other types of food poisoning (such as vomiting caused by *Bacillus cereus* emetic toxin), (c) inadequate collection of samples for laboratory analyses, (d) improper laboratory examination, and, (d) in many countries, unreported cases.

During a 5-year period (1993–1997) in the United States, bacterial pathogens accounted for 655 outbreaks involving 43,821 cases. Of those outbreaks, 42 outbreaks involving 1,413 cases, including one death, were attributed to staphylococcal toxin intoxication. [Figure 1](#) shows the comparative numbers of outbreaks and cases caused by staphylococcal foodborne illnesses from 1993 through 1997 in the United States (14).

### **B. Diagnosis of Human Illness**

In the diagnosis of staphylococcal foodborne illness, proper interviews with the victims and the gathering and analysis of epidemiological data are essential. Incriminated foods should be collected and examined for staphylococci. The presence of relatively large numbers of enterotoxigenic staphylococci is good circumstantial evidence that the food contains toxin. The most conclusive test is the linking of an illness with a specific food or, in cases where multiple vehicles exist, the detection of the toxin in the food sample(s). In cases where the food may have been treated to kill the staphylococci, as in pasteurization or heating, direct microscopic observation of the food may be an aid in the diagnosis. A number of serological methods for determining the enterotoxigenicity of *S. aureus* isolated from foods as well as methods for the separation and detection of toxins in foods have been developed and used successfully to aid in the diagnosis of the illness. Phage typing may also





**FIGURE 1** Comparative numbers of reported outbreaks and cases caused by staphylococcal species in the United States, 1993–1997.

be useful when viable staphylococci can be isolated from the incriminated food, from victims or from suspected carriers, such as food handlers. However, this approach can be limiting because there are strains of *S. aureus* that are not typable by this system. More recently, genetic fingerprinting techniques are being applied to characterize strains of staphylococci. Two of these approaches are pulse-field gel electrophoresis (PFGE) of DNA restriction fragment profiles (7) and DNA restriction fragment polymorphism of rRNA genes (ribotyping) (15).

### C. Vehicles of Transmission

Staphylococci exist in air, dust, sewage, water, milk, food, or on food equipment, environmental surfaces, humans, and animals. Humans and animals are the primary reservoirs. Staphylococci are present in the nasal passages and throats and on the hair and skin of 50% or more of healthy individuals. This incidence is even higher for those who associate with or who come in contact with sick individuals and hospital environments. Although food handlers are usually the main source of food contamination in food-poisoning outbreaks, equipment and environmental surfaces can also be sources of contamination with *S. aureus*. Human intoxication is caused by ingesting enterotoxins produced in food by some strains of *S. aureus*, usually because the food has not been kept hot enough (60°C, 140°F, or above) or cold enough (7.2°C, 45°F, or below).

### D. Foods Incriminated

Foods that are frequently incriminated in staphylococcal food poisoning include meat and meat products, poultry and egg products, salads such as egg, tuna, chicken, potato, and macaroni, bakery products such as cream-filled pastries, cream pies, and chocolate eclairs, sandwich fillings, and milk and dairy products. Foods that require considerable handling during preparation and that are kept at slightly elevated temperatures after preparation are frequently involved in staphylococcal food

**TABLE 2** Reported Vehicles of Transmission Causing Staphylococcal Foodborne Outbreaks in the United States, 1993–1997

Food type	Number of outbreaks					Total/food type
	1993	1994	1995	1996	1997	
Multiple vehicles	4	3	1	1	2	11
Ham	4		3			7
Beef	1			3		4
Turkey		2	2			4
Baked foods		1	1	1		3
Other salad (nonpoultry, fish, egg)	1				1	2
Mexican food		2				2
Chicken				1		1
Pork				1		1
Potato salad	1					1
Fruits/vegetables			1			1
Fish					1	1

Source: Ref. 14.

poisoning. The types of food incriminated in food-poisoning outbreaks in the United States are shown in Table 2 (14).

### E. Typical Food-Poisoning Outbreaks

Recently, 1364 children out of a total of 5824 who had eaten lunch served at 16 elementary schools in Texas became ill. The lunches were prepared in a central kitchen and transported to the schools by truck. Epidemiological studies revealed that 95% of the children who became ill had eaten chicken salad. The afternoon of the day preceding the lunch, frozen chickens were boiled for 3 hours. After cooking, the chickens were deboned, cooled to room temperature with a fan, ground into small pieces, placed into 12-inch-deep aluminum pans and stored overnight in a walk-in refrigerator. The following morning, the remaining ingredients of the salad were added and the mixture was blended with an electric mixer. The food was placed in thermal containers and transported to the various schools between 9:30 and 10:30 a.m. where it was kept at room temperature until it was served between 11:30 a.m. and noon. Bacteriological examination of the chicken salad revealed the presence of large numbers of *S. aureus*.

Contamination of the chicken probably occurred when it was deboned. The chicken was not cooled rapidly enough because it was stored in 12-inch-deep layers. Growth of the *Staphylococcus* probably occurred during the period when the food was kept in the warm classrooms. Prevention of this incident would have entailed screening the individuals who deboned the chicken for carriers of *Staphylococcus*, more rapid cooling of the chicken, and adequate refrigeration of the salad from the time of preparation until its consumption.

### F. Atypical Food-Poisoning Outbreaks (Thermally Processed Food)

In 1989, multiple staphylococcal foodborne diseases were associated with the consumption of canned mushrooms:

1. Starkville, Mississippi: On February 13, 22 people became ill with gastroenteritis several hours after eating at a university cafeteria. Symptoms included nausea, vomiting, diarrhea,

and abdominal cramps. Nine people were hospitalized. Canned mushrooms served with omelets and hamburgers were associated with illness. No deficiencies in food handling were found. Staphylococcal enterotoxin type A was identified in a sample of implicated mushrooms from the omelet bar and in unopened cans from the same lot.

2. Queens, New York: On February 28, 48 people became ill a median of 3 hours after eating lunch in a hospital employee cafeteria. One person was hospitalized. Canned mushrooms served at the salad bar were epidemiologically implicated. Two unopened cans of mushrooms from the same lot as the implicated can contained staphylococcal enterotoxin A.
3. McKeesport, Pennsylvania: On April 17, 12 people became ill with gastroenteritis a median of 2 hours after eating lunch or dinner at a restaurant. Two people were hospitalized. Canned mushrooms, consumed on pizza or with a parmigiana sauce, were associated with illness. No deficiencies were found in food preparation or storage. Staphylococcal enterotoxin was found in samples of remaining mushrooms and in unopened cans from the same lot.

## V. ISOLATION AND IDENTIFICATION

### A. Tests Used for Identification

Sometimes additional diagnostic features may be required to confirm *S. aureus* colonies because the inhibitors used may not completely prevent growth of other organisms, such as bacilli, micrococci, streptococci, and some yeasts. Microscopic morphology helps to differentiate bacilli, streptococci, and yeasts from staphylococci, which form irregular or grape-like clusters of cocci. Staphylococci may be further differentiated from streptococci on the basis of the catalase test, with the former being positive. Additional features are needed to differentiate staphylococci further from micrococci. Usually staphylococci are lysed by lysostaphin but not by lysozyme, and they can grow in the presence of 0.4 µg/mL of erythromycin. Micrococci are not lysed by lysostaphin, may be lysed by lysozyme, and will not grow in the presence of erythromycin. In a deep stab culture, micrococci will grow at the surface, whereas most staphylococci grow throughout the agar. Staphylococci will grow and produce acid from glucose and mannitol anaerobically, whereas micrococci do not. Staphylococcal cells contain teichoic acids in the cell wall and do not contain aliphatic hydrocarbons in the cell membrane, whereas the reverse is true with micrococci. Further, the G + C content (mole percentage) of staphylococci is 30–40% and 66–75% for micrococci. Testing for some of these features is difficult, time consuming, and expensive and usually not required for routine detection and enumeration procedures. Several commercially available miniaturized systems have been developed to speciate staphylococci. A number of commercially available nucleic acid and serological-based assays for the detection and confirmation of *S. aureus* are listed in [Table 3](#).

### B. Diagnostic Features

The principal diagnostic features of contemporary media include (a) the ability of *S. aureus* to grow in the presence of 7.5 or 10% NaCl, (b) the ability to grow in the presence of 0.01–0.05% lithium chloride, and 0.12–1.26% glycine, or 40 ng/mL polymyxin, (c) the ability of *S. aureus* to reduce potassium tellurite, producing black colonies, aerobically and anaerobically, (d) the colonial form, appearance, and size, (e) the pigmentations of colonies, (f) coagulase activity and acid production in a solid medium, (g) the ability of *S. aureus* to hydrolyze egg yolk, (h) the production of thermolysin, and (i) growth at 42–43°C on selective agar. Media used in the detection and enumeration of *S. aureus* may employ one or more of these diagnostic features.

#### 1. Media Selection

Enrichment isolation and direct plating are the most commonly used approaches for detecting and enumerating *S. aureus* in foods. Enrichment procedures may be selective or nonselective. Nonselective enrichment is useful for demonstrating the presence of injured cells whose growth is inhibited

**TABLE 3** Commercially Available Nucleic Acid and Serological-Based Assays for Detection and Confirmation of *S. aureus*

Trade name	Assay format	Company
AccuProbe	Probe <sup>a</sup>	Gen-Probe, San Diego, CA
GENE-TRAK	Probe	Gene-Trak, Hopkinton, MA
Staphylo-slide	Latex aggl.	Becton Dickinson, Cockeysville, MD
Aureus Test	Latex aggl.	Trisum, Taipei, Taiwan
<i>Staphylococcus aureus</i> Visual Immunoassay	ELISA	TECRA Diagnostics, Roseville, NSW, Australia
Staphaurex	Latex aggl.	Rhone Poulenc Glasgow, U.K.
Staphylase	Latex aggl.	Unipath—Oxoid Division Ogdensburg, NY
Slidex <sup>b</sup>	Latex/RBC aggl.	bioMérieux Marcy-l'Etoile, France
Mastastaph	Latex aggl.	Mast Laboratories
Staphytest	Latex aggl.	Unipath—Oxoid Division Ogdensburg, NY
Avistaph	Latex aggl.	Omega Diagnostics Ltd.
Staph Latex Test	Latex aggl.	Difco Laboratories Ltd. Detroit, MI
Bactident Staph	Latex	Merck

<sup>a</sup> Used for identification of pure culture isolates.

<sup>b</sup> A combined latex and hemagglutination test. RBC's sensitized with fibrinogen; latex particles sensitized with monoclonal antibodies to Protein A or immunogens on the surface of *S. aureus* strains.

Source: Updated, in part, from: Feng, P. 1998. FDA Bacteriological Analytical Manual, 8th Edition (Revision A/1998) AOAC International, Gaithersburg, MD.

by toxic components of selective enrichment media. Enumeration by enrichment isolation, or selective enrichment isolation, may be achieved by determining either an indicated number or the most probable number (MPN) of *S. aureus* present. Common MPN procedures use three or five tubes for each dilution.

For enumeration, samples may be applied to a variety of selective media in two main ways: surface spreading and pour plates used in direct plating procedures. Surface spreading is advantageous in that the form and appearance of surface colonies are somewhat more characteristic than the subsurface colonies encountered with pour plates. The principal advantage of pour plates is that greater sample volumes can be used.

Selective media employ various toxic chemicals, which are inhibitory for *S. aureus* to a varying extent as well as to competitive species. The adverse effect of selective agents is more acute in processed foods containing injured cells of *S. aureus*. A toxic medium may help prevent overgrowth of *S. aureus* by competing species.

## 2. Direct Plating Method

This method is suitable for the analysis of foods in which more than 100 *S. aureus* cells/g may be expected. The basic equipment, media, reagents, preparation of sample, and procedures for the

isolation and enumeration of staphylococci are described in the FDA Bacteriological Analytical Manual (17,18).

### 3. *Enrichment Isolation Method*

The most probable number method (19,20) is recommended for routine surveillance of products in which small numbers of *S. aureus* are expected and in foods expected to contain a large population of competing species.

## C. Differential Characteristics

*S. aureus* is differentiated from the other staphylococcal species by a combination of the following features: colonial morphology and pigmentation, production of coagulase, thermonuclease, acetone,  $\beta$ -galactosidase, phosphatase and  $\alpha$ -toxin (hemolysin), acid from mannitol, maltose, xylose, sucrose, and trehalose, novobiocin resistance, presence of ribitol teichoic acid, protein A, and clumping factor in the cell wall. The ultimate species identification may be established by DNA-DNA hybridization with reference strains. A nonisotopic DNA hybridization assay and a polymerase chain reaction (PCR) procedure have been used to successfully identify *S. aureus*.

### 1. *Coagulase*

The confirmation procedure most frequently used to establish the identity of *S. aureus* is the coagulase test (17). Coagulase is a substance that clots plasma of human and other animal species. Differences in suitability among plasmas from various animal species have been demonstrated. Human or rabbit plasma is most frequently used for coagulase testing and is available commercially. The use of pig plasma has sometimes been found advantageous, but it is not widely available. Coagulase production by *S. aureus* may be affected adversely by physical factors, such as culture storage condition, and pH of the medium. The extent to which the production of coagulase may be impaired by the toxic components of selective isolation media has not been demonstrated clearly.

Presence of clumping factor in cells is another unique feature of *S. aureus*. It can be used to distinguish tube-coagulase-positive *S. aureus* from other tube-coagulase-positive species such as *S. hyicus*. Clumping factor present in *S. aureus* cells binds to fibrinogen or fibrin present in human or rabbit plasma, resulting in agglutination of cells. This is referred to as slide coagulase, bound coagulation, or agglutination. Clumping of cells in this test is very rapid (<2 min), and the results are more clear-cut than 1+ or 2+ clotting in the tube coagulase test. Clumping factor can be detected using commercially available latex agglutination reagents. Anti-protein A immunoglobulin G (IgG) and fibrinogen are used to coat polystyrene latex beads to simultaneously bind protein A and coagulase, both of which are specific cell surface components of *S. aureus*. One latex kit was collaboratively studied by comparing a latex agglutination method to the coagulase test. The types of coagulase test reactions are shown in [Figure 2](#).

### 2. *Thermonuclease*

Thermonuclease (16) is also frequently used as a simple, rapid, and practical test for routine identification of *S. aureus*. Coagulase and heat-stable nuclease tests are very efficient for the identification of foodborne *S. aureus* strains isolated on Baird Parker agar. However, the use of the coagulase and/or thermonuclease test may result in erroneous species designation from a taxonomic standpoint. Two species, *S. intermedius* and *S. hyicus* subspecies *hyicus*, are both coagulase and thermonuclease positive. However, the latter species can easily be differentiated from *S. aureus* on the basis of the clumping factor test. Coagulase- and/or thermonuclease-negative staphylococci are being reported to be enterotoxigenic.

### 3. *Ancillary Tests*

Additional tests for the identification of *S. aureus* include catalase, anaerobic utilization of glucose and mannitol, and lysostaphin sensitivity (17).

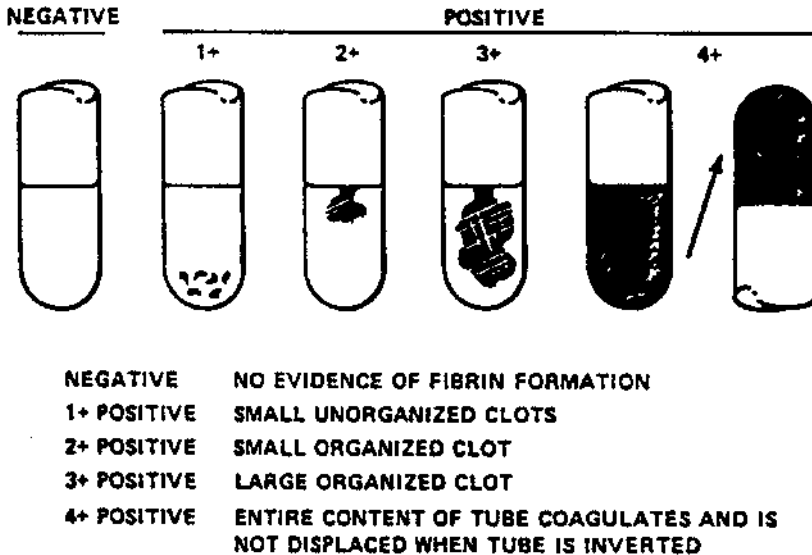


FIGURE 2 Types of coagulase reactions.

#### 4. Identification of Enterotoxins

The need to identify enterotoxins in foods encompasses basically two areas: (a) foods that have been incriminated in food-poisoning outbreaks and (b) foods that are suspected of containing enterotoxin. In the former case, the identification of enterotoxin in foods supports a staphylococcal food-poisoning outbreak or episode. In the latter case, the presence or absence of toxin will determine the marketability of the product. The latter cannot be overemphasized because it is difficult to prevent the presence of staphylococci in some types of foods. The isolation and determination of enterotoxigenicity of staphylococcal isolates in foods can serve as a signal of potential toxin formation if the food is time-temperature abused, which would allow for the proliferation of the organism. The two most common approaches involve biological or serological testing.

### D. Methods for Toxin Identification

#### 1. Biological Assays

Prior to the advent of serological identification of toxins, all toxins were identified by emetic responses in a monkey feeding assay (21). However, such assays had to be limited in quantity and possessed variable sensitivity, making interpretation sometimes difficult. In this method, the test sample is injected by catheter into the stomach of a young monkey. The animal is observed for 5 hours, and if vomiting occurs during the observation period, the sample is judged to contain toxin (1). While this animal assay is considered specific, a number of disadvantages exist (22). An alternative bioassay is through the intravenous injection of cats or kittens (23,24). However, other bacterial metabolites have been found to cause nonspecific emetic responses, although these nonspecific components can be neutralized or inactivated (22).

#### 2. Serological Methods

Most laboratory methods for the identification of the enterotoxins are based on the use of specific antibodies to each of the various toxin serotypes. While all of the enterotoxins are similar in composi-

tion and biological activity, they can be differentiated based on serology. Several serologically distinct types of enterotoxin have been characterized and designated as SEA, SEB, SEC (subtypes C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>), SED, and SEE. Approximately 5.0% of the staphylococcal foodborne outbreaks are caused by unidentified toxins (22). Their existence can be demonstrated by biological tests and are not serologically related to previously established toxin serotypes. More recently, a new enterotoxin, SEH, was identified, partially characterized (25), and a rapid method developed for its identification (26). A number of methods employing polyclonal or monoclonal antibodies have been used to identify and measure enterotoxins. Earlier developed methods utilized precipitation and agglutination approaches, while more recently developed methods employ tracer-labeled or tagging methods to increase assay sensitivity (9). Systems based on serological assays can, in general, be divided into a number of antigen-antibody reaction types: (a) gel immunodiffusion by direct precipitation or precipitation inhibition assays, (b) agglutination assays, and (c) tracer-labeled or tagged immunoassays. The most commonly used earlier developed methods have been described in a general way in reviews and in stepwise procedural detail by a number of investigators (1,2,9,22). Earlier developed classical methods, such as the microslide gel doubled diffusion test, have been described, in detail, in the FDA Bacteriological Analytical Manual and APHA Compendium of Methods for the Microbiological Examination of Foods (16). Some commercially available rapid methods for the identification of the enterotoxins are presented in Table 4 (27).

Several enzyme-linked immunosorbent assay (ELISA) methods have been proposed for the identification of staphylococcal enterotoxins and are currently the most commonly used methods. Of the competitive and noncompetitive ELISA-based methods, the noncompetitive, double antibody sandwich ELISA appears to be the most popular for routine toxin identification. With the noncompetitive ELISA, specific antibodies (polyclonal or monoclonal) are absorbed onto a solid support such as paper disks, polyvinyl or polystyrene in the form of polystyrene balls, plastic microtiter wells, plastic tubes, or other solid phase supports. The antibody absorbed onto the solid phase support is the capture antibody. The enterotoxin in samples are bound to the capture antibodies and, subsequently, detected by the addition of an enzyme-labeled secondary antibody, whose enzyme acts on a suitable substrate producing a color reaction. The intensity of the color reaction is proportional to the amount of toxin in the assay food extract or culture fluid. The advantages and limitations of some of the commercially available rapid methods have been reviewed in detail by Su and Wong (22). Of the rapid methods proposed for the identification staphylococcal enterotoxins in foods, only the microtiter plate polyvalent ELISA method has been studied exhaustively and approved by AOAC International (27). As a consequence, these methods should always be used with the recommended controls. A polyvalently configured automated enzyme-linked fluorescent immunoassay has also been developed and is commercially available. This multiparametric immunoanalyzer is highly sensitive because of the fluorescent tag. This is a labor-saving approach in that only the sample is added and the analyzer automatically completes the ELISA steps, providing printout data in approximately 80 minutes. Preliminary evaluation of this system shows that it is highly sensitive ( $\leq 0.5$ – $1.0$  ng/g) and, generally, specific. Other ELISA-based methods are dedicated to determining specific serotypes of staphylococcal enterotoxins. Some of these monovalently configured assays are also listed in Table 4. One such method, TECRA™, utilizes a single-specific serotype antibody as the capture antibody with polyvalent antibodies conjugated to the enzyme instead of each secondary antibody conjugated separately to the enzyme.

### 3. Toxin Production by *Staphylococci*

Determining the enterotoxigenicity of staphylococci by examining staphylococcal isolates for toxin production is helpful for identifying enterotoxin in foods and is desirable for examining strains isolated from various sources. A number of methods for the laboratory production of enterotoxins have been developed. Of the methods developed elsewhere for the laboratory production of enterotoxin, only the semisolid agar method is an AOAC International approved method (19). It is simple to perform and requires a minimum of items found in the routine analytical laboratory. To determine the presence of enterotoxin in culture fluid, any of the classical as well as rapid methods can be

**TABLE 4** Commercially Available Agglutination and ELISA-Based Methods for the Identification of Staphylococcal Enterotoxins (SET)

Kit name	Assay format	Company (distributor) <sup>a</sup>
TECRA-SET <sup>b</sup>	ELISA, polyvalent <sup>c</sup> (A-E) <sup>d</sup>	TECRA Diagnostics Roseville, NSW, Australia (International BioProducts Redmond, WA)
TECRA-SET	ELISA, monovalent <sup>e</sup> (A-E)	TECRA Diagnostics Roseville, NSW, Australia (International BioProducts Redmond, WA)
SET-RPLA <sup>f</sup>	Latex Agglutination Monovalent (A-D)	Unipath—Oxoid Division Ogdensburg, NY
VIDAS-SET	ELFA, <sup>g</sup> polyvalent (A-E)	bioMérieux-Vitek, Inc. Hazelwood, MO
Transia Tube-SET	ELISA, polyvalent (A-E)	TRANSIA Diffchamb, SA. Lyon, France (Gene-Trak Systems Hopkinton, MA)
Transia Plate-SET	ELISA, polyvalent (A-E)	TRANSIA Diffchamb, SA. Lyon, France (Gene-Trak Systems Hopkinton, MA)
SET-EIA	ELISA, Polystyrene ball, monovalent (A-D)	Diagnostische Laboratorien Bern, Switzerland (Toxin Technology Sarasota, FL)
Microtiter Plate-SET	ELISA, polyvalent (A-E)	W. Brommeli Bern, Switzerland
Ridascreen SET	ELISA, monovalent (A-E)	R. Biopharm GmbH Darmstadt, Germany (BioTek Instruments, Inc. Winooski, VT)

<sup>a</sup> USA distributor.

<sup>b</sup> AOAC International status—adopted “Official First” action.

<sup>c</sup> Does not distinguish between toxin serotypes.

<sup>d</sup> Identifiable toxin serotypes.

<sup>e</sup> Distinguishes between toxin serotypes.

<sup>f</sup> Reversed passive latex agglutination (RPLA).

<sup>g</sup> Enzyme-linked fluorescent immunoassay (ELFA).

*Source:* Updated, in part, from: Feng, P. 1998. FDA Bacteriological Analytical Manual, 8th Edition (Revision A/1998) AOAC International, Gaithersburg, MD.

utilized to determine the enterotoxigenicity of suspect staphylococcal isolates from foods or other sources. Although not approved by AOAC International, commercial kits generally recommend species broth media that are comparable in enterotoxin production to semisolid agar. The potential enterotoxigenicity of staphylococcal strains may also be determined by DNA hybridization techniques in cases where the nucleotide sequence has been determined. As a consequence, oligonucleotides can be synthesized and used as DNA probes to demonstrate that an enterotoxin gene exists,



in an isolate, although laboratory demonstration of toxin production is more direct and is a better signal for the possible presence of toxin in a suspect food. Determining the enterotoxigenicity of staphylococcal isolates is particularly important in determining prevalence of the various serotypes of toxin producers in foods and from other sources.

## **E. Toxin Identification in Foods**

The major problem of identifying enterotoxin in foods is the small amount that may be present in foods incriminated in foodborne outbreaks. The amount of enterotoxin that may be present in foods involved in food-poisoning outbreaks may be as little as 50 ng/g of food, although the normal amount of toxin in foods involved in food-poisoning outbreaks is easy to detect, since the amounts are frequently larger. As toxin can be readily identified in sources containing bacterial counts that are or were at some time  $\geq 10^6$  enterotoxigenic staphylococci organisms per gram of food, food samples containing such high counts are not acceptable. Marketable foods should contain no enterotoxin as demonstrated by rapid as well as classical methods.

Most outbreaks of staphylococcal intoxication are caused by foods that do not receive a high thermal treatment; staphylococci survive in sufficient numbers in these foods to form the toxin before the food is consumed. However, some heated foods have also been incriminated in illnesses that display the typical symptoms of intoxication. Foods that receive enough heat to render the bacterium nonviable and yet cause food poisoning have included boiled goat's milk, spray-dried milk, cooked sausage, and canned lobster bisque.

In two instances, the U.S. Food and Drug Administration (FDA) has taken regulatory action on staphylococcal enterotoxin-contaminated thermally processed foods. In 1982 thermally processed infant formula was incriminated in foodborne illness, and in 1989 mushrooms that received a higher than normal thermal treatment as a means of product preservation were implicated in staphylococcal foodborne illnesses (29).

In the food-poisoning episodes involving canned mushrooms, analysis of the product initially proved serologically negative, although there was a retention in toxicological activity in individuals who consumed the product, as indicated by symptoms that were consistent with staphylococcal intoxication. To determine the disparity between serological inactivity and human intoxication, studies were conducted to better understand the kinetics of thermal stress on the enterotoxin protein. In these experimental studies, it was determined that the enterotoxin underwent conformational changes, thus preventing antibody recognition because of toxin denaturation. Methods were developed to renature (reactivate) the heat-altered toxin utilizing urea or urea combined with zinc acetate (29–31). This renatured toxin could then be identified serologically. The utilization of urea or urea–zinc acetate to restore serological activity to heat-denatured enterotoxin has been confirmed by other investigators (32–34). The only practical way to eliminate future staphylococcal food poisoning outbreaks in thermally processed foods is to prevent the contamination and proliferation of enterotoxigenic staphylococci in foods before processing.

## **VI. TOXICOLOGICAL ACTIVITY OF ENTEROTOXINS**

The signs and symptoms of staphylococcal intoxication occur when foods containing enterotoxin are ingested in reasonably small amounts. However, death can occur if large amounts are ingested. The illness is particularly acute (1–7 hr) after the ingestion of toxin-contaminated foods (35), with recovery generally occurring in 1–2 days. The early signs are nausea and possibly abdominal cramping, with resulting primary observable symptoms of vomiting and diarrhea. Secondary symptoms may include retching, dehydration, sweating, weakness, and salivation. In severe cases, the victims may also exhibit headache, sweating, marked prostration, muscular cramping, and a subsequent drop in blood pressure. Fever may occur, or the body temperature may be subnormal. Blood and mucus may be observed in stools and vomit (36).

The complete effect of the staphylococcal enterotoxins on human beings has not been elucidated, since opportunities to study this type of illness in humans are very limited. The amount of toxin has not been precisely determined, although is estimated to be between 100 ng and 35 µg based on challenge studies and the evaluation of foods implicated in food poisoning outbreaks (36–38).

The true nature of the gastrointestinal syndrome is not completely understood. The vomiting reaction is initiated in the intestinal tract, although the specific binding of the enterotoxin to a site in the gastrointestinal system has not been demonstrated (1). It has been shown, however, that these toxins pass through the intestinal tract rapidly and are removed from the circulation by the kidneys in a reasonably short period of time (1). The enterotoxins could be considered neurotoxins based on their mode of action on the subcortical-vomiting center of the brain (39). This effect has been confirmed in monkeys rendered unresponsive to the enterotoxin emetic effects by detachment of the nerves to the brain proximal to the vomiting center (40). Other investigators have further demonstrated that orally administered enterotoxin (Type B) initiated a local neutral reflex in the gut of monkeys triggering the vomiting center, leading to vomiting and hypermobility (41). Additionally, there was no evidence of significant absorption of toxin or stimulation by a central-acting humoral mechanism. The activity of cysteinyl leukotrienes, patent medications of inflammation noted following exposure to these toxins, may be responsible for the enteritis and pseudomembranous enterocolitis in the intestinal tract of animals and humans, respectively (1).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE/TOXICITY

### A. Regulation of Virulence Determinants in *Staphylococcus aureus*

Successful colonization and persistence by *S. aureus* within an infected host requires coordinated, temporal expression of different staphylococcal virulence determinants. For instance, early in the infection, expression of the cell surface-associated “colonization factors,” which include protein A, fibronectin-binding protein, and collagen adhesin, are upregulated, while expression of postexponential phase-secreted exotoxins, which includes the staphylococcal enterotoxins, remains downregulated until the later stages of the infection. Presently, two separate, pleiotropically acting global regulons, the accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*), have been identified, which facilitate this required temporal expression (42–44).

The best characterized of these regulons is the *agr* locus. This locus consists of five genes contained within two divergent operons that are transcribed from three different promoters, P1, P2, and P3, located within the locus. The first of the operons consists of four open reading frames, *agrB*, *agrD*, *agrC*, and *agrA*, that encode a two-component signal-transducing, quorum-sensing system that positively regulates postexponential phase exotoxins while simultaneously negatively regulating the early phase, membrane-associated proteins (45). The P1 promoter is a weakly constitutive promoter that expresses AgrA. The P2 promoter transcribes the entire *agrBDCA* gene complex during the late exponential or early stationary phase of bacterial colonization to produce a transcript designated RNAII. From this transcript, *agrD* encodes a protein that is processed to an octapeptide pheromone and transported extracellularly. Currently, evidence is emerging that suggests that AgrB, whose predicted amino acid sequence indicates is a transmembrane protein, mediates the processing and secretion of AgrD (46,47). AgrA and AgrC are proteins that share many of the characteristics associated with other bacterial proteins of the two-component signal transduction systems. In *S. aureus*, AgrC is localized to the cell membrane and serves as the sensory component of the transducing system that is activated in a density-dependent manner by the auto-inducing AgrD octapeptide. AgrC then acts as the phosphate donor for the regulatory component of the system, AgrA. It is not currently understood how AgrA, which lacks any of the known intrinsic DNA-binding properties associated with transcriptional activators, influences transcriptional regulation of the *agr* locus, but it may interact with other proteins (i.e., SarA) to upregulate transcription at the P2 and P3 promoters (48). Importantly, studies have confirmed that each of these proteins is essential for this system to function, as inactivation of any one of them results in lower levels of transcription from the P3 promoter (49).

Transcription from the P3 promoter results in a 514-nucleotide transcript, designated RNAIII, that contains the open reading frame that encodes the 26-amino-acid residue staphylococcal hemolysin (*hld*) as well as a large amount of untranslated sequence. Studies performed with this operon cloned below an inducible promoter have demonstrated that an *agr*<sup>-</sup> mutant is restored to the *agr*<sup>+</sup> phenotype, indicating that RNAIII is the actual effector that downregulates the expression surface proteins while upregulating expression of the extracellular staphylococcal virulence determinants (50). Like AgrA, presently, it is not clearly understood how the regulation by RNAIII is mediated, but one speculation is that a RNA/protein complex mediates regulation at the transcriptional level. In contrast, however, one study has demonstrated that the RNAIII molecule binds to the 5' untranslated terminus of the mRNA transcript encoding the *S. aureus*  $\alpha$ -toxin (51), suggesting that RNAIII not only mediates regulation at the level of transcription, as previously postulated, but also at the level of translation as well.

In addition to *agr*, the *sar* genetic locus is also involved in both an *agr*-dependent and *agr*-independent regulation of the staphylococcal virulence determinants. This locus encodes for three overlapping transcripts that share a common 3' terminus but whose transcription is initiated from one of three distinct promoters, designated P1 (*sarA*), P3 (*sarC*), and P2 (*sarB*), that exists within the locus. Transcription from P1, the predominant promoter in the *sar* locus, produces the smallest transcript and is the main source of SarA, but because the transcripts are overlapping in nature, transcription from any of the promoters results in SarA expression. Immediately upstream of the P1 promoter is P3, which contains a small ORF designated ORF3, followed by P2, which contains another small ORF designated ORF4. Studies by Cheung and Projan using *sar* mutants revealed that *agr*-dependent regulation is mediated by SarA at the level of RNAIII transcription. Like *agr* mutations, insertional inactivation of *sarA* by transposon mutagenesis eliminates both RNAII and RNAIII induction, reducing staphylococcal virulence in animal models (52,53). Gel shift analysis has demonstrated that SarA interacts directly with the *agr* locus by binding the three high-affinity *cis*-acting sites at the P2 and P3 promoters of that operon (47). Interestingly, studies performed by Cheung and Projan (44) determined that the level of RNAIII expression in the isogenic *sar*<sup>-</sup> mutant is not restored to wild-type levels by *trans* complementation with SarA only. Complementation using DNA containing both the *sarB* and *sarC* (transcribed from P2 and P3, respectively) transcriptional units was required to fully restore RNAIII transcription in the *sar* mutant. This finding indicates that one or both of the two small open reading frames located upstream of the *sarA* P1 promoter are involved in the efficient transcriptional activation of the RNAIII operon by SarA. Unlike the *agr*-dependent pathway, which exerts its influence through regulation of RNAIII, the *agr*-independent regulation of virulence determinants by *sar* is mediated by direct binding of the SarA protein to the target gene (i.e., *spa* and *hla*) promoters to control gene expression (54).

The effect of environmental influences on the regulation of various virulence determinants in *S. aureus* has received considerable attention in the recent past. This work has produced evidence that oxygen, pH, osmolarity, glucose concentration, and temperature largely affect expression of virulence determinants. Unfortunately, little has been determined about the mechanism by which these influences are exerted. Just recently, however, studies by Yarwood et al. have implicated a genetic locus, referred to as the staphylococcal respiratory regulator (*srrAB*), that is homologous to the *Bacillus subtilis* *resDE* two-component system that has been implicated in the global regulation of aerobic and anaerobic metabolism in that organism (55). Moreover, this study has shown that the SrrA-SrrB two component system does influence virulence factor expression in response to environmental oxygen levels. Undoubtedly, as work continues on this newly described system, more insight into the mechanisms by which environmental signals regulate virulence factor expression will be attained.

## B. Staphylococcal Enterotoxin Genetics

Although the enterotoxins share many structural similarities as well as biological activities, the genes encoding each of these proteins vary significantly as to which genetic elements they are associated with and the manner in which they are regulated. For instance, *sea* and *see* are associated with

sometimes defective, lysogenic bacteriophage (56), while *sed* and *sej* are associated with an autonomously replicating plasmid (57). The genes encoding SEB and the SEC variants are generally accepted to be chromosomally located, but it is believed that they are associated with a discrete genetic element that remains to be fully characterized (58). Recently, *seg* and *sei* have been determined to be contained within an operon, designated “enterotoxin gene cluster” (*egc*), which contains an additional three open reading frames that encode the newly described enterotoxins L, K, and M (*sel*, *sek*, and *sem*, respectively) (59). Finding this polycistronic operon is a clear departure from the previous notion that all enterotoxins are expressed as a monocistronic unit from a promoter located immediately upstream from the open reading frame. Presently, the genetic location of *seh* has not been described.

Like the genetic elements on which the enterotoxin genes are found, factors that influence protein expression also vary significantly. In the case of SEB, SEC, and SED, regulation of protein expression is mediated via the *agr* locus described above. Similar to other *agr*-regulated extracellular exotoxins, the levels of SEB, SEC, and SED are low in the early log phase and greatest at the late log to stationary phase. Furthermore, consistent with *agr*-deficient strains (60,61), steady-state mRNA of each of these genes is greatly reduced in *agr*-deficient strains. Unlike *sed*, *sej*, which lies adjacent to and transcribed divergently from *sed*, is not mediated by the *agr* locus (57). Similarly, *sea* expression is not regulated via the *agr* mechanism. In contrast to SEB, SEA levels do not appear to be adversely affected in the *agr* mutant strain (62). Instead, like SEH (63), SEA expression may be affected by physiological factors, such as pH, aerobic conditions, and glucose concentration, suggesting the involvement of less characterized regulatory mediators, like the *srrA-srrB* system described above. Presently, regulation of the other more recently described toxins remains to be elucidated.

## VIII. PREVENTION/CONTROL MEASURES

Staphylococci are ubiquitous and are impossible to eliminate from the environment. The total destruction or significant reduction in the bacterial load in foods during growth, harvesting, processing, packaging, and storage prior to consumption has always been a general goal. However, the wide array of parameters for proliferation of foodborne pathogens is staggering. Some of the same methods for the control of organisms in the food supply are used separately or in combination in the preservation of foods. Staphylococci may be totally destroyed or injured when subjected to lethal or sublethal doses, respectively, of heat, cold, drying, irradiation, or chemicals. While total destruction of these organisms might be ideal, sublethal injury may occur, thus providing the organism an opportunity to recover and proliferate (9), if conditions are conducive. The phenomenon of sublethal injury and stressed cell rejuvenation has been examined by a number of investigators. Conditions that have been studied include heating, freezing and freeze-drying, irradiation, reduced water activity, and exposure to various chemicals such as acids and salts and have been reviewed previously (9). The effects of various nutritional and environmental factors on the growth of *S. aureus* with major emphasis on enterotoxin synthesis in foods and model systems have been reviewed previously (64). Occurrences of food-poisoning outbreaks have demonstrated that growth of staphylococcal species and subsequent enterotoxin synthesis are determined by a variety of nutritional and environmental factors, including temperature, pH, water activity, salt and sugar concentrations, bacterial load, bacterial competition, and atmospheric conditions.

To better understand the behavior of staphylococcal growth and toxin production in foods, a greater emphasis must be placed on the multiplicity of interactive factors involved in the proliferation of staphylococci in food matrices. Such characterization is necessary to make predictive microbiology a reality. The most frequent source of contamination of food is the food handler involved in preparing food for serving. Whenever a food is exposed to human handling, there is always a possibility that it will be contaminated. Not all strains of *S. aureus* and other species are enterotoxigenic, although 50–70% may be (12). To prevent food-poisoning outbreaks, it is necessary to keep foods either refrigerated (<10°C) or hot (>45°C) to prevent proliferation of the organism to such numbers

**TABLE 5** Causes of Foodborne Staphylococcal Outbreaks in the U.S.

Causes	Number of outbreaks
Improper holding temperatures	25
Poor personal hygiene	12
Inadequate cooking	3
Contaminated equipment	6
Food from unsafe source	4
Other	4

Source: Ref. 14.

( $10^5$  cells/g) necessary for detectable toxin formation. Additionally, foods should be refrigerated in shallow layers or small portions to facilitate quick cooling. While the bacterial inoculum size is important, time-temperature abuse and the nutritional composition of the contaminated food is of utmost importance. The leading factors contributing to staphylococcal food-poisoning outbreaks in the United States from 1993 to 1997 are listed in Table 5.

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# 5

## *Clostridium botulinum*

**Giovanna Franciosa and Paolo Aureli**

*Istituto Superiore della Sanità, Rome, Italy*

**Robert Schechter**

*California Department of Health Services, Berkeley, California, U.S.A.*

### **I. BACKGROUND**

In 1897 the Belgian physician Emile van Ermengem provided evidence of the etiology of foodborne botulism (1), a serious illness whose clinical signs and transmission through food, mainly poisonous sausage, or *botulus* in Latin, had been known for some time. He isolated similar anaerobic, spore-forming, rod-shaped microorganisms both from the clinical specimens of patients affected with botulism and from the improperly cured ham that they all had previously consumed. These microorganisms were capable of producing a heat- and alkali-labile substance that was toxic to various vertebrates upon feeding or injection. Then he concluded that the outbreak of alimentary intoxication under investigation was related to the “ham bacillus” and named it *Bacillus botulinus* (1). When the phylogenetic *Clostridium/Bacillus* subdivision was subsequently split on the basis of the metabolism (anaerobic or aerobic, respectively) of the different organisms, *Bacillus botulinus* was newly designated as *Clostridium botulinum* (2).

Since then, 100 years of extensive research into the microorganism, its toxin, and the clinical syndrome they cause to humans and animals have followed. The extreme potency of botulinum toxin has inspired the interest of eminent scientists and also caused a deep emotional impact on the public opinion: the toxin and organism have been suspected of being used as biological weapons (3). Recently, however, dramatic changes in the attitude towards this microorganism and its toxin occurred following the discovery of the therapeutic use of the botulinum toxin in very small doses against many involuntary muscle disorders (4). This is supported by the elucidation of the molecular mechanism of the toxin (5). These findings gave rise to new research and provided an impressive demonstration of how science can transform a poisonous substance into a curative medicine.

Despite a century of progress, hundreds of cases of foodborne botulism are still reported each year, and many more are presumed to occur where surveillance is poor. Most cases are due to mishandled home-canned foods contaminated with preformed botulinum toxin (6,7). Although commercial foods have had an excellent safety record for botulism, reflecting the general food processors’ awareness, the involvement of manufactured products—generally “new-generation” foods—and foods prepared in food-service establishments in some recent cases may have contributed to the reemergence of this microorganism as a foodborne pathogen (8–10). As a consequence, a potentially hazardous food is still defined as “one that can support the growth and toxin production of *C. botulinum*” (11), and food-safety measures primarily address the botulinum concern.



The recent isolation of botulinum toxin-producing clostridia other than *C. botulinum* from suspect cases of foodborne botulism (12,13) represents a new emergence: information on the requirements for growth and toxin production of these pathogens in foods and on inhibitory conditions to be applied is still needed for effective food safety assurance. Other bacteria capable of producing botulinum toxin may await discovery.

Finally, in addition to the “classic” food intoxication, which remains the most frequent form of botulism worldwide, other forms of the disease have more recently been elucidated, resulting from the *in vivo* elaboration of botulinum toxin by toxigenic clostridial spores, and hence regarded as toxin-mediated infections (14). This makes *C. botulinum* one of the most versatile bacterial pathogens and correlates to new public health concerns.

This chapter will focus on *C. botulinum*, its toxin, and botulism. Other clostridia producing botulinum toxin will be described as well. Emphasis will be given to the foodborne hazard from *C. botulinum* and to measures for controlling it in foods.

## II. CHARACTERISTICS

*C. botulinum* strains share morphological, metabolic, and genetic features with other members of the genus *Clostridium* (15).

### A. Morphology

*C. botulinum* are rod-shaped cells (2–22  $\mu\text{m}$  long and 0.5–2  $\mu\text{m}$  wide), motile by peritrichous flagella and capable of forming subterminal endospores, which confer on the rods the peculiar shape of a *closter*—Greek for spindle (Fig. 1). The location of spores within the cell and the level of spore production vary by strain. Spores, the most resistant bacterial forms existing in nature, allow the survival of the microorganism under restrictive environmental conditions, such as chemical-physical shocks and/or decreased availability of nutrients. Gram stain results are positive due to the thickness of the cell wall, mainly constituted by peptidoglycan of the meso-diaminopimelic acid direct-linked type, and to the lack of the outer envelope proper of gram-negative microorganisms.

### B. Metabolism

Lack of catalase and other enzymes, such as cytochrome oxidase and peroxidase, render all clostridia unable to use oxygen for ATP production, and they are therefore anaerobic. Some strains are more sensitive to oxygen, but most do not require strict anaerobic conditions for toxin production. Clostridia cannot use sulfate as a terminal electron acceptor in the oxidation of organic matter.

### C. DNA Base Composition

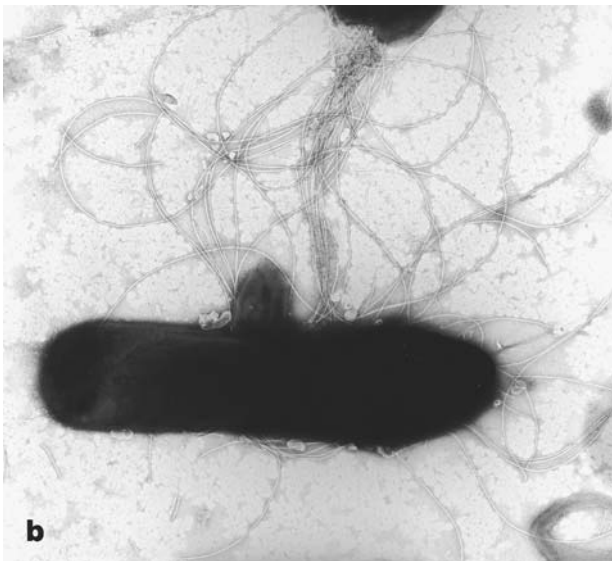
The genomes of *C. botulinum* strains contain a G + C ratio of 26–28%, which falls within the wider range (22–55%) of the genus.

Conventionally, the members of the species *C. botulinum* are distinguished from the other *Clostridium* spp. by their ability to synthesize powerful protein exotoxins (botulinum neurotoxins), responsible for the flaccid paralysis of botulism in humans and animals (16). Seven antigenic variants of the toxin have so far been identified and designated as types A to G in the chronological order of recognition (Table 1). They are similar in structure and pharmacological action (see Sec. VI) but differ from one another in their antigenicity, i.e., upon administration of the detoxified protein to humans or animals, immune response with production of antibodies against the native toxin occurs. The antisera specifically bind and neutralize the related toxins, with rare occurrences of cross-reactions, and are of important therapeutic and diagnostic value (17).

Most strains of *C. botulinum* produce a single botulinum neurotoxin and are classified as types on the basis of the toxin produced (16). Exceptions include *C. botulinum* types C and D, producing



(a)



(b)

**FIGURE 1** Photomicrographs of negative stained suspensions of *Clostridium botulinum* type A (ISS collection): (a) vegetative form (33,000 $\times$ ) and (b) sporulating cell (22,000 $\times$ ). (Photography by Lucilla Baldassarri, Laboratory of Ultrastructure, ISS.)

**TABLE 1** Milestones in the Discovery of Botulinum Neurotoxin Types

	Source of isolation	Form of botulism	Year of report	Location
<i>Clostridium botulinum</i> type				
A <sup>a</sup>	White beans	Foodborne	1904	Germany
B <sup>a</sup>	Salted ham	Foodborne	1897	Germany
C-alpha <sup>b</sup>	Fly larvae	Animal	1922	United States
C-beta <sup>b</sup>	Cattle	Animal		Australia
D	Cow carcass	Animal	1928	South Africa
E	Sturgeons	Foodborne	1936	Ukraine
	Herrings	Foodborne		Germany
F	Liver paste	Foodborne	1958	Denmark
G	Soil	—	1970	Argentina
<i>Clostridium botulinum</i> subtype				
Af	Soil	—	1970	Argentina
Ba	Stool	Infant	1981	United States
Ab	Pâté	Foodborne	1983	France
Bf	Stool	Infant	1987	United States
<i>C. baratii</i> type F	Stool	Infant	1985	United States
<i>C. butyricum</i> type E	Stool	Infant	1986	Italy
<i>Clostridium botulinum</i> A(B) <sup>c</sup>	Various	Various	1994	United States

<sup>a</sup> Presumptive designation was given to these strains after they were lost, on the basis of the characteristics described by the original authors (2).

<sup>b</sup> *C. botulinum* type C-alpha produces C neurotoxin plus C2 and C3 toxins; *C. botulinum* type C-beta does not form C neurotoxin, but just C2 (138).

<sup>c</sup> These strains only produce detectable amounts of type A botulinum toxin, even if they carry genes for both type A and type B neurotoxins (126).

Source: Refs. 2, 19.

minor amounts of type D and C neurotoxins, respectively, in addition to the dominant types (18), and rare strains that form a mixture of two BoNTs, one of which occurs in higher quantity. These latter strains are designated as subtypes, with a capital letter indicating the major toxin produced and a small letter for the minor toxin (Table 1) (19).

The selective advantage that these organisms might derive from the production of botulinum neurotoxins could be related either to the provision of an anaerobic substrate from the necrosis of the host tissues affected—suitable for the proliferation of the spores—or to the success in the interaction with competitive microbes and/or host immune system. In the absence of any evidence supporting the above speculations, other nondeterministic explanations are possible: toxins could either be formed as metabolic products or play some role in bacterial physiology (20,21).

The neurotoxin accumulates within the bacterial cell during multiplication, after the outgrowth of spores has started, and at the end of the growth phase it is externally released as a consequence of cellular lysis. The autolysis and release mechanisms are inefficient in some strains of *C. botulinum* that do not digest proteins (nonproteolytic strains). Moreover, toxins produced by these strains need cleavage by exogenous enzymes, such as trypsin, to be fully activated (22). Organisms with similar characteristics have been separated into four groups—I to IV—regardless of the toxin type produced (15). Other *Clostridium* species that show features similar to those of *C. botulinum*, except for their inability to produce botulinum neurotoxins, have been included in these cultural groups as nontoxicogenic equivalents (Fig. 2) (15).

PROTEOLYSIS	GELATINASE	LIPASE	LECITHINASE	FERMENTATION OF					METABOLIC <sup>a</sup> END PRODUCTS	TOXICITY	TOXIN TYPES	SPECIES	GROUP
				GLUCOSE	LACTOSE	SUCROSE	MANNITOL	MANNOSE					
+	+	+	-	+	-	-	-	-	A, iB, B, IV, PP	+	A, B, F	<i>C. botulinum</i> A, B, F	I
												<i>C. sporogenes</i>	
									A, iB, B, IV, PA	+	G	<i>C. botulinum</i> G	IV <sup>b</sup>
												<i>C. hastiforme</i> <i>C. subterminale</i>	
-	+	+	-	+	-	+	-	+	A, B	+	B, E, F	<i>C. botulinum</i> B, E, F	II
												(undetermined) <sup>c</sup>	
			+	+	-	-	-	+	A, P, B	+	C, D	<i>C. botulinum</i> C, D	III
												<i>C. novyi</i>	
	-	-	-	+	+	+	-	+	A, B	+	E	<i>C. butyricum</i> E	V
												<i>C. butyricum</i>	
			+	+	+	+	-	+	A, B	+	F	<i>C. baratii</i> F	VI
												<i>C. baratii</i>	

**FIGURE 2** Phenotypic key characteristics of clostridial cultural groups.

More recently, two additional groups of organisms (herein indicated as groups V and VI) have been included. The members of these groups produce botulinum toxins type E and F, respectively, and have been associated with human botulism (12,13,23–28). However, they differ from the other four cultural groups to such an extent that their initial isolation and identification was difficult. Because of their resemblance to clostridial characteristics other than botulinum, they have so far maintained their early designation of *C. butyricum* type E and *C. baratii* type F (23,29).

Whether it is more appropriate to preserve the name *C. botulinum* as “nomen periculosum” for such a heterogeneous group of bacteria on the strict basis of botulinum neurotoxin production or to reorganize the taxonomy by designating each cultural group—including the nontoxic derivatives—as a single species is still a matter of scientific discussion (30).

Some of the distinctive features of the cultural groups are listed in Figure 2 and Table 2. The biochemical markers used to distinguish between organisms of different groups include (31,32):

1. Proteolysis, evident when the strains are cultured in meat- or milk-based media. Proteolytic strains (groups I and IV) preferentially hydrolyze proteins and ferment amino acids in their metabolic pathways, while nonproteolytic strains (groups II, III, V, and VI) do not digest complex proteins.
2. Saccharolysis, observed by culturing the strains in sugar fermentation broths. Nonproteolytic strains acidify most carbohydrates for their energy production. Group I organisms

**TABLE 2** Some Physiological, Environmental, and Genetic Characteristics of Cultural Groups of Clostridia and Forms of Botulism Most Frequently Associated

	Group I	Group II	Group III	Group IV ( <i>C. argentinense</i> )	Group V ( <i>C. butyricum</i> )	Group VI ( <i>C. baratii</i> )
Growth temperature						
Minimum	10°C	3.3°C	15°C	20°C	10°C	20°C
Optimum	35–40°C	18–25°C	40°C	37°C	30–37°C	30–45°C
D <sub>100</sub> of spores <sup>a</sup>	25 min	<0.1 min				
D <sub>76.6</sub> of spores					2.3–2.5 min	
Inhibitory pH	4.6	5.0			5.0	
Minimum a <sub>w</sub>	0.935	0.97				
Inhibitory NaCl concentration	10%	5%				
Habitat	Soil	Wetland	Wetland	Soil		
Neurotoxin produced	A, B, F	B, E, F	C, D	G	E	F
Other toxins			C2, C3			
Neurotoxin gene location	Chromosome	Chromosome	Phage	Plasmid	Uncertain <sup>b</sup>	
Associated form of botulism	Foodborne; intestinal toxemia; wound	Foodborne; intestinal toxemia; wound	Animal	Uncertain <sup>c</sup>	Intestinal toxemia; foodborne	Intestinal toxemia

<sup>a</sup> D-value: time required to inactivate 90% of the population at 100 and 76.6°C.

<sup>b</sup> Both plasmid and chromosomal locations have been reported for neurotoxin E gene in the genome of these strains (152,153).

<sup>c</sup> Strains of *C. botulinum* type G have been isolated from SIDS (sudden infant death syndrome) cases, but their relation with illness has not been demonstrated (41).

only ferment glucose and a few other sugars, and those of group IV are exceptionally asaccharolytic.

3. Metabolic end products formed as a consequence of the energy metabolism. Complex mixtures of saturated and unsaturated organic acids that can be detected by gas-liquid chromatography are produced in culture fluids by proteolytic strains. The fermentation of carbohydrates by nonproteolytic strains primarily yields short-chain organic compounds, such as acetic and butyric acids.
4. Lipase and lecithinase reactions that can be seen on egg yolk agar plates, the former as a madreperlaceous layer surrounding the colonies and the latter as an opaque whitish halo of precipitation inside the agar. Until recently, the lipase reaction was considered a key characteristic for isolation of *C. botulinum* colonies. However, following recognition of groups IV–VI [*C. argentinense* (30), *C. butyricum* type E, *C. baratii* type F] that do not produce lipase, this reaction can no longer be considered as distinctive of botulinum toxigenic organisms. The lecithinase reaction is generally negative; only *C. baratii* and some group III organisms produce lecithinase.
5. Hemolysis of erythrocytes in blood agar plates is always negative for *C. butyricum* and *C. baratii* strains and strongly positive for group III *C. botulinum* strains, which produce large amounts of the hemolysin, botulinolysin, that has been shown to have a lethal effect on mice (33).

Other differential characteristics among groups have been reported for the patterns of synthesized bacteriocins (“boticins”) (16), antimicrobial susceptibilities (34), cell proteins (35), and cellular fatty acids (36).

All known *C. botulinum* strains producing botulinum toxin A belong to group I. All *C. botulinum* type E strains fall into group II, except for the lipase-negative strains (*C. butyricum* type E, group V). All strains of *C. botulinum* type G are classified as group IV (Fig. 2). Types B and F strains may show either proteolytic or nonproteolytic activity and are therefore included in groups I or II; the lipase-negative strains producing type F toxin are classified as *C. baratii* type F (group VI). Strains of group III simultaneously produce type C and D neurotoxins: botulinum neurotoxin C is the dominant toxin produced by type C strains, while type D strains produce toxin type D in major amounts. In addition, toxigenic organisms of group III form other nonbotulinum toxins (C2 and C3 toxins).

These groups also differ from one another in their physiological characteristics (Table 2). In general, the proteolytic strains of *C. botulinum* show a higher resistance than nonproteolytic ones. They are mesophiles and do not grow at temperatures lower than 10°C. As a consequence they are more frequently found in regions with temperate climates (37). Their spores are more resistant to heat and can also survive in more severe environmental conditions such as lower humidity, higher salt, and acidity.

Nonproteolytic strains of group II grow well at lower temperatures and are more widely diffused in cold regions and aquatic environments (37). On the other hand, *C. botulinum* group II spores show a higher sensitivity to heat and are inhibited by more restrictive values of pH,  $a_w$  (water activity), and salt concentration.

Organisms of group III are hard to isolate, since they need strict anaerobiosis, sporulate very poorly, and easily lose their phage-mediated toxigenicity (see Sec. VII) (38). They also require higher temperatures for growth (37–40°C), and this may be suggestive of life adapted in animal bodies; indeed, they seem to be obligate parasites of animals and birds. So far, they have only been associated with animal botulism, except for two cases of human botulism (39).

Few characteristics of group IV organisms have been studied in detail because, so far, they have never been confirmed as a cause of botulism, but solely isolated from Argentine soil (40) and from autopsy specimens (41).

The physiological characteristics of neurotoxigenic *C. butyricum* and *C. baratii* (groups V and VI) have not yet been adequately investigated, but they can be reasonably expected to overlap those

of the nontoxigenic strains of the species. Little is known about their environmental distribution. Attempts to detect *C. butyricum* type E in soil samples close to Rome, Italy, where these strains were first isolated, were unsuccessful (42). Conversely, *C. butyricum* type E has been isolated from lakeshore sediments in China (43), where an outbreak of *C. butyricum* type E foodborne botulism has been described (12).

The DNA sequence homologies detected by DNA/DNA hybridization experiments confirm a close genetic relationship of microorganisms within groups and, conversely, the unrelatedness between different groups. High degrees of genetic relatedness with the nontoxigenic clostridia species counterparts of each group have also been demonstrated (44,45). The distinction between groups is further supported by rRNA oligonucleotide cataloging (46,47).

Bacteriophages and plasmids have been isolated from all cultural groups (48–50), but only those observed in organisms of group III and IV have been correlated with toxigenicity (see Sec. VII). Most recently, preliminary results from genotyping techniques (PFGE and RAPD) have been promising for elucidating homologies and differences among strains (51,52).

### III. DISEASES

#### A. Foodborne Botulism

Although a minority of infant botulism cases are caused by eating honey containing spores of *C. botulinum* (53), this discussion is focused on foodborne botulism, which is caused by the direct consumption of botulinum neurotoxin in contaminated food. Because all forms of botulism are due to the systemic distribution of botulinum neurotoxin, much of this discussion also pertains to infant botulism and wound botulism. Almost all botulism in humans is due to neurotoxin type A, B, or E.

Botulism is a paralysis of symmetric, descending flaccidity, reflecting the progressive inhibition of the release of acetylcholine into the neuromuscular junction. The severity of the paralysis is proportional to the number of nerves that are intoxicated. An individual with mild botulism may complain of double vision and of difficulty in swallowing for a few days, while a severe case may become comatose, with complete paralysis and fixed, dilated pupils, for months.

Over half of foodborne botulism patients suffer from prodromal nausea or vomiting (54), which do not occur in infant or wound botulism. This gastrointestinal distress may be due to metabolites from *C. botulinum* or co-contaminants in the tainted food. Progressive cranial nerve palsies, a *sine qua non* of botulism, are among the first symptoms and signs to appear and the last to recede. These include double vision, dropping eyelids, slurred and muted speech, an inability to swallow food and saliva, gaze paralysis, and dilated pupils (mydriasis). Weakness soon becomes apparent below the neck, as the limbs become progressively paretic and hypotonic. Respiratory distress results from upper airway obstruction, aspiration, or respiratory paralysis. Mydriasis and diminished deep tendon reflexes may lag behind other findings. Constipation, urinary retention, and hypotension occur in more severe cases. At any stage of illness, the patient becomes easily fatigued. Recovery proceeds over weeks to months as motor axons sprout new endplates to replace their intoxicated predecessors (55).

#### B. Diagnosis

The flaccid paralysis of botulism is frequently misdiagnosed as due to Miller-Fisher disease or other variants of Guillain-Barré syndrome, myasthenia gravis, stroke, myelitis, including polio, brainstem encephalitis, or tick paralysis. Patients with milder symptoms have been misdiagnosed with psychiatric illness (56). The symptoms and electrophysiological findings of botulism most closely match Lambert-Eaton syndrome, in which antibodies to a lung carcinoma cross-react with presynaptic calcium channels (57). Botulism varies from other flaccid paralyzes in its prominent cranial nerve palsies disproportionate to milder weakness and hypotonia below the neck, in its symmetry, and in

an absence of sensory nerve damage; a tingling sensation (paresthesia) during early illness due to hyperventilation, rather than neuropathy, has been reported by a few patients.

The diagnosis of botulism is confirmed in the regional or national public health laboratory (see Sec. V. for methodologies). The electromyogram (EMG) and repetitive nerve stimulation can sometimes aid in distinguishing between causes of acute flaccid paralysis (58). The characteristic findings of botulism include normal nerve conduction velocity and sensory nerve function, brief, small amplitude motor potentials, and, most distinctively, an incremental response to repetitive stimulation often seen only at 50 Hz. Additional diagnostic procedures include examination of spinal fluid, which is unchanged in botulism, imaging of the brain, spine and chest to exclude hemorrhage, inflammation or neoplasm. A stool culture for *Campylobacter jejuni* is also performed as a precipitant of Guillain-Barré Syndrome, plus a test dose of edrophonium (Tensilon®) to briefly reverse paralytic symptoms in many patients with myasthenia gravis and a few with botulism. Assays are performed for auto-antibodies causing myasthenia gravis or Eaton-Lambert syndrome, as well as a close inspection of the skin, especially the scalp, for attached ticks.

### C. Therapy

Therapy for foodborne botulism consists of supportive care and passive immunotherapy with equine antitoxin. While administration of supportive care is less dependent on the diagnosis, optimal use of botulinum antitoxin requires an early clinical suspicion of botulism. Unless it is clear that the patient is already improving from maximal paralysis, botulinum antitoxin should be given as soon as the clinical diagnosis is made, as early administration will minimize subsequent nerve damage and severity of disease (59). Approximately 9% of recipients in the United States have displayed an apparent hypersensitivity to equine antitoxin (60). Clinicians caring for patients with suspected foodborne botulism should notify public health authorities immediately, both to obtain antitoxin and to initiate epidemiological investigation to protect the community from the contaminated food.

The supportive care for botulism includes feeding by enteral tube or parenteral nutrition, respiratory toilet, mechanical ventilation, and the treatment of secondary infections. Hospitalization may last from weeks to many months. With contemporary measures of intensive care, the mortality and sequelae of botulism have diminished. The percentage of foodborne botulism cases dying in the United States fell from 25% during 1950–1959 to 6% during 1990–1996, with a similar decrease occurring for botulism caused by each toxin type (61).

## IV. EPIDEMIOLOGY

### A. Foodborne Botulism: Regional Variation

The global epidemiology of foodborne botulism has been shaped by regional diet and soil ecology. Perhaps any food can cause botulism if it is contaminated with a neurotoxic clostridium, processed and stored under permissive conditions, and undercooked before consumption. Despite this potential, a majority of botulism is caused by a minority of foods (Table 3), reflecting in any region those culturally preferred foods in which botulinum neurotoxin is produced and persists. Similarly, a single neurotoxin type causes most foodborne botulism in a given region, usually the predominant type capable of causing human botulism found in that region's soil. Hauschild (8) and Dodds (62) have compiled comprehensive global reviews of the epidemiology of botulism and the soil microbiology of *C. botulinum*.

The etymology of botulism may be more apparent in its Polish translation, *kielbasianym*, as it is arguable that more people have eaten a kielbasa, or polish sausage, than know that the ancient Romans called their sausages *botuli*. Of any nation, the most foodborne botulism is diagnosed in Poland due to the contamination of home-canned pork, ham, and sausage with type B toxin. From 1988 to 1992 there was a median of 314 cases per year of foodborne botulism reported in Poland (63) compared to 24 cases in the United States (64). The remainder of Central and Eastern Europe



**TABLE 3** Patterns in Global Epidemiology of Foodborne Botulism

	Predominant neurotoxin type	Predominant contaminated food
Americas		
Canada, Alaska	E	Fermented fish and aquatic mammals
Continental United States.		
Western	A	Vegetables and fruits
Eastern	B	Vegetables and fruits
Argentina	A	Vegetables and fruits
Eurasia		
Scandinavia	E	Fish and aquatic mammals
Central and eastern Europe	B	Meat
Poland		
Italy, Spain	B	Vegetables and fruits
Iran	E	Fish eggs
Fomer Soviet Union	A, E	Fish
	B	Ham, mushrooms
China	A	Fermented soy beans
Japan	E	Fermented fish

Source: Refs. 8, 54, 64.

shares a predominance of type B botulism due to meat products (8). While type B is also the most common type of foodborne botulism in Italy and Spain, the implicated foods in these southern European countries are usually home-canned vegetables and capsicums (peppers) (7,8).

Vegetables and fruits also are the most frequent vehicle of foodborne botulism in the continental United States, with the toxin type varying by region. Of laboratory-confirmed outbreaks of foodborne botulism in the United States from 1899 to 1989, 57 (69%) of 83 due to type B neurotoxin occurred east of the 95th meridian, while 254 (86%) of 297 due to type A occurred to the west (54,64). In his systematic soil survey, Smith (65) found 73% of type B isolates from soils collected in the eastern United States and 88% of type A isolates from the western United States, corroborating earlier results of Meyer and Dubovsky (66). Although produce prepared at home has caused most outbreaks of botulism in the United States, recent outbreaks due to foods prepared in restaurants have accounted for a disproportionately large number of cases (6).

In the Americas south of the United States, almost all reports of foodborne botulism have originated from Argentina, where 70 outbreaks were noted from 1922 to 1997 (67). The epidemiology in Argentina is similar to the western United States. Home-canned fruits and vegetables have caused most outbreaks. All but six laboratory-confirmed outbreaks through 1997 were caused exclusively by type A toxin. Over 70% of typable neurotoxicogenic strains isolated in Argentina produced type A toxin (68).

From 1958 to 1989, a mean of 90 foodborne botulism cases was diagnosed each year in China (69). Fermented legumes, usually strong-smelling soybean curd (*chou-doufu*), caused over 60% of these cases. Over 75% of typed cases were due to type A neurotoxin, and 80% were diagnosed in a single northwestern province, where type A *C. botulinum* was the most prevalent neurotoxicogenic clostridium isolated in the soil.

Type E *C. botulinum* has been cultured from a variety of aquatic sediments and inhabitants. It has been the predominant type isolated in Canada and Alaska, found in up to 74% of coastal soil samples (70). Type E botulism in humans has a strong association with the consumption of fermented fish and aquatic animals. Type E toxin consumed in aquatic foods caused 81% of foodborne botulism

outbreaks in Alaska through 1989 (54,64) and 97% of outbreaks in Canada from 1994 to 1997 (71). Type E is also the most frequent type of foodborne botulism in Japan (72), Iran (73), and Scandinavia (8). To reiterate, the specific foods associated with type E botulism reflect regional cuisine. For the native peoples of Canada and Alaska, frequent vehicles are fermented seal, whale, and the eggs and heads of salmon (74). In Japan, two related fermented fish dishes, *izushi* and *kirikomi*, have caused nearly all reported cases (8). Other vehicles have been salt-cured fish and fish eggs in Iran, seal meat in Greenland, and herring and half-fermented trout (*rakfisk*) in continental Scandinavia.

*Cervelas*, thick pork sausage akin to mortadella, caused an outbreak of 60 cases of type E botulism in Madagascar (75), while ham caused smaller outbreaks in France (8), the former Soviet Union (76), and Argentina (77). Type E botulism due to neurotoxicogenic *C. butyricum* was first described in Italian infant botulism cases (24), for whom the source of spores was not identified. An outbreak of type E botulism in eastern China in 1994 was caused by a paste of fermented soybeans and winter melon contaminated with *C. butyricum* (12,78). Investigation of this outbreak led to the discovery that two prior outbreaks in China of type E botulism due to fermented soybeans were caused by *C. butyricum* (12). Gram (legume) flour was the source of toxigenic *C. butyricum* implicated in a 1996 outbreak of botulism in India (13). The only type E botulism outbreak in the United States not associated with aquatic foods occurred in California in 1941 (79). Botulism was caused by toxic imported Yugoslavian mushrooms, from which no organism was ever cultured. Could these cases have been caused by *C. butyricum*, subsequently identified as an agent of botulism in Italy, located across the Adriatic Sea from Yugoslavia? Routine microbial procedures at that time may not have been capable of detecting the lipase-negative *C. butyricum* as a source of neurotoxin. In any outbreak of type E botulism where the suspected food contains a vegetable or fruit grown in Eurasia, care should be taken to search for toxigenic *C. butyricum*.

Cases of foodborne botulism from type F *C. botulinum*, while very rare, have been caused by liver paste in Denmark (80), venison jerky in California (81) and pickled cucumbers in Argentina (67). In some regions there is a seasonal occurrence of foodborne botulism reflecting those months when home-preserved foods are consumed more frequently (8).

## B. Infant Botulism

Infant botulism results from intestinal colonization with a *Clostridium*-producing botulinum neurotoxin. The precise source of clostridia causing infant botulism is usually not known. *C. botulinum* producing the same type of neurotoxin has been isolated both from affected infants and from soil and dust samples within their homes (53). Testing of food has been unable to identify a dietary vehicle for infant botulism with the prominent exception of honey, fed to a minority of infant botulism cases. Indigenous honey has been linked by epidemiology and microbiology to type A infant botulism in Japan (82) and Argentina (83), type B in Italy (84), and types A and B in the United States (53). These toxin types match the prevailing types causing foodborne botulism in these nations with the exception of Japan, where botulism is primarily caused by aquatic, not terrestrial food. Regional variations may become increasingly less relevant, as honey purchased in today's market is often a blend of product from multiple sites, even from one or more distant continents; two recent Scandinavian cases of infant botulism were fed honey imported from as far as Argentina (85,86). Because of this risk of contamination with spores, honey should not be given to infants under 1 year of age. The United Kingdom has set an admirable precedent by requiring this recommendation on the label of all honey sold within its borders.

Contaminated corn syrup has never been definitively linked to any case of infant botulism. Initial studies prior to 1982 by the U.S. Food and Drug Administration (FDA) found contamination with *C. botulinum* spores in 13 (1.3%) of 1001 market samples of corn syrups (87). The major producer of corn syrups in the United States intermittently detected low levels of spores in their corn syrups (88). In response, they initiated proprietary processing changes to avoid contamination. Subsequent studies by FDA (89) and by Health and Welfare Canada (90) did not detect *C. botulinum* in over 650 samples of corn syrups.

In many societies, herbal teas or infusions are given to infants for colic and other common ailments. No spores were detected in herbal teas given to some of the first cases of infant botulism recognized in California (91). Satorres et al. recently prepared infusions from 100 samples of medicinal herbs used in western Argentina (92). They isolated type A *C. botulinum* from one sample of *Lippia turbinata* (commonly *poleo*) and detected toxicity in mice suggestive of botulinum toxin from one sample each of three additional herbs.

When attempting to link an item from the baby's diet with the subsequent diagnosis of infant botulism, one must carefully compare the timing of feedings with the onset of symptoms. For instance, both corn syrup and honey have been given to treat constipation caused by infant botulism. Herbal teas have been given to infants believed to have colic, who instead were distraught from hunger caused by the feeding paralysis of early infant botulism.

### **C. Susceptibility to Botulism**

Immunity to botulinum neurotoxin in the population appears to be very rare. A sample of human intravenous immune globulin, pooled from thousands of plasma donors, was unable to neutralize botulinum neurotoxins A and B at the level of 1 mouse LD<sub>50</sub> (93). This absence of immunity probably reflects both the rarity of botulism and the extreme potency of the botulinum neurotoxins. If an analogy can be made with tetanospasmin, a closely related but slightly less potent clostridial neurotoxin, a single lethal dose of botulinum neurotoxin may be smaller than an immunizing dose (94). This supposition may apply to foodborne botulism, which results from a limited ingestion of neurotoxin. There are at least two unfortunate individuals who have had two episodes of foodborne botulism caused by the same neurotoxin, type B in Norway (94) and type E in Alaska (95). Neutralizing antibody was detected only in those survivors of type E foodborne botulism who had received equine antitoxin, and antibody levels in survivors treated with antitoxin did not increase 14 days after a single immunization with type E botulinum toxoid (96).

In contrast, antibody to neurotoxin has been detected in a small number of patients receiving multiple exposures to neurotoxin, whether in adult intestinal colonization botulism (97), infant botulism (98), or after receiving larger doses of injected type A botulinum toxin as therapy (99). As few belong to these groups exposed repeatedly to neurotoxin, few persons, if any, would not develop botulism if exposed to sufficient neurotoxin. One exception may be laboratory workers immunized for occupational protection with the investigational pentavalent (ABCDE) botulinum toxoid vaccine, distributed in the United States by the federal Centers for Disease Control and Prevention (CDC) (100).

## **V. ISOLATION AND IDENTIFICATION**

### **A. Botulinum Neurotoxin Detection**

The confirmation of the clinical diagnosis of botulism is most effectively achieved by detection of the botulinum toxin in the clinical specimens from patients (61). Blood serum collected from patients before administration of the therapeutic polyvalent antiserum, and feces are routinely tested for botulinum toxin. Constipation from botulism can be an impediment to diagnosis: an enema of sterile water may be required to obtain an adequate fecal sample. Other clinical samples that can be analyzed for botulinum neurotoxins are vomitus, gastric contents, or autopsy specimens submitted from fatal cases.

The food source is also identified by demonstration of botulinum toxin. Failure in detecting preformed toxin in the foods consumed before illness might be presumptive of toxico-infectious botulism—wound (101) or intestinal toxemia botulism (102)—which, however, must be supported by additional epidemiological and laboratory findings. The persistence of botulinum toxin in the feces of both infants and adults clearly indicates intractestinal toxin production by the toxigenic microorganism and is consistent with intestinal toxemia botulism.

Toxicity testing is not generally performed on foods consumed by babies less than a year old who present with signs of botulism. While some of these foods might be the vehicle of spores, preformed toxin has never been detected, consistent with their composition and processing (103). Environmental samples are not usually analyzed for botulinum toxin, either. However, the demonstration of botulinum toxin in enrichment cultures from environmental, as well as from clinical and food samples not toxic before enrichment, is evidence of the presence of neurotoxic spores. These are also identified by testing botulinum toxin production in culture supernatants, since there are no other phenotypic characteristics unique to the species (Fig. 2). Botulinum toxin detection remains the most conclusive test to demonstrate contamination with *C. botulinum* spores and is fundamental in investigations of all forms of botulism.

The large variety of antigenic structures and the extreme lethality of botulinum toxins require highly versatile methods capable of detecting very low amounts of substances. Early detection of botulinum neurotoxin is necessary for the avoidance of contaminated food by the public. The conventional method for the detection of botulinum toxin involves the use of laboratory animals, which is impractical, costly, and time consuming, since up to 4 days are necessary for responses. In Europe, animal testing is restricted by law (104). Many alternative *in vitro* tests for botulinum toxin have been devised. Some of the newest tests are comparable in sensitivity and specificity to the standard *in vivo* assay. The advantages and disadvantages of these methodologies will be briefly addressed.

### 1. *In Vivo* Assays

The mouse bioassay is universally accepted as the method of choice for the detection of botulinum toxin because of its high specificity and sensitivity (2 LD<sub>50</sub>/mL): one mouse lethal dose is equivalent to approximately 20 pg of crystalline botulinum toxin (105). The same procedure is followed with all types of samples, except that solid samples require a preliminary extraction with gelatin phosphate buffer to solubilize the botulinum toxin. Test fluids are subjected to either centrifugation or membrane filtration to exclude the presence of microorganisms and consequent nonspecific deaths due to infections in mice. Diagnostic polyvalent (A–F) and monovalent botulinum antisera are used in the mouse neutralization tests. These antisera are only available from a few suppliers because limited demand makes their industrial production infeasible (3). Small volumes of the sample fluids are injected intraperitoneally (IP) into pairs of mice, either as such or mixed with polyvalent antiserum. Trypsinization has been reported to enhance the low toxicity of some samples. Mice are observed at time intervals for 4 days. Those injected with the untreated or trypsinized toxic fluids show the first symptoms of botulism (in sequence: ruffled fur, weakness of limbs, gasping for breath) within 10 hours. Death due to respiratory paralysis generally occurs 24 hours after injection. A longer time can elapse depending on the amount and type of toxin administered. Mice receiving samples mixed with botulinum polyvalent antitoxin survive. Occasionally, the polyvalent antiserum does not prevent death in mice. In these cases, one of the following hypotheses must be considered: (a) contamination of samples with lethal nonbotulinum substances; (b) excess of toxin in the sample; (c) botulinum toxin not as yet recognized. Two- or fivefold dilutions of the samples are generally sufficient to rule out the first two. The heat lability of the toxin can be demonstrated by injecting another pair of mice with samples previously heated at 100°C for 10 minutes; serum samples cannot be heated due to coagulation.

Once toxicity has been demonstrated, the mouse neutralization test with monospecific botulinum antitoxins can be performed to identify toxin type: mice will be “protected” by the antitoxin type-specific for the botulinum toxin involved. The simultaneous presence of two different botulinum toxins may rarely account for excessive toxicity of samples, and a mixture of two monovalent antitoxins will be necessary to achieve complete neutralization (19).

For quantitative determination of the toxin level, serial dilutions of the samples are injected intraperitoneally into groups of four mice. Survival and death are recorded after 4 days and the median lethal dose (LD<sub>50</sub>) is calculated according to Reed and Muench (106). Intravenous injections shorten the times for determination of LD<sub>50</sub> values and require fewer animals, since a single dilution is tested and values are extrapolated from standard curves (107). Lethality of a sample may also

be determined as minimum lethal dose (MLD), which is the ultimate dilution that causes death of all animals injected.

## 2. *In Vitro Assays*

When a large number of samples has to be analyzed, as, for instance, in microbial risk evaluation studies for food quality assurance programs, a fast alternative for the detection of botulinum toxins is offered by *in vitro* assays. Methods currently available have recently been reviewed by Hatheway and Ferreira (105). Most of them depend on the immunogenic properties of the toxins, i.e., on their reaction to type-specific antibodies. They can be broadly divided into four categories: precipitation assays, agglutination assays, and radio- (RIA) and enzyme (mostly ELISA) immunoassays.

*Precipitation assays* rely on the formation of immune complexes that can be observed directly in agar gels as in immunodiffusion (108), capillary tube diffusion (109), toxic colony overlay (110) assays, or after molecular separation as in counterimmunoelectrophoresis (111). Although very quick and easy to perform, the minimum sensitivity reported for these assays—100 mouse IP LD<sub>50</sub>/mL—is too low for analysis of food and clinical samples. Moreover, problems of cross-reactivity between different serotypes are encountered which can cause false results.

*Agglutination assays* are based on the ability of red blood cells or latex particles coated with toxin or antitoxin to agglutinate with the corresponding antitoxin or toxin. In the presence of the test sample, the cells or latex particles are cross-linked and can be easily visualized (112,113). Cross-reaction between type A and B toxins, due to the use of polyclonal antibodies raised against the large toxin complexes that share nontoxic molecules, interferes with the sensitivity of the tests.

*Radioimmunoassays* were originally developed to increase the sensitivity of earlier tests (114). However, RIA were abandoned in favor of EIA or ELISA methods that are based on the same principles, but avoid the hazards of radioisotopes.

Several *enzyme-linked immunosorbent assay* variants have been designed for the detection of botulinum toxin, e.g., sandwich (115), double-sandwich (116,117), and immunoblot (118) procedures. Improvements have been achieved through production of antibodies against more purified toxins, production of monoclonal antibodies, enhanced detection systems, simplified and faster schemes, increased reproducibility, and automation.

One recent commercially available ELISA, the enzyme-linked coagulation assay (119), has a high sensitivity (<1 LD<sub>50</sub>/mL) that is achieved through amplified signals based on the cascade reaction of blood coagulation. This test entails three stages. In stage I, the botulinum neurotoxin binds to horse IgG specific for the neurotoxin; the antibody is conjugated to Russell's viper venom factor X-activating enzyme. In stage II, a mixture of coagulation factors is added: if the complex is present, thrombin will be formed. Then, alkaline phosphatase labeled fibrinogen is added and the presence of thrombin will cause the hydrolysis of fibrinogen to fibrin. In stage III, fibrin is detected by addition of a substrate for alkaline phosphatase. This assay has successfully been applied to the detection of the toxin in foods with results absolutely comparable to the mouse bioassay (120), but its applicability to patients sera samples is still to be verified.

*Optical fiber-based biosensors* have recently been proposed as a fast, specific, and sensitive immunoassay for the detection of botulinum neurotoxins (121). Antitoxins are immobilized on the surface of fibers to capture neurotoxin. A second fluorescently labeled antibody is added and the fluorescence emission generated upon laser excitation is detected by a photodiode. Despite the high speed of responses, its use in food analyses will depend on the cost and availability of technical equipment.

A major drawback of immunoassays is that they detect antigenicity rather than toxicity. Hence, limits of detection should be expressed in terms of toxin mass, rather than biological activity, and conversion tables for each toxin type would be convenient for this purpose.

Innovative approaches are based on the detection of the recently elucidated zinc endopeptidase activity of botulinum neurotoxins by the use of *synaptic vesicles* and *synthetic peptides* (122,123). These methods measure the biological activity of the botulinum toxin rather than its immunogenicity and are suitable for both diagnostic and pharmacological assessments.

## B. Organism Detection

Mild botulism deriving from exposure to low levels of botulinum toxin may lead to failure in detecting the toxin in clinical samples. A negative result for toxin in these specimens, however, is not sufficient for excluding the diagnosis of botulism if it is not supported by the research of spores. Detection of the neurotoxic organisms from the biological samples is even more conclusive in investigations of botulism other than foodborne. The repeated recovery of neurotoxic organisms from the feces of patients over extended periods of time supports the hypothesis of intestinal colonization, while isolation from an injured tissue implies wound infection. Serum is not cultured because botulism patients are presumed to not have bacteremia, an assumption that has not been adequately tested in wound botulism.

Recovery of the toxigenic organisms, but not the toxin, from foods is inconclusive. However, according to the HACCP (hazard analysis and critical control points) system, raw ingredients and foods collected at all stages of preparation and processing in a food-production chain must be checked for contamination with *C. botulinum* spores in order to identify those points at which control is essential to minimize the risk of the final product (see Sec. VIII). Checking foods for contamination may help in tracing the source of neurotoxic spores causing intestinal toxemia. Environmental samples can be analyzed for the presence of neurotoxic organisms as well.

### 1. Conventional Methodology

Portions of foods, samples from patients, or environmental swabs are seeded into specific broth cultures. Suspensions are heat-shocked (70 or 80°C for 10 min) in order to inactivate the vegetative forms of other contaminating bacteria and to promote spore outgrowth. Incubation at 30 and 35°C under anaerobiosis follows, optimally for 5 days. Broth cultures are then centrifuged and supernatants tested for the presence of botulinum toxin. If toxin is demonstrated, isolation of the neurotoxic organism in pure culture is achieved by streaking toxic cultures on egg yolk agar (EYA) plates. This medium is specifically formulated to recognize the lipase and lecithinase reactions (see Sec. II). After incubation under anaerobiosis, single colonies may be picked up, retransferred into broth, and assayed for toxin after appropriate incubation. When required, enumeration of spores is carried out by the MPN (most probable number) test (124).

### 2. Alternative Assays

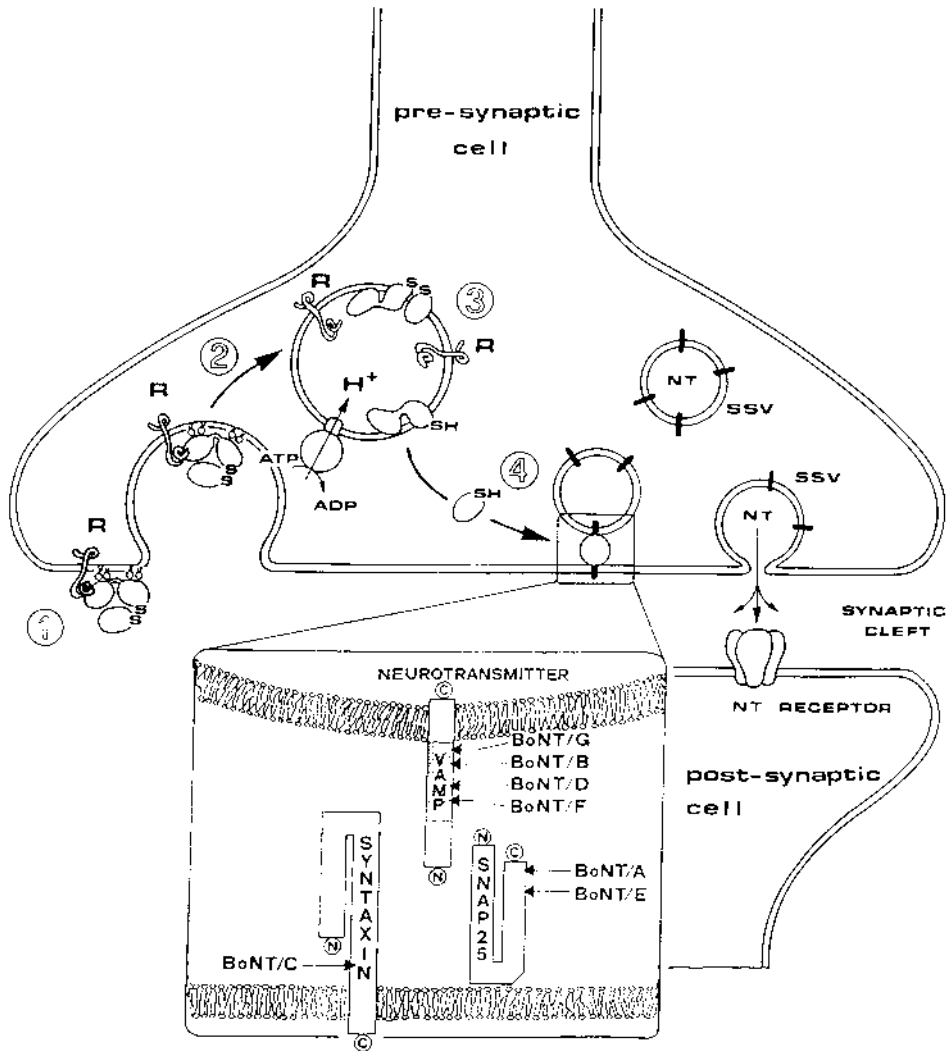
A number of assays based on the *in vitro* enzymatic amplification (PCR) of specific fragments from the different neurotoxin genes have been described (125–129). Positive PCR reactions are conducive to the presence of the neurotoxic organism in the sample analyzed. Foods and clinical and environmental samples may be tested by PCR directly. Although most of the tests described in the literature are fast, sensitive, and specific, the primary drawback of this methodology is that it confirms the presence of the organisms, but provides no evidence of bacterial viability or toxin production. Previous enrichment of the samples is hence necessary to show spore multiplication, while toxicity testing remains the only way to demonstrate toxin formation. Single toxigenic colonies from agar plates are successfully identified by PCR, even if the actual production of the toxin should be confirmed (130).

## VI. PATHOGENICITY

### A. Botulinum Neurotoxins

Botulinum neurotoxins (BoNTs) are among the most poisonous substances existing in nature, the lethal dose for humans being estimated around 1 ng/kg of body weight (131). Their high potency is due to the specific action on the neuroexocytosis process: indeed, they block the neurotransmitter acetylcholine (ACh) release at the peripheral neuromuscular junctions, thus affecting a vital course for vertebrates (132). All seven serotypes (BoNT/A–G) have common structures, which correlate

to their similar mode of action. They are synthesized as biologically inactive polypeptides of 150 kDa molecular weight, in combination with other large nontoxic proteins: some of these show hemagglutinin (HA) activity, while others do not (nontoxic-nonHA components), but all are believed to protect BoNT from acids and proteases present in the stomach of the host (133). In an alkaline environment, like that of the small intestine, BoNT dissociates from the nontoxic proteins and is absorbed into the bloodstream. At an unidentified stage, an intrachain disulfide bridge located one third of the way from the amino-terminal end of the 150 kDa polypeptide is proteolytically nicked.



**FIGURE 3** Molecular mechanism of botulinum neurotoxins (BoNTs) (top panel): (1) toxin binding to specific receptors (R) on the presynaptic membrane; (2) internalization of the toxin-receptor complex inside vesicles; (3) reduction of the disulfide bond between the L and H chains, and translocation of the L chain into the cytosol; (4) zinc-endopeptidase activity. Target proteins for the catalytic activity of the different BoNT types and their location are specified in the lower panel. SSV: small synaptic vesicles containing neurotransmitter (NT). (Courtesy of Cesare Montecucco, University of Padua, Italy.)

A dichain active form results from such cleavage: the two chains [heavy (H), 100 kDa; light (L), 50 kDa] remain linked by a single disulfide bridge and noncovalent bonds (134). Study of the crystal structure of BoNT/A has recently revealed the tridimensional folding of the chains (135). Three 50 kDa distinct structural and functional domains have been individuated. The first domain (carboxyl-terminus of the H chain) recognizes specific receptors on the presynaptic membranes of the nerve cells (*cell binding*) (136). Then, internalization of BoNT enveloped in membrane vesicles follows (*endocytosis*). Subsequently to intracellular acidification, a double event occurs inside the vesicles: (a) reduction of the disulfide bond, disconnecting the L and H chains; (b) formation by the amino-terminus domain of the H chain of trans-membrane pores suitable for the transfer of the L chain (catalytic domain) into the cytosol (*membrane translocation*) (5).

High amino acid sequence homology exists among the different types of BoNT, especially at the level of the L chains: one of the most conserved regions is a zinc-binding amino acid sequence common to most metalloproteases. The L domains of all BoNT serotypes bind one or two atoms of zinc, and possess *zinc endopeptidases activity* (137). They selectively cleave different short peptides of target proteins that are, respectively, VAMP (or synaptobrevin) for BoNT/B, -/D, -/F, -/G; SNAP-25 for BoNT/A and -/E; syntaxin for BoNT/C (Fig. 3). All three target proteins are implicated in the docking/fusion of the Ach-containing vesicles with the presynaptic membrane. Thus, their cleavage impairs the release of the neurotransmitter at the terminal ends, blocks nerve pulse transmission and ultimately produces the flaccid paralysis of botulism (5).

## B. ADP-Ribosylating Toxins (C2 and C3 Toxins)

Some *C. botulinum* C and D strains produce nonbotulinum enzymatic toxins, C2 and C3 toxins, along with type C and D neurotoxins (138).

C2 toxin is composed of two subunits: a binding component recognizing specific receptors on a variety of eukaryotic cells, and an enzyme component that ADP-ribosylates intracellular non-muscular G actin. This protein normally forms the microfilaments of the cytoskeleton; its ADP-ribosylation causes loss of the cell shape, an increase in vascular permeability, and enterotoxic and cytotoxic effects. C2 toxin may help the diffusion of the botulinum neurotoxin in the body by increasing vascular permeability, but it does not produce the symptoms of botulism. Although it is lethal in mice, its clinical significance for humans is unclear (139).

C3 toxin acts by ADP-ribosylating low molecular mass GTP-binding proteins of the Rho family, found in many types of host cells. The GTP-binding proteins regulate the eukaryotic cells metabolism and so C3 toxin might interfere with this regulation. However, the total lack of lethality makes the pathological significance of C3 toxin even more uncertain than that of C2 (139).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

Since recombinant DNA technologies became available, the structural genes encoding all seven BoNT serotypes have been sequenced (140–146). The determination of the nucleotide sequences of BoNT genes representative of all serotypes has revealed many similarities, such as a high [A + T] content (72–75%) and identical translational start and stop codons. Moreover, the comparison between the derived amino acid sequences shows a high degree of homology in regions involved in similar structure/function, e.g., the two cysteine residues of the disulfide bridge linking the L and H chains and the zinc-binding motif essential for the catalytic activity of the toxins. On the contrary, sequence divergence in the carboxyl terminus of the H chains seems to account for the specific binding to different nerve ending receptors (147).

Sequencing of the BoNT/E and -/F genes from neurotoxigenic *C. butyricum* and *C. baratii* has shown high relatedness with the corresponding BoNT genes from *C. botulinum* strains, respectively, of 97% and 70–74% (148,149). It is assumed that a lower degree of homology reflects an earlier transfer of the gene in the evolutionary scale (147).



The possibility that BoNT genes are mobile and may have been transferred among progenitor strains through DNA vectors seems to be confirmed by other observations: (a) phenotypically and genotypically similar clostridia strains may or not carry BoNT genes (i.e., toxigenic versus nontoxigenic strains of the same cultural groups; see Sec. II); (b) strains of different cultural groups can produce the same BoNT type (e.g., BoNT/B and -/F are produced both by proteolytic and nonproteolytic strains of *C. botulinum*); (c) some strains of *C. botulinum* are able to form two BoNT types concomitantly (*C. botulinum* subtypes); (d) some strains of *C. botulinum* type A contain a silent BoNT/B gene in their genome [*C. botulinum* A(B)] (126,150).

So far, only BoNT/C, -/D, and -/G genes have been associated with extrachromosomal elements (Table 2). BoNT/C and -/D genes are located on separate bacteriophages; when cured of their phages, strains C and D still produce C2 toxin, indicating that the gene for this toxin is not phage encoded (38). BoNT/G gene has been associated with a 76 Mda plasmid harbored in all type G strains: loss of this plasmid results in the cessation of toxin production (38). The plasmid location of BoNT/G gene has been confirmed by gene probes and PCR studies (151). Failure in associating the other BoNT genes to extrachromosomal elements has led to the conclusion that they are located on the bacterial chromosome (Table 2). Attempts to identify the mechanism of transfer involved in the acquisition of the BoNT/E gene from neurotoxicogenic *C. butyricum* strains have led to discordant results, suggesting either plasmid or chromosomal locations (152,153).

## VIII. CONTROL MEASURES

Although botulism is increasingly rare in developed countries, the food industry remains deeply concerned about the threat to public health from food contamination with *C. botulinum*. Botulism may result when a food (a) contains spores that survive processing or that contaminate after processing; (b) by composition or storage favors outgrowth and toxin production by *C. botulinum*; (c) is eaten without sufficient cooking to inactivate preformed neurotoxin.

The primary objective of the food-processing industry is to achieve the widest possible margin of safety against outgrowth and toxin production by *C. botulinum*. Safety margins are estimated on the basis of the rate of occurrence of *C. botulinum* spores in the foods and the capacity of the processing and storage system to destroy or inhibit the organism. At present, food processors are dealing with this situation by developing hazard analysis systems for critical control points (HACCP) in their food-production plants.

### A. Distribution of Spores in Foods

Raw products are naturally contaminated with *C. botulinum*. Several studies have demonstrated that this organism is ubiquitous in soil. The factors determining type distribution are as yet unclear (62). Organic fertilizers of animal origin and sewage sediments contribute to soil contamination (154). It is therefore not surprising that foods of vegetable origin are contaminated with this microorganism. *C. botulinum* spores, generally types A and B, have been detected in red capsicum, apricots (155), carrots, onions (156), potatoes, parsley, spinach (157), garlic (158), cabbages (159), and cultivated mushrooms (160,161). Spore numbers in mushrooms range from <0.08–0.16/100 g (11) to 41/100 g (160).

Terrestrial sediments present in the aquatic environment may contaminate fish with nonproteolytic *C. botulinum* spores. However, the aquatic environment is optimal for the survival of the type E spore. The incidence of *C. botulinum* type E in aquatic sediments appears to result from aquatic carrion (162). Spores of nonproteolytic strains, type E in particular, have been found in bred trout (163–165), salmon (166), whitefish (167), catfish and sardines (168). The level of contamination in fish products can be quite high (169), estimated around 17 spores/100 g (170).

Meats can also be contaminated with *C. botulinum* spores, more frequently pork rather than beef, mutton, or poultry. This may be due to the ingestion by pigs of soil along with their feed.

Contamination levels for red meats, though based on limited data and methods, range from 0.04 to 2.2 spores/kg (171).

Clostridia are rarely present in raw milk. However, when the hygienic conditions of harvesting are poor and milk cows are fed on silage, their number increases considerably. The clostridia affecting the organoleptic quality of cheese have been widely studied, but little is known about the occurrence of *C. botulinum* spores in raw milk, probably because milk-derived products have rarely been associated with cases of botulism. The level of contamination of raw milk has been estimated at <1 spore/kg in Canadian milk (172); 1 spore type B/100 mL has been detected in 5.5% of the lots furnished by 35 Italian dairies (P. Aureli and G. Franciosa, 1998, unpublished data). Their milk produced the mascarpone (typical Italian spread process cheese) that was responsible for a botulism outbreak in Italy in 1996 (10).

Spores of *C. botulinum* types A and B occur naturally in other products meant for human consumption such as honey (85,171,173,174), natural sweeteners (87,175), and herbs for infusion (92), but none of these products have so far been involved in cases of foodborne botulism. The contamination levels for honey have been estimated between 55 and 60 spores/g (176).

## B. Inactivation of *C. botulinum* Spores in Foods

Clostridial spores remain viable for long periods of time, even under conditions unfavorable to their growth. If safety cannot be guaranteed through intrinsic factors (pH,  $a_w$ , Eh, microbial antagonism, preservatives) or extrinsic factors (T and shelf life), foods must undergo specific treatments in order to destroy contaminating spores. Sterilization by heat is the most common method used. In general, microbes are destroyed in an exponential fashion; the initial number of a population exposed to heat diminishes at a constant rate per unit of time. Spore resistance to heat is conventionally designated as a D-value (decimal reduction time), i.e., the time necessary to kill 90% of the population at a specified temperature. D-values for *C. botulinum* show inter- and intratype variations. The spores of proteolytic strains types A and B show a greater resistance to heat ( $D_{112^\circ\text{C}} = 1.23$  min) than spores of nonproteolytic strains types B, E, and F ( $D_{80^\circ\text{C}} = 0.6\text{--}1.25$  min) (177). The International Commission on Microbiological Specifications for Foods (ICSMF) has published a comprehensive list of D-values obtained with laboratory substrates and foods for both proteolytic and nonproteolytic *C. botulinum* spores (178).

The temperature increment that reduces the D-value to 1/10 is called the z-value (expressed in °C). In industrial treatments z-values of 10°C are adopted for spores of the most resistant *C. botulinum* strains (177), even though the actual value for different strains may vary by several degrees (179). The D-values at 76.6°C for two strains of *C. butyricum* type E (strains 5262 and 5520) suspended in phosphate buffer (pH 7.0) are 2.5 and 2.3 minutes, respectively. The z-values for the same strains are 12.6 and 13 (180).

The food industry ensures the safety of low-acid products packaged in hermetically sealed containers by subjecting them to a minimum thermal process called 12 D, i.e., heating at 121°C for 3 minutes to reduce the number of the *C. botulinum* spores that can grow in the product to a value less than  $10^{-12}$  per portion. For foods that must be marketed under refrigeration, a 4–6 D process is considered sufficient.

The term  $F_0$  is frequently used to indicate the time in minutes at 121°C required to kill a microorganism with a known heat resistance. For proteolytic *C. botulinum* spores, the value of  $F_{0_{121^\circ\text{C}}}$  is 3. There are several factors that influence the heat resistance of spores. Heat resistance increases as water activity ( $a_w$ ) decreases (181), while spores are more sensitive to heating at extreme pH values (182). Fat and protein are known to protect *C. botulinum* spores when heated (183). Sublethal heat treatments due to insufficient temperature or exposure time may induce the germination of dormant spores. This phenomenon led to an unsuccessful trial of double pasteurization of vacuum-packaged potatoes. Unfortunately, *C. botulinum* spores remained viable even after the second pasteurization (184). Besides sterilization, the UHT (ultra high temperature) process (up to

137.8°C for 2 seconds) used in the milk industry renders the product safe. *C. botulinum* spores experimentally inoculated in milk were killed at only 125°C for 5 seconds (172).

It is possible to destroy *C. botulinum* spores by using ionizing radiation such as x-rays or gamma rays from the radioactive isotope Co-60. However, *C. botulinum* spores of all types have been shown to be radiation resistant at the doses recommended for food preservation (185). As an alternative to food preservation by thermal inactivation, high-pressure processing (HPP), pulsed electrical field technology, and microwave heating in UHT treatment have also been recently tested, but no data are as yet available on their effect on *C. botulinum* spores.

### **C. Inactivation of the Toxin in Food**

In his initial studies a century ago, van Ermengem demonstrated that the toxic substance responsible for foodborne botulism was heat sensitive (1). Botulinum toxins A, B, E, and F are inactivated by heating at 79°C for 20 minutes or 85°C for 5 minutes (186). Thermal inactivation of the toxin is not a linear function. Substances in food such as divalent cations and organic acid anions protect the toxin from heat. The toxin is stable at pH 5. Ionizing radiation at doses set for food preservation has no effect on their toxicity.

### **D. Sanitation in Plant Environment**

This procedure involves inactivation of the spores on utensils, machinery, packaging materials, in water used to wash or transport raw foods, and in water used to cool heat-sterilized cans.

Chlorine and chlorine compounds are widely used by the food-processing industry because they are highly sporecidal in the absence of organic material. *C. botulinum* spore resistance to chlorine varies according to strain (178). In general, the most heat-resistant strains require longer exposure times. For the sanitation of machinery, solutions containing 100–200 ppm of hypochlorite are applied for at least 2 minutes. *C. botulinum* spores are inactivated by chlorine dioxide, ozone (187), and ethylene oxide (188), a cyclic ether that is a gas at room temperature and is usually used to sterilize dry food. Hydrogen peroxide, used for the aseptic packaging of foods such as milk and eggs, inactivates *C. botulinum* spores (189), but it is not the primary method to control *C. botulinum*.

### **E. Control of Growth and Toxin Production**

The safety of certain foods is achieved by using one or more methods to inhibit the growth of *C. botulinum* and toxin production. This is the best solution for foods with a high humidity content that cannot be treated at temperatures high enough to kill *C. botulinum* spores without altering their organoleptic characteristics.

#### *1. Control Through Food Storage Temperature*

As the lowest growth temperatures for proteolytic and nonproteolytic strains of *C. botulinum* are 10°C (190) and 3.3°C (191), respectively, refrigerated (4–8°C) storage cannot be the sole protection against botulism. In nonproteolytic strains, growth and toxin production in food may occur when the shelf life of the product is sufficiently long. Proteolytic strains grow in food when temperature abuse occurs during storage. Proteolytic strains have been shown to produce neurotoxin after one week at 15°C or after 2–3 days at 20°C (190). At the same storage temperature, the higher the number of bacteria inoculated, the earlier the production of the toxin (192,193).

#### *2. Control Through pH*

The minimum pH value required for most proteolytic strains to grow is 4.6 (188), but for several strains it may be over 5.0 (177). For nonproteolytic strains the limiting pH is above 5.0. The inhibition of spore outgrowth and toxin production in high-moisture, low-protein foods such as vegetables is achieved by the addition of acidulants to obtain an equilibrium pH of 4.6. However, the inhibiting

action of pH is neutralized if molds, yeasts (194), or bacilli (195) grow in food because their presence increases pH (effect of metabiosis). In high-protein foods, control using pH is counteracted by the buffering activity of proteins (196). In cured meats, starters, either natural or added to the product, ferment rapidly to prevent toxin production. The risk of *C. botulinum* growing in fish is higher because fish ferment more slowly and have a low carbohydrate concentration, delaying acidification. Fish are rendered safe by the additional measures of salt or refrigerated storage. In dairy products, pH plays a decisive role in the control of *C. botulinum*. Fermented milks have never been involved in botulism cases.

### 3. Control Through $a_w$ or NaCl

Growth and toxin production are influenced by the quantity of free water available for metabolic activity. Salt, like other solutes such as potassium chloride, saccharose, or lactose, reduces  $a_w$  values. While proteolytic strains are incapable of growing at  $a_w = 0.935$ , a condition obtained with a 10% NaCl solution in the substrate, nonproteolytic strains require a higher value ( $a_w$  0.970), which is obtained with a 5% NaCl solution (177). However, curing at higher temperatures does not prevent the production of botulinum toxin in home-made raw ham (197). In fish, a NaCl content of 5% in the aqueous phase if products are refrigerated, or of 10% if they are stored at room temperature, is enough to prevent the risk of botulism. Since the effect of salt is influenced by pH, the amount of NaCl used for these products can be greatly reduced by decreasing pH values (198).

### 4. Control Through Additives

Nitrite has long been used in the meat-processing industry to inhibit outgrowth and toxin production. Its efficacy, however, depends on the complex interaction of several other factors (pH,  $a_w$ , T, etc.) (177). The risks of carcinogenicity and teratogenicity posed by nitrosamines, resulting from the reaction of nitrite with amines, have spurred the search for alternatives to permit the reduction or removal of nitrite. Sorbic acid and its salts are capable of delaying outgrowth and toxin production in several types of cured meat (199). Their action increases as pH decreases; the inhibiting effect depends on the concentration of undissociated sorbic acid. As a secondary function, polyphosphates also enhance other inhibiting techniques (200,201). Ascorbic acid can reduce the requirement or nitrite in meat (202), and liquid smoke can reduce the salting of fish (203), while *C. botulinum* growth and toxin formation remain inhibited. Essential oils (garlic, onion, black pepper, cloves, origanum), or alcohol extracts (nutmeg, garlic, rosemary, thyme, sage) of several aromatic plants have been found to inhibit spore germination or vegetative growth (204–206). The presence of essential oils was inadequate to prevent botulism from garlic in oil-fried onions in the United States and from pesto sauce containing garlic and basil in Italy (L. Fenicia and P. Aureli unpublished data, 1997). A good safety margin against *C. botulinum* may also be obtained by adding natural preservatives, or biopreservatives, such as lactic acid bacteria or their purified metabolites, bacteriocines. Biopreservatives decrease pH either by transforming carbohydrates into organic acids, lactic acid in particular, or through production of acidic metabolites such as carbon dioxide, oxygen peroxide, carbon anhydride, and bacteriocines. Nisin, a well-known biopreservative used in vegetables and spread cheeses, has an indirect antibotulinal effect. Nisin permits a reduction in thermal treatments and in salt and phosphate levels, which in turn increases the water concentration of products stored at room temperature (207). Lysozyme has been proposed to control *C. botulinum* growth, but it has been recently demonstrated that the presence of this enzyme increases the risk of growth of nonproteolytic *C. botulinum* strains (208).

### 5. Control Through Combined Factors

In the preparation of several foods, the growth of *C. botulinum* growth is controlled by using a combination of different factors: pH,  $a_w$ , and preservatives coupled with various processes and storage conditions. This approach has also been adopted to reduce the risk of *C. botulinum* growth in fresh, minimally processed foods refrigerated for extended duration, which pose a greater risk of intoxication if abused. There are several studies of combined control methods for various foods.

These results are only valid for the specific products or testing conditions and may not be extrapolated to other foods. Yet another recent approach utilizes predictive models to quantify the effects of the different factors that influence *C. botulinum* growth and toxin production (209).

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# 6

## *Clostridium perfringens*

**Bruce A. McClane**

*University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.*

### I. BACKGROUND

*Clostridium perfringens* is ideally suited for its role as a major foodborne pathogen (1,2). The presence of this bacterium in soil and feces (both human and animal) provides ample opportunity to contaminate foods. Once established in foods, the rapid doubling time (<10 min) of *C. perfringens* allows quick attainment of pathogenic levels. The spores and vegetative cells of this bacterium are relatively heat-resistant, facilitating its survival in incompletely cooked foods (for further discussion see Secs. IV and VI). Finally, *C. perfringens* produces two toxins that are active in the human gastrointestinal (GI) tract and can thereby induce human foodborne illnesses. These two toxins are *C. perfringens* enterotoxin (CPE), the toxin responsible for the symptoms of *C. perfringens* type A food poisoning, and  $\beta$ -toxin, which is the toxin primarily responsible for the symptoms of necrotizing enteritis.

### II. CHARACTERISTICS

*Clostridium perfringens* is a nonmotile, encapsulated, endospore-forming, gram-positive rod. It is classified as an anaerobe since no colonies arise when agar plates streaked with this bacterium are incubated in air (1,2). However, *C. perfringens* is considerably more aerotolerant than most other anaerobes; in fact, only a modest reduction in oxidation-reduction potential (Eh) is necessary for initiating its growth (1,2). Importantly, most foods have a sufficiently low Eh to support the growth of *C. perfringens* (1,2).

Several other factors also influence the growth of *C. perfringens* (for review, see Refs. 1, 2). Growth of this bacterium can occur between pH 5 and 8.3, but is optimal at pH 6–7. The lowest water activity ( $a_w$ ) supporting the growth of *C. perfringens* is  $\sim 0.95$ . The growth of this bacterium is optimal between 43 and 45°C, but continues up to at least 50°C. The spores and vegetative cells of *C. perfringens* type A food poisoning isolates appear to be especially heat-tolerant (see Secs. VI and VIII), e.g., the spores of food poisoning isolates often survive exposure to boiling for 15 minutes or longer (3). Growth of *C. perfringens* vegetative cells is also affected by low temperatures, slowing noticeably below 15°C and stopping altogether by  $\sim 6^\circ\text{C}$  (1,2).

The effectiveness of curing agents for inhibiting the growth of *C. perfringens* in foods is controversial. Older studies (4) had suggested that growth of this bacterium is only inhibited when curing salts are used at commercially unacceptable levels. However, some evidence (1,2) now suggests that commercial levels of curing salts can be inhibitory for *C. perfringens* growth, particularly when combined with other preservation factors (such as heating and nonneutral pHs). Perhaps the best, although still indirect, evidence for the effectiveness of curing salts at commercial levels is the relatively uncommon involvement of commercially cured foods as uncommon food vehicles for *C. perfringens* type A food poisoning outbreaks (1,2).

**TABLE 1** Toxin Typing of *Clostridium perfringens*

<i>C. perfringens</i> toxin type	Toxin produced			
	$\alpha$	$\beta$	$\epsilon$	$\iota$
A	+	–	–	–
B	+	+	+	–
C	+	+	–	–
D	+	–	+	–
E	+	–	–	+

*C. perfringens* isolates are commonly classified into one of five types (A–E), depending upon their ability to express  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\iota$  toxins (see Table 1). Each *C. perfringens* foodborne illness is associated with a specific type of *C. perfringens* isolate; as evident from its name, *C. perfringens* type A food poisoning almost always involves CPE-producing type A isolates (2), while necrotic enteritis is usually caused by type C isolates, which produce  $\beta$ -toxin (5).

### III. DISEASES

#### A. *Clostridium perfringens* Type A Food Poisoning

Of the two human foodborne illnesses caused by this bacterium, *C. perfringens* type A food poisoning is by far more common, particularly in industrialized countries. The latest statistics compiled by the U.S. Centers for Disease Control and Prevention (CDC) indicate that *C. perfringens* type A food poisoning currently ranks as the third most commonly reported foodborne illness in the United States (6). Estimates indicate that more than 250,000 Americans become sickened with this illness each year, resulting in approximately 10 deaths and economic losses exceeding \$120 million dollars (7,8).

The most common symptoms (2) of *C. perfringens* type A food poisoning are diarrhea and abdominal cramps, which start about 8–16 hours after ingestion of contaminated food. These symptoms continue for 12–24 hours before self-resolving in most healthy individuals. In the elderly or debilitated, this illness is sometimes fatal.

#### B. Necrotic Enteritis

Relative to *C. perfringens* type A food poisoning, necrotic enteritis (also known as enteritis necroticans) is not only more rare, but also typically more serious (5,9). Necrotic enteritis is characterized by severe abdominal pain, bloody diarrhea, vomiting, and obstruction of the intestinal mucosa. Those symptoms develop 1–5 days after a victim has consumed food contaminated with *C. perfringens* type C isolates.

The severity of necrotic enteritis varies. Milder cases may recover without treatment, but more severe cases (particularly those involving intestinal obstruction) often require surgery (see Sec. VIII). In the absence of such medical treatment, the more severe cases can be rapidly fatal.

### IV. EPIDEMIOLOGY

#### A. *Clostridium perfringens* Type A Food Poisoning

Temperature abuse is the single most important factor contributing to the occurrence of a *C. perfringens* type A food poisoning outbreak (2). Incomplete cooking is implicated in ~30% of *C.*

*perfringens* type A outbreaks, while improper holding of foods contributes to nearly 100% of *C. perfringens* type A outbreaks (6).

Most *C. perfringens* type A food poisoning outbreaks are large (averaging ~50–100 people) and occur in institutionalized settings (6). These epidemiological characteristics can be attributed to at least two factors. First, institutions often prepare foods in advance and then hold those foods for later serving, which favors the growth of *C. perfringens* if the food is temperature-abused during the holding process. Second, given that most *C. perfringens* type A food poisoning cases present with relatively mild and nondistinct symptoms, public health authorities usually pursue laboratory confirmation (see Sec. V) of a *C. perfringens* type A food poisoning outbreak only when large numbers of people are sickened in a single outbreak.

*C. perfringens* type A food poisoning occurs throughout the year; however, this illness is somewhat more prevalent during the summer, when higher ambient temperatures favor temperature abuse during food holding (6). The most common food vehicles for this food poisoning are meats (especially beef and poultry), meat-containing products (e.g., stews and gravies), and Mexican foods (6).

Although people sickened with *C. perfringens* type A food poisoning develop elevated serum IgG responses against CPE (10), there is no evidence that prior exposure provides any protective immunity against future bouts of this illness.

## **B. Necrotic Enteritis**

Most (but perhaps not all) cases of necrotic enteritis caused by *C. perfringens* type C are foodborne and involve malnourished individuals (5). Therefore, it is not surprising that this illness was first recognized in post–World War II Germany, where it was referred to as *Darmbrand* (“bowel fire”). *C. perfringens* type C was later shown to be the cause of endemic necrotizing enteritis (locally referred to as Pig-Bel) in the highlands of Papua New Guinea, where the local population subsists primarily on a protein-poor diet rich in sweet potatoes (see Sec. VI for further discussion). Foodborne necrotic enteritis still occurs in several regions of Southeast Asia, e.g., Vietnam. It is notable that, as in Papua New Guinea, people in these other endemic regions also subsist on a protein-poor diet that is rich in sweet potatoes.

Necrotic enteritis has been best studied in Papua New Guinea (5), where it usually develops following the ingestion of pork contaminated with *C. perfringens* type C isolates (often present in pigs). The affected patients usually present with severe abdominal pain that begins 12 hours to several days after consumption of the contaminated pork. Other symptoms of this illness include vomiting, diarrhea (often bloody), and a distended bowel.

In endemic areas such as Papua New Guinea, foodborne necrotic enteritis usually affects children under the age of 13, but not newborn infants (2,5). This epidemiological pattern suggests that exposure to type C isolates of *C. perfringens* provides subsequent immunity.

## **V. ISOLATION AND IDENTIFICATION**

### **A. *Clostridium perfringens* Type A Food Poisoning**

Clinical and epidemiological criteria such as incubation time, symptoms, and type/history of food vehicle are often the first criteria applied towards identifying an outbreak of this food poisoning. However, since other food poisonings (particularly the diarrheal form of *Bacillus cereus* food poisoning) share similar clinical/epidemiological features with *C. perfringens* type A food poisoning, laboratory approaches are necessary to rigorously confirm an outbreak of this illness.

The CDC currently accepts (6) several laboratory criteria for confirming the occurrence of a *C. perfringens* type A food poisoning outbreak: (a) demonstrating the presence of  $10^5$  *C. perfringens* organisms/g of stool from two or more persons, (b) demonstrating the presence of  $10^5$  *C. perfringens* organisms/g of epidemiologically implicated food, or (c) demonstrating the presence of CPE in the feces of several people involved in a food poisoning outbreak (demonstrating that CPE is present



in the feces of multiple victims of a single food poisoning outbreak is important since CPE-positive *C. perfringens* strains also cause nonfoodborne human gastrointestinal diseases, such as antibiotic-associated diarrhea).

Procedures for isolating *C. perfringens* from food or feces are described in detail in the U.S. Food and Drug Administration *Bacteriologic Analytical Manual*, 8th ed., but will now be briefly summarized. An implicated food or fecal sample is streaked onto a relatively selective agar, such as tryptose-sulfite-cycloserine. After anaerobic incubation, representative colonies showing the characteristic appearance of *C. perfringens* are selected for confirmatory testing. Some confirmatory tests commonly used to identify *C. perfringens* include motility (*C. perfringens* is nonmotile), nitrate reduction (*C. perfringens* reduces nitrates to nitrites), lactose fermentation (*C. perfringens* ferments lactose to acids and gas), gelatin hydrolysis (*C. perfringens* liquefies gelatin), and Gram stain (*C. perfringens* is a gram-positive rod).

Several types of serological tests are now commercially available for fecal CPE detection (2). These include both a reversed passive latex agglutination assay (Oxoid) and an ELISA (TechLab). Fecal CPE testing should always be performed as soon as possible after the onset of diarrhea in order to ensure maximum reliability (11).

## B. Necrotizing Enteritis

Because foodborne necrotizing enteritis is relatively uncommon and typically occurs in poor, isolated, rural areas lacking good medical care, cases of this illness may go undiagnosed despite their severity. When recognized, necrotic enteritis is usually diagnosed on the basis of clinical/epidemiological grounds, which include such features as patient history, symptomatology, characteristic x-ray results revealing intestinal obstruction, and laparotomy results showing areas of necrosis on the serosal surface of the bowel (9). No currently available laboratory test is capable of providing a rapid diagnosis of this disease; however, demonstrating that *C. perfringens* type C isolates are present in the intestinal contents of an ill person (using culture on selective agar and subsequent toxin typing assays) is helpful for confirming diagnoses made on clinical grounds (9).

## VI. PATHOGENESIS

### A. *Clostridium perfringens* Type A Food Poisoning

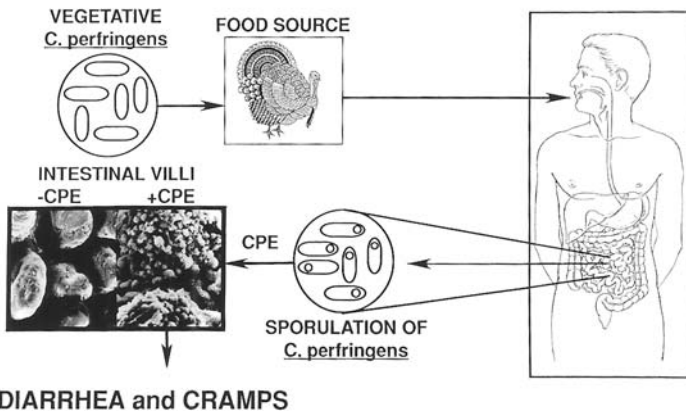
A person contracts this foodborne illness (Fig. 1) after ingesting a food (usually a meat or meat-containing product) that has become heavily contaminated with vegetative cells of a CPE-positive type A strain of *C. perfringens*. The infective dose required for initiating this disease is  $>10^6$ – $10^7$  CPE-positive vegetative cells/g of food (2).

While many of the ingested vegetative cells die when they encounter the acidity of the stomach, some survive to passage into the small intestines, where they quickly multiply and then start to sporulate. What triggers this intestinal sporulation is not yet completely clear, but some evidence suggests that it results from the *in vivo* exposure of the *C. perfringens* vegetative cells to bile or stomach acid (12,12a). Other recent studies (13) suggest that this *in vivo* sporulation may be promoted by a low  $M_r$  factor produced by *C. perfringens* itself (perhaps following exposure to acids or bile?).

It is during this intestinal sporulation that large amounts of CPE are expressed (see Sec. VII). Since CPE is responsible for the clinical symptoms of *C. perfringens* type A food poisoning (see next paragraph), the fact that CPE is produced *in vivo* means that this food poisoning is a true foodborne infection, rather than a foodborne intoxication involving ingestion of a preformed toxin already present in food.

After expression, the enterotoxin is not secreted. Instead, it remains in the cytoplasm of the sporulating cell (14). Such high levels of CPE often accumulate in the cytoplasm of sporulating cells that CPE-containing inclusion bodies form (1,2). The enterotoxin is finally released into the

## CLOSTRIDIUM PERFRINGENS FOOD POISONING



**FIGURE 1** The pathogenesis of *C. perfringens* type A food poisoning. Contaminated foods (usually a beef or poultry product) containing large numbers of vegetative cells of a chromosomal *cpe* isolate of *C. perfringens* are ingested. These bacteria then multiply and sporulate in the small intestines. The sporulating cells produce an enterotoxin (CPE), which damages the small intestine. That intestinal damage induces the fluid and electrolyte losses responsible for diarrhea. (From Ref. 67.)

intestinal lumen when the mother cell releases its mature endospore by lysing at the completion of sporulation.

Substantial evidence now supports CPE as the major (if not only) toxin responsible for the symptoms of *C. perfringens* type A food poisoning. For example, epidemiological studies have demonstrated that CPE is present in the feces of virtually all *C. perfringens* type A food poisoning victims (10,11), usually at levels sufficient to cause gastrointestinal effects in animal models (15). Furthermore, ingestion of purified CPE by human volunteers fully reproduces the gastrointestinal symptoms of this food poisoning (16). Finally, recent studies have revealed that specific inactivation of the *cpe* gene in a human food poisoning isolate is sufficient to completely eliminate the gastrointestinal virulence of that isolate in animal models (17).

Once released into the intestinal lumen, the enterotoxin quickly binds to receptors present on the intestinal mucosa. Studies with animal models indicate the presence of CPE receptor(s) throughout both the small intestine and colon (18). It is now well established that this receptor(s) is proteinaceous, although the identity and number of different CPE receptor(s) mediating *in vivo* disease remains unsettled. Recent expression cloning studies (19–21) have demonstrated that certain claudins (a recently discovered family of ~22 kDa proteins found in epithelial tight junctions) can bind CPE and convey a cytotoxic response *in vitro*. Whether those claudins also mediate, in whole or in part, the intestinal pathophysiological response to CPE has not yet been established. In fact, some biochemical studies (22–24) support the possibility of alternative intestinal receptor(s) for CPE. Those biochemical studies showed that binding of CPE to intestinal brush border membranes results in rapid localization of the enterotoxin in a small (~90 kDa) complex that also contains an ~50 kDa eukaryotic protein. It remains to be determined whether that ~50 kDa eukaryotic protein (a) represents a CPE receptor, (b) is a protein that associates with CPE after the toxin has already bound to a claudin (or other) receptor, or (c) functions (together with other proteins, e.g., certain claudins) as a coreceptor for binding CPE to the intestines.

Localization of CPE in the ~90 kDa small complex is not sufficient to trigger a biological response (22,25). Instead, CPE toxicity occurs only when the small complex interacts with additional eukaryotic proteins to form even larger complexes in host cell plasma membranes (22,25–29). In isolated CaCo-2 human colonic carcinoma cells, at least three such larger CPE complexes have now

been identified (29): (a) an ~135 kDa complex, (b) an ~155 kDa complex, and (c) an ~200 kDa CPE complex containing the tight junction protein occludin. Some evidence suggests the presence of certain claudin(s) in one or more of these larger CPE complexes (19).

Formation of the larger CPE complexes has several biological consequences. First, their presence in enterocytes induces a rapid breakdown in the normal permeability properties of the plasma membrane (30). This effect causes a net influx of small (<200 daltons) molecules into the cytoplasm, resulting in cell death from either osmotic lysis or metabolic shut-down. Recent studies by Singh and McClane (29a) strongly suggest that formation of the ~155 kDa CPE complex is sufficient to trigger both CPE-induced membrane permeability alterations and death of the CPE-treated cell. Some evidence suggests that the ~155 kDa complex may correspond to a plasma membrane pore that permeabilizes plasma membranes and kills mammalian cells. For example, it has been shown that, when localized in large complex material, the enterotoxin closely associates with plasma membranes (31), which is consistent with the toxin inserting into plasma membranes as part of a pore.

The death of enterocytes from CPE-induced membrane permeability alterations produces histopathological damage to the small intestines (15,18,32–34). Animal model studies indicate that this small intestinal histopathological damage, which most notably includes epithelial cell desquamation (particularly at villi tips), is a prerequisite for obtaining CPE-induced fluid and electrolyte losses from the GI tract, i.e., for causing diarrhea. For example, studies have demonstrated (32) that only those CPE doses causing histopathological damage are capable of producing fluid and electrolyte losses from the rabbit ileum. Furthermore, the development of histopathological damage has been shown to closely coincide with the onset of fluid/electrolyte losses in the CPE-treated rabbit ileum (15). Finally, it has been reported (18) that, while CPE binds well to the rabbit colon, it does not induce either histopathological damage or fluid/electrolyte losses in that organ (whether CPE affects the human colon has not yet been studied).

Recent studies by Singh and McClane (29a) using Transwell cultures of polarized CaCo-2 cells suggest that the ~200 kDa complex develops more slowly than the ~155 kDa complex. One explanation for this observation is that CPE cannot access occludin [as required for formation of the ~200 kDa complex (29)] until CPE-induced cytotoxic effects (mediated by the ~155 kDa complex) have damaged the epithelium. Although formation of the ~200 kDa complex is relatively slow, it still may contribute to CPE action in vivo. For example, since the ~200 kDa complex contains CPE and tight junction structural components (including occludin and, possibly, certain claudins), formation of the ~200 kDa complex could conceivably damage tight junctions [as has been observed in CPE-treated liver (35)] and thereby trigger paracellular permeability alterations [as have been observed in MDCK cells treated with a CPE fragment (36)] that might contribute to the diarrhea associated with *C. perfringens* type A food poisoning.

## **B. Necrotic Enteritis**

The early steps in the pathogenesis of foodborne necrotizing enteritis have been most extensively studied in the highlands area of Papua New Guinea (5), where this illness starts with the consumption of food (usually pork) contaminated with type C isolates of *C. perfringens*. Presumably these contaminated foods contain significant numbers of *C. perfringens* cells in order for some vegetative cells to survive their transit through the acidity of the stomach.

Once present in the small intestines, the type C isolates apparently adhere to the mucosa, where they multiply (5). Necrotizing enteritis is considered primarily a disease of the jejunum, although the ileum may also be affected; the colon is not affected (5). During their in vivo multiplication, the type C vegetative cells express, and then secrete,  $\beta$ -toxin.

$\beta$ -Toxin is easily inactivated by intestinal proteases, such as trypsin (37). This susceptibility of  $\beta$ -toxin to proteases helps explain why necrotizing enteritis is rare in most human populations. However, the “high-risk population,” i.e., malnourished individuals on a protein-poor diet, are more susceptible to this illness because they produce significantly reduced amounts of intestinal proteases (5).

Another important factor also contributes to the endemic nature of foodborne necrotic enteritis in the Papua New Guinea highlands. The dietary staple in this region, i.e., sweet potato, is very rich in a trypsin inhibitor (5). The ingestion of high levels of sweet potato trypsin inhibitor by people on a low-protein diet is a key contributor to the pathogenesis of necrotizing enteritis since it inactivates the low trypsin levels present in the New Guinea highlanders, thereby rendering those individuals sensitive to the effects of  $\beta$ -toxin.

A wealth of evidence implicates  $\beta$ -toxin as the toxin responsible for most/all symptoms of Pig-Bel (9). For example, serological studies have shown that  $\beta$ -toxin antibody titers are elevated in surviving patients. Furthermore, the symptoms of necrotic enteritis can be reproduced by injecting purified  $\beta$ -toxin into animals. However, the most persuasive argument for the importance of  $\beta$  toxin in this illness is the clinical efficacy of a  $\beta$ -toxoid vaccine in Papua New Guinea (see Sec. VIII).

$\beta$ -Toxin is a potent toxin, with an LD<sub>50</sub> for mice of <100  $\mu$ g/kg (5). While the molecular action of  $\beta$ -toxin is still incompletely understood, emerging evidence suggests that  $\beta$ -toxin (like CPE) acts by altering the normal plasma membrane permeability properties of mammalian cells (38).  $\beta$ -Toxin has been shown to oligomerize (38), which suggests that the membrane permeability alterations in  $\beta$ -toxin-treated cells might result from toxin oligomers forming a pore in plasma membranes.

Presumably,  $\beta$ -toxin kills cells in the intestinal epithelium via these membrane permeability alterations, with that effect leading to the intestinal necrosis that characterizes this illness.

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

### A. *Clostridium perfringens* Type A Food Poisoning

To date, only two genetic traits have been linked to the virulence of *C. perfringens* food poisoning isolates: the ability to produce CPE (see Sec. VI) and the ability to form heat-resistant spores (and perhaps somewhat more heat-tolerant vegetative cells).

#### 1. Heat Resistance

A recent study reported that the spores of *cpe*-positive food poisoning isolates can, on average, survive boiling for over 30 minutes; in contrast, spores of *cpe*-positive *C. perfringens* nonfoodborne human GI disease isolates only survive boiling for about 1 minute (3). That same study also determined that at 55°C the vegetative cells of food poisoning isolates survive about twice as long as vegetative cells of *cpe*-positive nonfoodborne GI disease isolates.

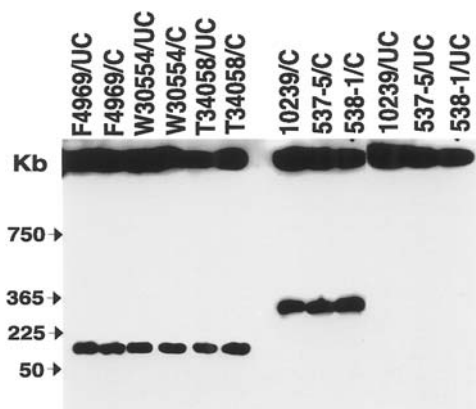
As briefly mentioned in Sec. II, the enhanced heat resistance of food poisoning isolates could contribute to food poisoning by facilitating survival of those isolates in incompletely cooked and/or in improperly held foods. The genetic or physiological basis for the greater heat resistance of the spores/vegetative cells from *C. perfringens* food poisoning isolates has not yet been determined.

#### 2. CPE Expression and Structure/Function Relationships

As described below, understanding of both CPE structure versus function relationships and *cpe* genetics/expression gained considerable momentum when the intact *cpe* gene of food poisoning isolate NCTC 8239 was cloned and sequenced in the early 1990s (14).

*a. CPE Structure/Function Relationships.* Sequencing of the cloned *cpe* gene from NCTC 8239 (14) determined that native CPE is comprised of a single 319-amino-acid polypeptide (35,317 M<sub>r</sub>) and also showed that the primary sequence of the enterotoxin is unique except for some limited shared homology (of unknown significance) with the nonneurotoxic Antp 70/C1 protein that is produced by some strains of *Clostridium botulinum*.

Exploiting the availability of the cloned *cpe* gene, recombinant DNA approaches clearly mapped receptor-binding activity to the 30 C-terminal amino acids of CPE and localized biological activity to amino acids 45–116 of the native toxin (25,26,39,40). The N-terminal 45 amino acids of native CPE were shown to be unnecessary for biological activity (26). In fact, removal of those sequences was found to increase CPE biological activity approximately twofold (26); a similar CPE activation



**FIGURE 2** Evidence that the *cpe* gene is chromosomal in food poisoning isolates, but is present on a plasmid in nonfoodborne human gastrointestinal disease isolates. The figure shows DNA from food poisoning isolates (10239, 537, and 538) and antibiotic-associated diarrhea isolates (F4969, W30554, and T34058) that was either cut (C) or uncut (UC) with *I-CeuI* before pulsed field gel electrophoresis and hybridization with a *cpe*-specific DNA probe. Because of the small size of plasmid DNA vs. chromosomal DNA, some *cpe*-containing DNA enters the pulsed-field gels even without restriction enzyme digestion in samples where the *cpe* gene is located on a plasmid. However, when the *cpe* gene is chromosomal, *cpe*-containing DNA only enters pulsed-field gels after a sample has been digested with restriction enzymes, such as *I-CeuI*. (Note: Since *I-CeuI* cuts only chromosomal DNA, the migration of plasmid-borne *cpe*-containing DNA is unaffected by digestion with that enzyme.) Thus, it can be concluded that antibiotic-associated diarrhea isolates F4969, W30554, and T34058 carry a plasmid-borne copy of the *cpe* gene, while isolates 10239, 537, and 538 have chromosomal *cpe* genes. (From Ref. 49 with permission.)

effect may occur in vivo, since intestinal proteases can also remove limited N-terminal amino acids from CPE and thereby produce an activated toxin (41,42).

*b. Molecular Aspects of cpe Genetics and Expression.* Using probes and primers derived from the cloned *cpe* gene, epidemiological surveys (43–45) determined that only a small fraction (<5%) of the global *C. perfringens* population carries the *cpe* gene. Most, but not all, of these *cpe*-positive isolates classify as type A (see Table 1), explaining (at least in large part) why nearly all CPE-associated human disease involves *cpe*-positive isolates belonging to type A.

Recent studies (3,17,46–49) also established that the *cpe* gene is present on the chromosome of food poisoning isolates but is located on a plasmid in nonfoodborne human GI disease isolates (see Fig. 2). Sequencing analyses determined that the *cpe* ORF sequence is identical in both the nonfoodborne human disease isolates carrying a plasmid *cpe* gene and the food poisoning isolates carrying a chromosomal *cpe* gene (48).

Other recent studies (50) demonstrated that, in vitro, the *cpe* plasmid of nonfoodborne GI disease isolates can be conjugatively transferred to other *C. perfringens* isolates. If the *cpe* plasmid can be similarly transferred in vivo to *cpe*-negative strains of *C. perfringens* found in the intestinal flora, that might explain why CPE-associated nonfoodborne human GI diseases is often a more prolonged and severe disease than *C. perfringens* type A food poisoning (51).

Considerable evidence suggests that, despite its chromosomal location, the *cpe* gene of food poisoning isolates is also associated with mobile genetic elements. For example, early pulsed-field gel electrophoresis (PFGE) studies by Cole's group (52) showed that the *cpe* gene of food poisoning isolate NCTC 8798 maps to a highly variable region of the *C. perfringens* chromosome, suggesting that the *cpe* gene is present on a mobile genetic element that has integrated into a "hot spot" on the *C. perfringens* chromosome. More recently, sequencing analyses of food poisoning strain NCTC

8239 detected putative insertion sequences lying upstream and downstream of the *cpe* gene (53,54). Based upon those findings, it has been proposed (53,54) that the *cpe* gene of NCTC 8239 is located on a 6.3 kb transposon that has integrated into the chromosome.

Duncan's group first suggested that CPE expression is sporulation-associated; for example, they reported that mutants of a *C. perfringens* food poisoning strain with a block at stage 0 of sporulation also fail to produce any CPE (55). The putative association between sporulation and CPE expression was subsequently confirmed, for both chromosomal *cpe* isolates and plasmid *cpe* isolates, by studies using rigorous Western blot approaches (14,48). Those Western blot studies revealed that sporulating cultures of food poisoning strain NCTC 8239 produce at least 1300-fold more CPE during sporulation versus vegetative growth.

The molecular basis for the highly regulated expression of CPE is now coming under study. When a low-copy shuttle vector carrying the cloned *cpe* gene was introduced into several different *cpe*-negative *C. perfringens* strains, the resultant transformants were found to express CPE during sporulation but not during vegetative growth (56). This finding strongly suggests that most (or all) sporulation-capable *C. perfringens* isolates produce some (or all) of the regulatory factor(s) responsible for the normal, sporulation-associated expression of CPE. Thus, it appears that some (or all) regulators of *cpe* expression also modulate the expression of other *C. perfringens* genes.

One such potential "global" regulator, Hpr, has already been implicated in the modulation of CPE expression (57). Hpr-like binding sequences have been identified both upstream and downstream of the *cpe* ORF. Furthermore, DNA from many *C. perfringens* strains have been shown to hybridize an *hpr*-specific gene probe. Since the Hpr protein is known to repress gene expression during exponential growth of *Bacillus subtilis*, it is conceivable that a clostridial Hpr could repress CPE expression during vegetative growth of *C. perfringens*.

Kinetic studies demonstrated that CPE expression starts soon after the induction of sporulation and then increases throughout the next 6–8 hours (58,59). By 6–8 hours of sporulation, CPE represents more than 15% of the total protein present in many sporulating *C. perfringens* cells (14). At least in vitro, both chromosomal *cpe* isolates and plasmid *cpe* isolates appear to produce similar levels of CPE during sporulation (48).

RNA slot blot and Northern blot studies (56,58) indicated that CPE expression is regulated at the transcriptional level, i.e., *cpe* message is detectable during sporulation, but not during vegetative growth. The ~1.2 kb size of *cpe* mRNA (56) suggests the *cpe* gene is transcribed as a monocistronic message. This suggestion is further supported by (a) the presence of a stem-loop structure, with features of a rho-independent transcriptional terminator, immediately downstream of the *cpe* ORF (14), and (b) primer extension analyses, RNase T2 protection assays, and deletion mutagenesis results (58,60) indicating that *cpe* mRNA transcription initiates immediately upstream of the *cpe* ORF. Those transcription initiation studies also identified at least three different *cpe* transcription start sites (named P1, P2, and P3). Interestingly, P1 shares some homology with SigK-dependent promoters, while P2 and P3 share some homology with SigE-dependent promoters. Since SigK and SigE are sporulation-associated sigma factors in *B. subtilis*, clostridial homologs of these alternative sigma factors might play an important role in regulating CPE expression, i.e., perhaps these alternative sigma factors are the global regulators that turn on CPE expression during sporulation.

A final important question about CPE expression should be addressed: Why do some *C. perfringens* strains produce so much CPE? One contributing factor to high-level CPE expression could be the stability of *cpe* mRNA. An older study (61) reported that the functional half-life of the *cpe* message is ~58 minutes, which is exceptionally long for a prokaryotic message. This putative *cpe* message stability might be due, at least in part, to the presence of the stem-loop structure downstream of the *cpe* ORF (14); similar stem loops are known to contribute to stability of other mRNAs.

## B. Necrotic Enteritis

Thus far only two pathogenic properties have been implicated in the pathogenesis of necrotic enteritis: the adhesion of *C. perfringens* type C isolates to the intestinal mucosa and the production of  $\beta$ -toxin by those adherent isolates (5).

Unfortunately, little is currently known about the genetic or physiological basis by which type C isolates adhere to the intestines.

With respect to  $\beta$ -toxin, early studies (62) had strongly suggested that the *cpb* gene encoding  $\beta$ -toxin is present on a large plasmid present in type C and type D isolates of *C. perfringens*. More recent studies (63,64) using molecular approaches have confirmed that finding and also indicated that the plasmid-borne *cpb* gene is associated with an insertion sequence. While it seems clear that maximal  $\beta$ -toxin expression occurs during the exponential phase of vegetative cell growth (5), the molecular regulation of  $\beta$ -toxin synthesis has not yet been intensively studied.

The cloning and sequencing of the *cpb* gene in the 1990s (63,64) provided some important insights into both  $\beta$ -toxin's action and structure/function relationship. Results from those cloning studies indicate that  $\beta$ -toxin is first expressed as a single polypeptide consisting of 336 amino acids. An N-terminal signal peptide of 27 amino acids is then removed during secretion, yielding a mature toxin of 309 amino acids (34,861 M<sub>r</sub>). As mentioned earlier (Sec. VI), recent evidence (38) suggests that this  $\beta$ -toxin monomer can oligomerize, with that oligomerization process possibly contributing to pathogenesis.

Sequencing studies of the cloned *cpb* gene (63) also revealed that this toxin shares ~28% homology with another spore-forming toxin, *Staphylococcus aureus*  $\alpha$ -toxin. Based upon this shared sequence homology and results from previous studies mapping *S. aureus*  $\alpha$ -toxin structure/function relationships, site-directed mutagenesis was performed on *cpb* (64). Those mutagenesis studies produced results supporting the hypothesis that *C. perfringens*  $\beta$ -toxin and *S. aureus*  $\alpha$ -toxin share partial, although not complete, similarity in their structure and action. The previously mentioned findings (38) indicating that, like *S. aureus*  $\alpha$ -toxin,  $\beta$ -toxin can oligomerize and affect membrane permeability properties in mammalian cells, provide some additional evidence that these two toxins share some similarities in their action and structure.

Additional mutagenesis experiments (64) demonstrated that the single cysteine residue located at residue 265 of mature  $\beta$ -toxin is not important for action. However, those same mutagenesis studies did indicate that the region surrounding that cysteine 265 residue may be important for maintaining a proper secondary structure for  $\beta$ -toxin activity.

## VIII. CONTROL MEASURES

### A. *Clostridium perfringens* Type A Food Poisoning

While a possible CPE toxoid vaccine candidate has been identified (65), the relatively mild nature of most cases of *C. perfringens* type A food poisoning precludes the need for widespread vaccination. Therefore, the most effective method for controlling this foodborne illness is to prevent the contamination of foods with pathogenic levels of chromosomal *cpe* isolates.

Prevention is best achieved by two approaches. First, it is critical to thoroughly cook foods. This is particularly true for large meats (such as turkey and beef roasts) where it is difficult to generate the high internal temperatures necessary to kill the heat-resistant spores of chromosomal *cpe* isolates. Second, cooked foods must be quickly cooled and stored at conditions that do not support the growth of *C. perfringens* vegetative cells (e.g., under refrigeration or at temperatures >70°C).

Since *C. perfringens* type A food poisoning typically self-resolves within 12–24 hours, treatment of this illness is only symptomatic, i.e., antimicrobial therapy is not necessary.

### B. Necrotic Enteritis

It is possible to actively immunize people against  $\beta$ -toxin using a type C toxoid (5). The effectiveness of this vaccine is now well documented: when this vaccine was introduced into Papua New Guinea in 1980, an 80% reduction in necrotizing enteritis cases soon followed (5).

Active cases of necrotic enteritis are usually managed by combining antimicrobial therapy (pen-

icillin, chloramphenicol, and metronidazole have all been used against this illness), intravenous fluids, and (in many cases) surgical resection of the affected intestinal segment to remove necrotic tissue and bowel obstruction (9). Most patients are managed conservatively for 1–2 days before surgery is attempted; more severe cases often require more rapid surgical intervention (9).

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# 7

## *Listeria monocytogenes*

Atin R. Datta

*U.S. Food and Drug Administration, Rockville, Maryland, U.S.A.*

### I. BACKGROUND

*Listeria monocytogenes* (*Lm*), the causative agent of human and animal listeriosis, has become one of the most studied microorganisms in the last 20 years. Although the organism was reported as early as 1891, Murray first described this organism as a causative agent for animal disease in 1926. Since the pathogen caused monocytosis as one of the symptoms, the organism was named *Bacterium monocytogenes* and later changed to *Listerella monocytogenes* in honor of Lord Lister. The name *Listeria monocytogenes* was finally accepted in 1940. The first reported isolation of the predecessors of *Listeria* goes back to 1929. However, interest in this organism rapidly grew following a series of foodborne outbreaks in the 1980s. The high mortality rate involved in these outbreaks has drawn the attention of people involved in food safety. A tremendous amount of knowledge has been accumulated in the area of listeriosis and about the organism *Lm*. Several in-depth reviews on *Listeria* and listeriosis have been published in the last 10 years (1–4). The current article is intended to provide an up-to-date and comprehensive account of human listeriosis and its causative agent *Lm*. Because of space limitation, wherever possible, individual references have been replaced by an appropriate review article. For specific in-depth information, readers are advised to consult individual references, including review articles.

### II. CHARACTERISTICS

#### A. Physical Description and Growth Conditions

*Lm* is a gram-positive, micro-aerophilic, non-spore-forming rod, measuring 0.4–0.5  $\mu\text{m}$  in diameter and 0.5–2  $\mu\text{m}$  in length. In 3- to 5-day-old cultures, long filamentous structures are often encountered. *Lm* is actively motile by means of four peritrichous flagella. The tumbling motility is characteristic of *Listeria* and often used as a conventional marker for *Listeria* identification. The degree of motility is temperature dependent. Motility is best expressed when growth temperature is between 20 and 25°C. At 37°C and higher, flagellin production is considerably reduced, thereby reducing motility (1,2). *Lm* is not a fastidious organism and grows well in most common nutrient media, including brain heart infusion broth (BHI), trypticase-soy broth with 0.6% yeast extract (TSBYE), Luria broth (LB), etc. Optimum growth temperature is 35–37°C, although *Lm* grows reasonably well at temperatures as low as 4°C. The growth rate reduces as the growth temperature decreases. The pH range for the optimum growth is 5–9. On agar media, *Lm* colonies are translucent with a characteristic blue-green sheen when viewed by obliquely transmitted light. On sheep blood agar at 37°C, colonies are slightly smaller than on TSBYE or BHI agar and produce a weak zone of hemolysis. *Lm* can also be cultured in defined synthetic and semi-synthetic media, but growth rates are much slower than in nutrient-rich media (1,2).

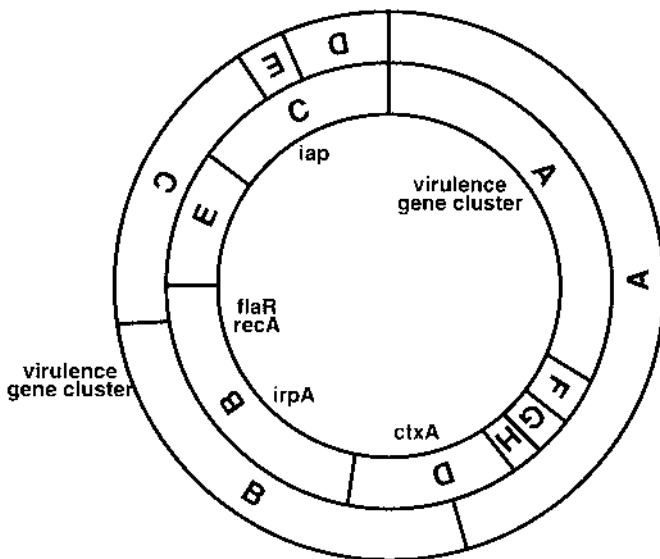
## B. Taxonomy and Biochemical Properties

*Lm* has been classified together with *Lactobacillus*, *Erysipelothrix* under the genus *Listeria* in the family Corynebacteriaceae. According to the 1984 edition of Bergey's *Manual of Systematic Bacteriology*, the genus consisted of eight species: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. murrayi*, *L. grayi*, *L. welshemiri*, and *L. denitrificans*. Based on the numerical taxonomy, DNA homology, and 16S ribosomal RNA sequences, *L. denitrificans* was transferred to a separate genus *Jonesia* as *J. denitrificans*. More recently, *L. murrayi* and *L. grayi* have been merged into one species, *L. grayi* (3,5).

*Lm* is catalase positive, ferments rhamnose, dextrose, esculin, and maltose, but does not ferment xylose and manitol. These biochemical properties are used for the differentiation of *Lm* from other members of the genus *Listeria* and from related organisms like *Erysipelothrix*, *Kurthia*, *Brochothrix*, and *Streptococcus*. *Lm* is also differentiated from other hemolysin-producing *Listeria*—*L. ivanovii* and *L. seeligeri*—by CAMP reaction. In this test, unlike other hemolysin-producing *Listeriae*, *Lm* produces a synergistic zone of hemolysis in presence of *Staphylococcus aureus* hemolysin on sheep blood agar (1,2).

## C. Genome and Plasmid

*Lm* contains a single, circular chromosome with 36–42% G + C content (3). Based on macrorestriction enzyme (NotI and SseI) fragment length analysis, the total length of a *Lm* serotype 1/2c strain genome was calculated to be 3150 kb (6). NotI and AscI restriction fragment length analysis of ScottA (serotype 4b) (7) and EGD (serotype 1/2a) (8) strains also resulted in similar genome sizes. All these groups used PFGE and DNA hybridization data to create a circular physical map of *Lm* and identified several gene locations on these maps. Based on the data published by these three groups, a schematic diagram of the *Lm* physical genomic map is presented in Figure 1. From this figure it is clear that although the total length of the genome is similar, the location of NotI fragments in the Scott A strain is different from the ones obtained from LO28 (1/2c) and EGD (1/2a) strains.



**FIGURE 1** Diagrammatic representation of NotI restriction fragments in *L. monocytogenes* chromosomes. Outside circle represents Scott A chromosome (8), while the inside circle represents 1/2c (6) and 1/2a (7) chromosomes. Fragments containing the virulence gene clusters also contain *inlA* and *inlB* genes.

The position of virulent gene clusters is also similar in LO28 and EGD strains, while it is different in Scott A strain. Whether these differences between serotype 1 and 4 really represents distant genetic relatedness or not requires further studies and sequencing information of some conserved genes. Recently, Glasser et al. have published (9) the complete genome sequences of an *Lm* serotype 1/2a (GenBank/EMBL accession number AL592022) and an *L. innocua*, serotype 6a strain (GenBank/EMBL accession number AL592102). Chromosome sizes and G+C content were similar in both these strains. While the majority of the coding sequences were similar, the *Lm* strain contained 270 (294 kb DNA including 10 kb virulence locus) and the *L. innocua* strain contained 149 (195 kb DNA) strain specific genes. The Institute of Genomic Research (TIGR) in Maryland is currently sequencing a 4b strain of *Lm*.

Several reports indicated the presence of plasmids of different sizes in *Lm* strains (9a–11). Some of these plasmids are cryptic (9a), while others carry antibiotic resistance (11) and cadmium resistance genes (10). No association of these plasmids with *Lm* virulence has been established so far.

## D. Typing

Based on somatic and flagellar antigens, Patterson first proposed four serotypes to classify all *Listeriae*. Later, Donker-Voit and Seeliger expanded the scheme into seven serotypes, some containing subtypes, e.g., 4a, 4b, 4c, etc. (1–3). The serotypes are not restricted to particular species of *Listeria* except serotype 5, is only reported in *L. ivanovii*. Although serotyping provides a useful and convenient way to classify *Lm* strains, its usefulness in human listeriosis cases is severely limited by the finding that about 95% of all clinical *Lm* isolates belong to 1/2a, 1/2b, and 4b (1–3). To overcome this problem and to aid in the outbreak investigation, several other typing schemes, including phage typing, isoenzyme typing, monocine typing, and plasmid typing, were developed. With the advent of recombinant DNA technology, several DNA-based typing methods were developed. All of these methods are based on DNA restriction fragment length polymorphism (RFLP). Of these DNA-based methods, RFLP by pulsed field gel electrophoresis (PFGE) is the most widely used for typing *Lm* isolates. The other methods are ribotyping, random amplification of polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) (12). Several workers compared different typing methods and concluded that RAPD, PFGE, and ribotyping probably provide maximum discrimination (13,14). The PFGE patterns of *Lm* have been extensively used in epidemiological investigations throughout the world (15). In the United States, a computer-based PFGE pattern matching and early warning system called PulseNet has been developed to quickly identify outbreak strains including *Lm* (16). Although DNA sequencing can provide the ultimate discrimination between isolates, its usefulness in epidemiological investigation is yet to be proved.

## E. Survival and Growth

*Lm* has been isolated from a variety of foods containing dairy, meat, seafood, eggs, and vegetables (1,2). These products include processed foods that require cooking or minimum preparation or are ready to be consumed. Because all cooking processes require some amount of heating, it is generally accepted that cooked food will contain the least amount of viable listeriae. *Lm* can grow at 2.5–44°C, with a temperature optimum of 35–37°C. The growth rate at low temperatures commonly used for storage depends on nature of food. In general, high-moisture food (e.g., soft cheese, milk) would support *Lm* growth with a doubling time of 1–2 days (1,2). Although experimentally *Lm* was not found to be unusually heat resistant, the involvement of pasteurized milk in an outbreak in the United States created great interest in studying the heat resistance of this organism and reevaluation of the commercial milk pasteurization process to make sure that the current pasteurization process is adequate for killing. Data collected by different researchers using a variety of experimental conditions indicated that *Lm* is not unusually thermotolerant and that current milk pasteurization protocol is adequate (17). However postpasteurization contamination followed by storage at 4–6°C could enhance the chances of *Lm* contamination. Although *Lm* rarely grows at a temperature below 0°C, the survival of *Lm* in foods stored below 0°C has been reported. In one study (18) a Mexican-

style soft cheese was found to contain more than a million organisms per gram of food even after the cheese was stored at  $-70^{\circ}\text{C}$  for 3 years. The survival at low temperature depends on the amount of moisture, pH, and osmolarity of the food (19,20). Several studies have reported the genes and physiological conditions that can alter the thermotolerance (21,22), cryotolerance (23), and osmotolerance (24–26) of *Lm*. Of particular interest is the description of heat-shock process and heat-shock genes in thermotolerance (27,28). Several researchers identified genes that alter the survival and growth of *Lm* at low temperatures (29,30). Further studies are needed to understand the mechanisms and factors that control the ability of *Lm* to grow and survive at low temperatures.

*Lm* has been isolated from various acidic foods including sausage, cheese, cole slaw, etc. Experimentally, *Lm* was found to grow at pH 5–9 in cabbage (1,2). Several investigators studied the acid tolerance of *Lm* under experimental conditions in order to understand *Lm* survival in low-pH foods and in the stomach and intestine (31–33). In TSBYE, *Lm* was found to be very sensitive to low pH;  $<0.0001\%$  of *Lm* survived when exposed to pH 3.0 at  $37^{\circ}\text{C}$  for 1 hour, in contrast to 100% survival of *Shigella* and *Escherichia coli* O157:H7 under identical condition (33). From these and other studies, it was argued that *Lm* would probably be inactivated more easily in stomach and intestinal pH than *Shigella* and *E. coli* O157:H7 and hence would have a higher infective dose than the other two pathogens. The acid tolerance of *Lm* is inducible; prior exposure to low pH resulted in higher tolerance and vice versa (31–33). The implication of this finding is that *Lm* in low-pH food would probably have lower infective doses than *Lm* in neutral- or high-pH foods. Several workers identified genes that control the acid tolerance of *Lm* (34–38); some of these genes are also involved in *Lm* virulence (36–38).

### III. DISEASE

Human listeriosis is one of the few foodborne diseases where the fatality rate often reaches as high as 30–40%. Based on the susceptible population, the human listeriosis can be classified into two groups: adult and neonatal listeriosis. Most adult listeriosis cases are reported to have underlying diseases leading to immunocompromised state, although a few outbreaks involving “healthy” individuals have also been reported (3,4). These reports and the high incidence of listeriosis cases among people suffering from acquired immunodeficiency syndrome (AIDS) indicate that T-cell-mediated immunity plays an important role in human listeriosis. This conclusion has also been supported by various experimental studies. Symptoms of adult listeriosis can be classified into two phases. During the initial enteric phase, patients experience mild flu-like symptoms with occasional diarrhea. The enteric phase occurs within 1–2 days of ingestion of contaminated food. Pregnant woman infected with *Lm* may have an early onset of flu-like symptoms, which are often misdiagnosed as influenza. Although most pregnant women recover without any complications, maternal infection can lead to neonatal infection. The invasive phase of adult listeriosis is characterized by septicemia, meningitis, and endocarditis. The average incubation time varies from 2 to 4 weeks after the day of infection. Although early diagnosis can be successfully treated with antibiotics, the fatality rate of untreated or late-treated meningitis cases is often as high as 70%. Listeria infection can also cause abscess of brain and liver. Several recent outbreaks have demonstrated that *Lm* infection can result in febrile gastroenteritis and the organisms can be isolated from stool specimens (4). As with other foodborne gastroenteritis, the disease is self-limiting, with early onset and very high attack rate (70–80%).

Neonatal listeriosis accounts for almost 40% of all human listeriosis cases (2,4). Early-onset listeriosis with incubation time of 1–2 days can result in abortion, stillbirth, or premature delivery of a severely infected infant. *Lm* can be cultured from the blood, cerebrospinal fluid (CSF), and skin of the affected fetus and also from the placenta. This form of infection, resulting in pustular skin lesions, is called granulomatous infantiseptica. Late-onset listeriosis occurs 7–20 days after delivery, with meningitis as the primary symptom. Organisms can readily be isolated from the CSF of the affected child. Even with the availability of antibiotic therapy, 10% of late-onset cases die because of the advanced stage of the illness. It is likely that some of the cases became infected during passage through the genital tract.

Current knowledge of the infective dose (ID) of *Lm* is very sketchy. Based on our understanding of the disease process, the virulence mechanisms, and the physiology of the organism, it is clear that ID depends on several factors. Also important in this process are the host factors, which modulate the susceptibility to *Lm* infection. Based on epidemiological studies, the foods implicated in various outbreaks often contained  $10^3$ – $10^6$  CFU/g. Several animal studies aiming to obtain minimum ID resulted in variable numbers. The ID<sub>50</sub> in orally fed mice was found to be  $10^3$ – $10^8$  in various studies (2). This number could be reduced by 1–2 log if the gastric pH was elevated before the oral inoculation (4). This study provided credence to the generally accepted hypothesis that in foodborne diseases, the stomach pH provides a first line of defense and bacterial tolerance to low pH plays an important role in determining the ID of that particular organism. In vitro studies showed that *Lm* has much lower acid tolerance than *Shigella* and *E. coli* O157:H7 (33). Based on these acid-tolerance studies, one could probably argue that *Lm* will have a much higher ID than *Shigella* or *E. coli* O157:H7. Lowering of ID in a mice model was also noticed with treatments that suppress immune response. In nonhuman primates, at least  $10^9$  organisms were needed for the development of any noticeable symptom (2). A pregnant monkey model is currently being evaluated to obtain data about how pregnancy affects ID. Recently Lecuit et al. (39) reported the use of transgenic mice expressing human E-cadherin as a model for listeriosis. Further work is needed to evaluate the usefulness of such model.

#### IV. EPIDEMIOLOGY

Listeriosis cases have been reported from almost all the parts of the world. The current estimation of the incidence of listeriosis is 2–15 cases per million of population (40). These numbers include both sporadic and outbreak cases. It is clear that the precision of the estimation depends on accurate diagnosis and reporting. The estimation of sporadic cases in the United States was obtained by an active surveillance system called FoodNet. The system covers 20.5 million people in several states. Results from recently published FoodNet studies estimate 2518 cases of listeriosis per year, with a case fatality of about 500. This number is approximated to be 10 cases per million of population (40). A vast majority of the sporadic listeriosis cases were associated with the consumption of deli-type meat and cheese products (41). In contrast, the outbreak cases were linked to several different kind of foods.

A look at major listeriosis outbreaks (Table 1) indicates several interesting features. These

**TABLE 1** Summary of Major Foodborne Listeriosis Outbreaks

Year	Location	Number of cases (% mortality)	Serotype	Implicated food
1980–81	Canada	41(34)	4b	Coleslaw
1983	Massachusetts	49(29)	4b	Pasteurized milk
1984	Switzerland	57(32)	4b	Soft cheese
1985	California	142(34)	4b	Jalisco cheese
1987–89	UK	823(?)	4b, 4bX	Pate
1992	France	38(32)	4b	Rillettes (pork)
1993	Italy	39(0)	1/2b	Rice salad
1994	Illinois	45(0)	1/2b	Chocolate milk
1997	Italy	1566(0)	4b	Corn salad
1998–99	USA	101(21)	4b	Hot dogs, deli meats
1998–99	Finland	25(24)	3a	Butter
1999	France	32(31)	—	Pork tongue
2000	USA	29(7)	1/2a	Deli turkey meat



include (a) the variety of foods implicated, (b) the symptoms associated, and (c) the serotypes of *Lm* involved. *Lm* has been isolated virtually from all kinds of food, including those of plant and animal origin. Because *Lm* can survive in a wide range of environmental conditions, it is not surprising that it is recovered from soil, water, and food-processing environments. However, to be able to cause disease, it is expected that a particular food and its storage condition must provide a suitable environment for survival and, at times, growth. It is clear from Table 1 that all foods associated with these outbreaks were consumed without further processing or after minimum heat treatment (e.g., hot dogs) and that many of these foods provide a suitable environment for growth (e.g., soft cheese).

Although all the serotypes of *Lm* are virulent and considered potential pathogens, outbreak investigations clearly show that the predominant serotypes are 1/2a, 1/2b, and 4b. This is also true of sporadic cases. The recent outbreak in Finland involving patients in a hospital setting is the only known case involving a serotype other than 1 or 4 (42). It is not clear why other serotypes are underrepresented among clinical isolates. It is possible that serotypes other than 1 and 4 survive poorly in food and/or inside the host. It is also likely that strains belonging to these serotypes lack some essential function needed to establish a successful infection.

As discussed earlier, the most common symptoms of listeriosis are septicemia and meningitis, with occasional flu-like symptoms at the very early phase of infection. However, in several outbreaks of listeriosis, the symptoms were restricted to febrile gastroenteritis (see Table 1). These outbreaks were characterized by high attack rates (70–80%), no mortality, and early onset (within 24 hours of ingestion of contaminated food) involving healthy adults (43). Thus, it appears that *Lm* infection can result either in gastroenteritis or in classical symptoms of septicemia, meningitis, etc., depending on the *Lm* strain and/or the infected population. A recent study (44) indicated the presence of unique sequence differences between isolates involved in invasive (septicemia, meningitis, etc.) and noninvasive (gastroenteritis) isolates. Because gastroenteritis is not commonly associated with *Lm* infection, it is not clear how many sporadic gastroenteritis cases are due to *Lm* infection.

## V. PATHOGENICITY

The genus *Listeria* consists of seven species, of which *Lm* is the only human pathogen. Although two other species—*L. ivanovii* and *L. seeligeri*—also contain known virulent factors, these species were rarely associated with human illnesses. *L. ivanovii* causes animal listeriosis (1,2). Beside species difference and strain variability, host factors play a very important role in pathogenicity. Most cases of adult listeriosis are associated with some underlying disease condition compromising the immune system. AIDS patients have been reported to have a 100- to 300-fold higher incidence of listeriosis than the rest of the population (4). Recent reports describing human infections associated with *L. ivanovii* (45) and *L. murrayi/grayi* (46) in patients suffering from AIDS and Hodgkin's disease again emphasize the importance of host immunity in human listeriosis.

A successful foodborne *Lm* infection requires that the organisms survive in the stomach and intestine and attach to and invade the intestinal cells. Once internalized, the organism then must survive the cellular immune response before multiplication and cell-to-cell spread in susceptible organs. Our understanding of all these steps of *Lm* pathogenicity has been greatly enhanced in recent years by studies with animal models, in vitro cell culture studies, and identification and characterization of mutants and genes affecting different steps during the infection process (3,4). Animal studies were conducted by both intraperitoneal and intragastric routes. Although a majority of studies was carried out following intraperitoneal injection, inoculation through the oral route provides more similarity with the foodborne disease. Following ingestion of *Lm*, bacteria probably enter through the cells in Peyer's patches through either M (macrophage) or E (epithelial) cells. Once internalized, *Lm* survives and replicates inside the cytoplasm and eventually is transmitted via blood to lymph node, spleen, and liver. At that point, within several hours most of the organisms are destroyed by the cellular immune system. Survivors then begin to multiply exponentially inside

macrophages for 2–3 days until the host clears the infection. This is achieved by the development of cellular immunity against specific antigens of *Lm*. Humoral antibody does not play a role in the development of host immunity against *Lm* infection. In animals where cellular immunity is not developed, the infection spreads through blood, lymph, and crosses the blood-brain barrier and placental barrier in the case of a pregnant animal. This ensures a more severe and occasionally fatal form of listeriosis, unless prevented.

## A. In Vitro Studies

Several in vitro studies have been developed to study the infection process of *Lm*. *Lm* has been shown to invade macrophage, fibroblast, hepatocyte, and epithelial cell lines. Studies from different laboratories using electron microscopy have elucidated the broad steps of *Lm* infection process from invasion to multiplication to cell-to-cell spread (47). The first step in this process is internalization by professional phagocytes (e.g., macrophage) or by nonprofessional phagocytes (e.g., hepatocyte). Several minutes after entry, bacteria are found inside vacuoles. Within the first hour of internalization, most (80–90%) bacteria are killed, probably by the combined effect of low pH, oxidative radicals, and hydrolytic enzymes. Lysis of vacuolar membrane results in the release of surviving bacteria into the cytosol, where they multiply, with a doubling time of 40–60 minutes, depending on cell lines. The intracytoplasmic bacteria then surround themselves with cellular actin molecules, which then start forming actin-like filaments from one pole of these organisms. The polymerization of actin filaments results in the formation of comet tail–like structure. The formation of actin tails propels bacteria towards cell membrane at a speed (0.1–1  $\mu\text{m}/\text{sec}$ ) proportional to the length of actin tail. The tail can be as long as 40  $\mu\text{m}$ . Movement of bacteria to the plasma membrane results in formation of a protrusion into the neighboring cells (48). Each protrusion containing one bacterium then is internalized in the neighboring cell, creating a double membrane vacuole containing a single organism. Lysis of double membrane releases the organism, which then undergoes multiplication and cell-to-cell spread as before. The entire process takes about 5 hours. The cell-to-cell spread thus achieved without leaving the cell explains why the circulating antibodies are of little significance to combat listerial infection.

With the advent of recombinant DNA technology combined with in vivo and in vitro studies, it was possible to dissect various steps of infection process in terms of involvement of specific genes and their products in these steps. Although the picture is far from complete, with the help of mutants defective in virulence it is now possible to assign several genes to different stages of the infection process (47). A list of virulence-associated genes and their properties is shown in [Table 2](#).

The first step in the process is the internalization of *Lm* within M or E cells. In vitro studies have resulted in the identification of several gene products collectively termed as internalins because their involvement with the internalization processes (47). InlA, a product of the *inlA* gene, is required to invade epithelial cells, whereas InlB, a product of the *inlB* gene, is involved in hepatocyte invasion (50). A recent study has shown that InlB is also required for the adhesion and invasion of human umbilical vein endothelial cells (51). The entry occurred over the entire surface of the endothelial cells. Reduced growth of *inlA* and *inlB* mutants in spleen and liver of infected animals clearly demonstrated the role of these genes in *Lm* pathogenicity. InlA and InlB are surface proteins, coded by two adjacent genes *inlA* and *inlB*, respectively, located in a region separated from the 10 kb chromosomal region called virulence gene cluster or *vgc*, harboring the rest of the known virulent genes (47). The *inlA* gene codes for an 800-amino-acid protein, while the *inlB* gene codes for a 630-amino-acid protein. Both proteins have homologous signal sequence and C-terminal ends containing leucine-rich repeats. E-cadherin, a transmembrane glycopeptide, acts as an InlA receptor on the mammalian cell surface (47). The identity of the InlB receptor is still unknown. The presence of residual invasion in mutants deleted for both *inlA* and *inlB* genes indicates the involvement of yet other unknown invasion genes. Recently several groups have reported the identification and partial characterization of additional internalin like genes in *Lm* (52–54). Although *Lm* strains carrying mutations in *irpA* (*inlC*) or strains carrying deletion in the *inlGHE* region exhibited reduced viru-

**TABLE 2** Properties of Some Known Virulence-Associated *Listeria monocytogenes* Genes

Gene	Property
<i>hly</i>	58 kDa SH-activated hemolysin; mutants are avirulent, unable to lyse phagolysosomal membrane and unable to multiply; invasion is normal; involved in antigen processing, apoptosis
<i>plcA</i>	Phosphatidyl inositol specific phospholipase C 38-kd protein; polar mutation results in reduced virulence
<i>plcB</i>	Lecithinase; 29 and 32 kDa polypeptides
<i>mpl</i>	Lecithinase-specific metalloprotease; mutants are of reduced virulence and produced only 32 kDa lecithinase polypeptide
<i>actA</i>	90 kDa surface protein; mutants do not form plaques in mouse fibroblast, do not nucleate actin polymerization
<i>inlA</i>	Internalin; 88 kDa protein (744 amino acids); mutants do not invade cultured epithelial cells
<i>inlB</i>	Internalin; 65 kDa protein (630 amino acids) invasion protein; gene product involved in hepatic phases of infection, replication.
<i>inlC(irpA)</i>	30 kDa protein; homologous to InlA, InlB proteins. Mutants are of reduced virulence
<i>prfA</i>	Transcriptional activator of <i>hly</i> , <i>plcA</i> , <i>plcB</i> , <i>actA</i> , <i>inlA</i> , <i>inlB</i> , and <i>mpl</i> 27 kDa DNA-binding protein, member of CAP/FnR family
<i>imt</i>	Mutant is defective in intracellular motility; actin polymerization is normal but cannot rearrange to generate movement; avirulent
<i>oppA</i>	62 kDa oligopeptide-binding protein; involved in intracellular survival and growth
<i>clpC</i>	100 kDa transcriptional modifier; involved in adhesion and invasion, modulates expression of <i>inlA</i> , <i>inlB</i> , and <i>actA</i>
<i>iap</i>	Extracellular invasion-associated protein, also known as p60 (60 kDa molecular weight protein); mutants are defective in fibroblast entry; reduced virulence in mice

lence in animal models, their role in the invasion process remained unclear. A major secreted protein of *Lm*, p60 has been shown to be necessary for adhesion and invasion of 3T6 mouse fibroblast cells but not for Caco-2 epithelial cells (49). The 60 kDa protein is coded by a *iap* gene that has been mapped and found to be separated from the virulent gene cluster in *Lm* chromosome (Fig. 1). Rough mutants impaired in p60 synthesis are defective in virulence, produce long chains separated by double septa. Additionally, mutations in the *iap* gene were associated with decreased viability and reduced lecithinase activity (55).

Following internalization, *Lm* becomes trapped inside vacuoles. Survival of *Lm* inside these vacuoles results from concomitant lysis of the vacuolar membrane releasing the *Lm* in cytoplasm. Numerous studies had indicated that the lysis of vacuolar membrane is achieved by a 58 kDa sulfhydryl (SH)-activated, pore-forming extracellular protein called listeriolysin, similar to hemolysins found in *Streptococcus* and *Clostridium perfringens* (2,3,49). Listeriolysin is coded by the *hlyA* gene located in the virulence gene cluster. Mutants defective in listeriolysin production or mutants producing listeriolysin lacking PEST-like sequence (P, Pro; E, Glu; S, Ser; T, Thr) are also defective in intracellular multiplication and virulence (3,49,56). Besides its pivotal role in lysis of vacuolar membrane, listeriolysin is also involved in antigen processing (57) and apoptosis (58). Besides listeriolysin, products of *plcA*, *plcB*, *mpl*, and *inlB* (Table 2) are also necessary for lysis of primary vacuole and release of *Lm* into cytoplasm (59–61). Intracellular multiplication is a prerequisite for successful *Lm* infection. Following release from the vacuoles, *Lm* starts multiplying immediately. Cytoplasmic content is rich in all the ingredients necessary to support *Lm* multiplication. This is confirmed by the finding that the auxotrophic mutants requiring a variety of metabolites showed very little effect on intracellular multiplication and virulence (62). An oligopeptide-binding protein,

OppA, has been shown to be involved in survival and growth in organs infected with *Lm* (63). Whether the effect is due to reduced uptake of peptides inside the cell or some other function is not known. The *iap* gene product p60 has also been implicated in intracellular growth (64).

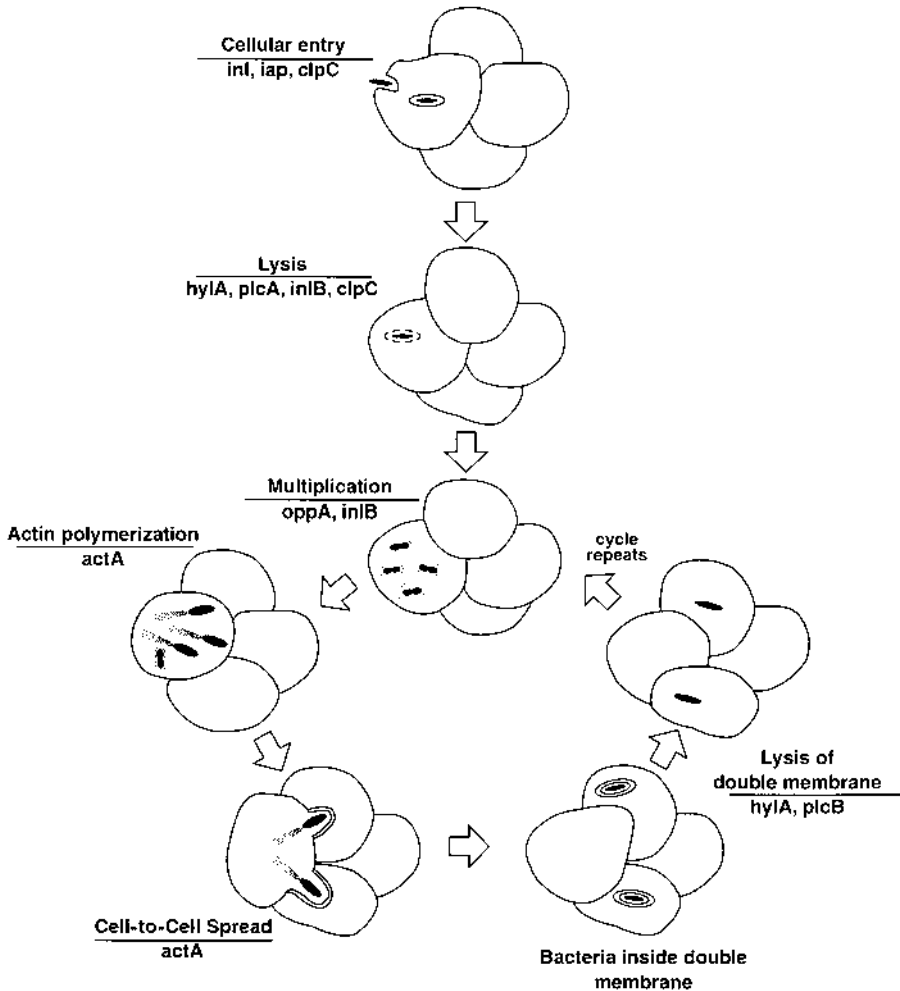
## B. Cell-to-Cell Spread

*Lm* can spread from cell to cell without leaving the cell. The first step in this process is the polymerization of actin molecules surrounding the cell. Polymerization starts at one of the two poles of the organism through the concerted effect of *actA* gene product and some host cellular components. The formation of an actin tail at one pole leads to movement of the bacterium toward the cell surface, which ultimately leads to formation of protrusion-like structures in the neighboring cells. These protrusion-like structures, also called listeriopods, contain single bacterium surrounded by double membrane—one from the donor cell and other from the recipient cell. The listeriopods then turned into double membrane vacuoles containing *Lm* in the neighboring cell. The double membranes then are lysed, releasing the organisms, which then start to multiply, and the cycle repeats (3) (Fig. 2). In the last several years extensive use of transposon-induced mutation of *Lm*, electron microscopy, and video microscopy have led to the elucidation of this process in great detail. Although the intracellular bacteria are surrounded by actin clouds, the polymerization process was found to be dependent on the presence of ActA protein, a product of *actA* gene located with other virulence genes in the virulence gene cluster (49). The 97 kDa ActA protein is needed for actin nucleation on bacterial surface. The distribution of ActA protein on bacterial surface is asymmetrical—weak in one pole and dense in the opposite pole. The dense actin pole acts as a site for actin tail formation. The process of polymerization of actin in one pole provides the propelling power of the organism to move away from the point of actin tail formation (65). Several studies indicated that ActA is the only bacterial protein required for actin polymerization, and in the absence of ActA the organism does not move from cell to cell and is nonvirulent (47). A nonvirulent *Lm* mutant defective in intracellular motility (*Imt*) induced actin polymerization but could not rearrange to form actin tail to generate movement resulting in entrapment in actin filaments (66). This seems to be the only indication that actin polymerization and actin nucleation to produce tail can be separated by different mutations. The *Imt* mutant was also less virulent in the mouse virulence assay. The gene and the product responsible for defective *Imt* phenotype are still unknown. Besides these bacterial products, several host factors also play an important role in actin polymerization and tail formation. Two cytoskeletal proteins, namely profilin (67) and VASP (vasodilator-stimulated phosphoprotein) (68), have been found to play a crucial role in actin-based motility. The proline-rich repeats (PRR) of ActA are responsible for VASP binding as well as colocalization of profilin on bacterial surface. However about 30% of wild-type movement is dependent not on the presence of PRR but on long repeats in the ActA molecule (47).

Lysis of double membrane takes place through the actions of lecithinase and listeriolysin. Lecithinase, also known as phospholipase C (PL-PLC), is a product of the *plcB* gene collocated with the other virulence genes in the *Lm* chromosome. The lecithinase enzyme is produced as a precursor (32 kDa) cleaved by a metalloprotease (*Mpl*) to generate the active polypeptide (29 kDa) (69). The *mpl* gene coding for the metalloprotease has been identified and sequenced. This Zn-dependent protease can also degrade actin. Studies with *mpl*, *plcA*, and *plcB* mutants indicated that PL-PLC could also be activated by some host factor, probably a cysteine protease (69). Besides its role in activation of PL-PLC, *Mpl* protein may play other important roles in *Lm* infection.

## VI. REGULATION OF VIRULENCE GENE EXPRESSION

It is clear from the above discussion that the entire process of infection—from entry to multiplication to cell-to-cell spread—represents a very coordinated interaction of several bacterial and host factors. Genetic studies indicated that most of the known virulence-related genes of *Lm* reside in a cluster,



**FIGURE 2** Schematic representation of different steps of *L. monocytogenes* infection and known virulence genes associated with this process. This diagram is based on the original diagram of *L. monocytogenes* cell-to-cell spread proposed by Tilney and Portnoy (48).

within a 10 kb DNA fragment (6,7,8,49). The positioning of these genes in one small fragment of DNA suggested that the expression of these genes be probably regulated by some common mechanism. One of these regulatory genes is *prfA*, located immediately upstream from the *plcA*–*plcB* gene cluster (*plcA*–*hlyA*–*mpl*–*actA*–*plcB*). *PrfA* is a 27 kDa protein that acts as a transcriptional activator for genes in *plcA*–*plcB* cluster and also for *inlA*–*inlB* genes located about 240 kb upstream of the *vgc* locus (8). The protein binds to consensus sequence (*PrfA* box) located at –35 region of the genes leading to activation of these genes. The *PrfA* protein structurally resembles that of *E. coli* cAMP receptor protein CRP and other members of the CRP–FNN family of transcription regulators (70). *PrfA* not only activates virulent gene transcription, it also upshifts its own transcription. Several environmental factors including temperature, pH, stress, and medium composition control the transcription of *prfA*, thereby controlling expression of all the *prfA*-regulated genes (3,71). For example, *prfA* expression is greatly reduced at low temperature (15–20°C) compared to 37°C, the

human body temperature. This is interesting in view of the fact that at environmental temperature (15–20°C) most virulence genes are turned off because the gene products have very little role in bacterial survival and multiplication in the environment; they are turned on when they infect animal host (37°C). Mutations in *prfA* rendered *Lm* completely avirulent, indicating a role of this important regulatory gene in virulence. In vitro studies have shown that *prfA* is upregulated when *Lm* interacts with mammalian cells, again implicating the pivotal role PrfA in *Lm* pathogenicity (72). Recently a member of the Clp heat-shock protein family (ATPases), *clpC*, has been implicated in *Lm* infection (73). Mutation analysis had shown that the 100 kDa ClpC protein is associated with the expression of *inlA*, *inlB*, and *actA* genes. The reduction in transcription of these genes in absence of *clpC* may explain why the *clpC* mutants were defective in cell adhesion and invasion. The mechanism by which ClpC exerts its pleiotrophic effect is yet to be determined. Another Clp-like protein, ClpP, was also found to be required for survival inside macrophage and virulence in mouse model (74).

Timely and coordinated expression of the virulence factors is a prerequisite for successful infection. *Lm* has been isolated from various foods providing diverse matrices, which might influence *Lm* pathogenicity. Of all the environmental factors, pH probably plays one of the most important roles in virulence. Increased acid tolerance, either by induction or by mutation, has been shown to affect virulence either by increasing survival in stomach and intestinal pH or by increasing invasion in macrophage and enterocyte or by increasing survival in macrophage (37,38). Lowering of intracellular pH has been associated with Mpl activation of pro PC-PLC (75). Using green fluorescent protein–tagged *act-plcB* genes it was shown that expression of *actA* and *plcB* takes place only after *Lm* escapes from the vacuole into cytoplasm (76). This indicates that either some cytoplasmic factor is necessary for the expression of these genes or that alteration of some property, e.g., pH may be necessary for expression of these virulence genes. It is also known that under iron stress conditions, *actA* gene expression increased 10-fold within enterocytes (77).

## VII. DETECTION AND ENUMERATION

Isolation, identification, and enumeration of *Lm* present several challenges. In order to develop a suitable isolation protocol, one has to consider the complexity of food matrices and environment in which *Lm* may be present. *Lm* is normally present in low numbers in foods. To be able to detect such low numbers, one must have enrichment steps suitable for the organism. *Lm* is often associated with organisms belonging to genus *Listeria* as well as with members of other genera. Enrichment media and protocol thus should favor the growth *Lm* over other competitive microflora.

Several selective enrichment media are currently used for isolation of *Lm* from food and other products (78). These media contain a combination of antibacterial and antifungal substances at concentrations that have minimal effect on *Listeria* while suppressing growth of competitive microflora. *Listeria* enrichment broth (LEB), developed by FDA, is tryptic soy broth supplemented with 0.6% yeast extract, nalidixic acid, acriflavine and cyclohexamide—recommended for use with all foods except meat and meat products. Although the medium provides good selective enrichment for *Listeria*, it is not suitable for stressed (injured) *Lm*, which might be present in processed foods. To provide repair of stressed *Lm* in food, the current FDA protocol recommends addition of antibiotics and antifungal agent after 4 hours of incubation in TSBYE (79). Once the selective agents are added, the enrichment is carried out for 24 and 48 hours, after which the enrichment broth is screened for the presence of *Listeria*. The U.S. Department of Agriculture uses a modified version of an enrichment medium originally developed at the University of Vermont, called UVM1 broth, for meat and poultry products. The food samples are incubated in UVM1 for 24 hours followed by a 1 : 10 dilution of the primary enrichment culture to a secondary enrichment broth called Fraser broth minus ferric ammonium citrate. Nalidixic acid and acriflavine are the inhibitory substances in UVM1, while nalidixic acid and lithium chloride are the inhibitory ingredients in Fraser broth. Esculin is added to both UVM1 and Fraser broth, which gives a dark color when hydrolyzed by *Listeria* in the culture. Following enrichment, the cultures are screened either by commercial kits and/or by streaking the

content on selective agar media (LPM, PALCAM, Oxford, modified Oxford, etc.) followed by screening of suspected colonies by a combination of biochemical and sometimes genetic tests. The selective agars also contain combination antimicrobials (Lithium chloride, moxalactam, etc.) to suppress the growth of non-*Listeria* organisms (78). Some selective agar media combine antimicrobials with fluorogenic or chromogenic substrates for species identification (80,81). Final discrimination between seven species of *Listeria* and confirmation of *Lm* is achieved by a combination of biochemical reactions, hemolysis, and CAMP tests.

It is clear from the above discussion that the process, from contaminated food to confirmation of *Lm*, can easily take 1–2 weeks. As increasing numbers of listeriosis cases are reported throughout the world, tremendous efforts are being made to develop rapid and sensitive detection methods (82). These methods can be broadly categorized in three groups.

1. Development of miniaturized testing strips, which provide the necessary biochemical reaction profile within couple of hours. These strips use a miniaturized format of the standard biochemical tests earlier used for characterization of bacterium. Final confirmation still requires hemolysin and CAMP tests for identification of *Lm*.
2. Antibody-based assays use either polyclonal or monoclonal antibody directed against one of several cell surface antigens of *Listeria*. Although most of the commercial kits are for *Listeria*, a few kits specific for *Lm* have recently been evaluated (83,84). All the kits require some sort of enrichment so that the number of target molecules is at least between  $10^5$ – $10^6$ . Performance varies from kit to kit and is also dependent upon the food matrices. Validation of each kit with a particular product is thus very important before one uses it. Nonetheless, the screening of foods by using these kits could be very useful in routine inspection of large numbers of samples.
3. DNA-based methods utilize nucleotide sequence specificity of *Listeria* or *Lm* genes (85). The probe-based detection techniques use a colony hybridization format, which can provide identification and direct enumeration in the same assay. As more and more information about virulence genes became available, specific probes against these genes were also becoming useful in the identification of *Lm*. Several commercial kits also used DNA hybridization technology for the detection of *Listeria* and *Lm*. With the advent of polymerase chain reaction (PCR) technology, several methods to detect and identify *Lm* in foods were developed. Most of these methods used virulence gene sequences for target amplification. The PCR-based methods can detect as few as 10 CFU/mL without any enrichment step, thus completing the analysis within several hours.

Results from numerous surveys indicated that *Lm* concentration in foods and environmental samples are low. This creates special challenges for *Lm* detection. Besides liquid enrichment, several reports indicated the use of immunomagnetic beads (86) and buoyant-density gradient for concentration of *Listeria/Lm* in foods. While immunomagnetic separation concentrates *Lm* from other microorganisms, the density gradient separation is intended to separate microorganisms from the food matrices that might be interfering with either liquid enrichment or PCR detection. The current regimen of liquid enrichment suffers from several drawbacks. First, it cannot discriminate *Lm* from other *Listeria* species; in fact, there are reports that many conventional enrichment media would preferentially support *L. innocua* growth better than *Lm*, thus creating a problem of *Lm* detection from foods contaminated with both *Lm* and *L. innocua*. Second, many non-*Listeria* organisms may grow well in the standard *Listeria* enrichment broth, creating problems of further detection of *Lm* in the enrichment media. Specific amplification and detection by PCR is successful to some extent, although the presence of PCR-inhibitory material and the inability to discriminate between viable and nonviable organisms limits the use of PCR for direct detection of *Lm* in foods. Enumeration of *Lm* is becoming more and more of a necessity for determination of infective dose and risk assessment. Unfortunately, few methods are currently available to enumerate *Lm* in foods. DNA probe colony hybridization in its different forms still seems to be the best method to directly enumerate

*Lm* in foods (85). As the initial numbers are often very low, some kind of physical concentration without multiplication seems to be appropriate for this effort. Recent development of quantitative PCR also provides a way to enumerate *Lm* in food, although these methods would benefit again from some kind of physical means to concentrate the organisms and separate the interfering food matrices before PCR (87–89).

## VIII. CONTROL MEASURES

The incidence of listeriosis can be controlled by controlling our intake of foods contaminated with *Lm*. In that *Lm* infects mostly immunocompromised and elderly and neonates, lowering the exposure of this vulnerable population should decrease the incidence of listeriosis. Several basic principles can be adopted to reduce the exposure to *Lm*.

Farm products should be produced under conditions that minimize *Lm* concentrations in these products. Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) should be strictly adhered to in order to reduce processing plant contamination. Implementation of Hazard Analysis of Critical Control Points (HACCP) plans in manufacturing facilities should be also useful to reduce in-plant contamination.

Once food is produced, any process that kills *Lm* should eliminate and/or reduce the *Lm* burden of food. Pasteurization of milk and other foods is an example of postproduction treatment that eliminates *Lm* and other pathogenic bacteria. A postproduction kill step to reduce *Lm* is, however, not applicable to many foods. Several other experimental protocols are currently under investigation. These include irradiation (90), hydrostatic pressure, and ozone treatment (91). All these processes have benefits and problems and therefore need to be evaluated on a case-by-case basis. However, foods undergoing postproduction treatment need to be handled and stored appropriately to avoid further contamination.

Control measures to prevent survival and growth of *Lm* in food is another way to decrease *Lm* load in our food supply (92,93). These measures can be adopted in addition to the measures mentioned in earlier paragraphs. Although *Lm* survives and grows relatively well at refrigeration temperature, the use of low temperature is still the most practical and economic way to store food. The doubling time of *Lm* at refrigeration temperature is a function of water content, osmolarity, and other ingredients of food (20). High osmolarity and low water content adversely affect *Lm* growth. These factors should be considered before one uses refrigeration as an effective means to control *Lm* in food. Use of chemicals is mostly restricted to those chemicals that change either the osmolarity or the pH of the food. As *Lm* is very sensitive to low pH, wherever possible acidic pH should be considered to restrict *Lm* survival and growth. Recently a group of chemicals called bacteriocins have been shown to be effective against *Lm* growth and survival (94). Bacteriocins are small polypeptides obtained from mostly gram-positive bacteria. Nisin is the most well-characterized bacteriocin that has been shown to affect the cell wall permeability of the organisms, thus altering the physicochemical balance leading to growth arrestation and cell death. In the United States, nisin has been approved as a Generally Recognized as Safe (GRAS) substance for some cheese products and has been shown to reduce *Lm* tolerance to low pH, thus raising the possibility that a combination of moderately low pH and a bacteriocin like nisin may be useful for storage of food (33,95). Further research is needed to make this possibility a practical method.

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# 8

## *Escherichia coli*

**Carlos Eslava, Jorge Villaseca, Ulises Hernandez,  
and Alejandro Cravioto**

*Universidad Nacional Autónoma de México, Mexico City, Mexico*

### I. BACKGROUND

Infectious diseases that spread through food, so-called foodborne illnesses, are a common, distressing, and sometimes life-threatening problem for millions of people in the United States and around the world (Headrick and Tollefson, 1998). Foodborne disease is caused by consuming contaminated foods or beverages. Many different disease-causing microbes, or pathogens, can contaminate foods, creating many different foodborne infections (Ryan et al., 1996). More than 250 different foodborne diseases have been described, with most of these diseases being infections caused by a variety of bacteria, viruses, and parasites (Baird-Parker, 1994). Other diseases are caused by poisonous elements in the form of harmful toxins (Notermans and Wernars, 1991; Notermans and Tatini 1993) or chemicals that have contaminated the food, for example, poisonous mushrooms. These varying diseases have many different symptoms, meaning that there is no one “syndrome” that can be described as foodborne illness. The microbe or toxin enters the body through the gastrointestinal tract, often the site of the first symptoms, with nausea, vomiting, abdominal cramps, and diarrhea being common symptoms in many foodborne diseases.

The epidemiology of microbial foodborne diseases has changed over the last 10 years. This is due not only to an increase in susceptibility of the human population to diseases or changes in lifestyles, including more adventurous eating, more convenience foods, and less time devoted to food preparation, but also to the emergence of newly recognized foodborne pathogens (Keskimäki et al., 1998). The gastrointestinal tract is an easy target for different pathogens that are ingested with food, and this is the principal reason why bowel infections are among the most common human infectious diseases.

*Escherichia coli* is just one of many bacteria that can cause diarrhea. The association between *E. coli* and intestinal infections was suspected by Escherich in 1885, when the bacteria were first identified. It was not until 1945, however, that Bray and other researchers demonstrated its involvement in gastroenteritis. *E. coli* associated with enteric diarrheal disease includes strains of many different serotypes, categorized into five major groups according to their virulence mechanisms: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC), and enteroaggregative (EAEC). Other strains, namely diffusely adherent *E. coli* (DAEC), are less well established as pathogens (Giron et al., 1991; Nataro and Kaper, 1998).

### II. CHARACTERISTICS

*E. coli* belongs to the Enterobacteriaceae family. It is a short gram-negative, non-spore-forming, usually with flagellae that are peritrichous, and fimbriate bacillus. A capsule or microcapsule is

often present. *E. coli* is the main facultative anaerobe in the large intestine. This bacteria colonizes the gastrointestinal tract during the first hours of life. The function of *E. coli* as part of intestinal flora has been linked to nutrition as a source of vitamins. Although regarded as part of the flora of the human intestinal tract, several highly adapted *E. coli* clones have evolved and developed the ability to cause disease in several areas of the human body. Most of these diseases are related to mucosal surfaces. Sometimes, however, mucosal colonization of the intestine and urinary tract may be asymptomatic.

### **III. DISEASES**

#### **A. Enteropathogenic *E. coli***

EPEC infection usually results in watery diarrhea accompanied by vomiting and fever, but in some cases there is protracted chronic enteritis. EPEC is traditionally associated with outbreaks in maternity units and child daycare centers, although outbreaks in adults are also common.

#### **B. Enterotoxigenic *E. coli***

ETEC are a common cause of infectious diarrhea (Black, 1993), especially in tropical climates. ETEC produces a watery diarrhea associated with cramps and a low-grade or no fever (Cohen and Giannella, 1995). Diarrhea caused by ETEC has a lot in common with cholera; both result from ingestion of rather large inocula of bacteria, which then colonize the small intestine and produce toxins that cause net secretion into intestinal lumen.

#### **C. Enteroinvasive *E. coli***

EIEC infection produces a disease similar to that caused by *Shigella*. The diarrhea is initially acute and watery, accompanied by fever and abdominal cramps, and then may progress to a colonic phase with bloody and mucoid stools. EIEC infection has been traced to contaminated foods and water and can also spread from person to person.

#### **D. Enterohemorrhagic *E. coli***

The EHEC group causes severe bloody diarrhea (hemorrhagic colitis), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (Karmali et al., 1983; Greatorex and Thorne, 1994) although sometimes the infection causes only diarrhea or no symptoms. In the United States, hemolytic uremic syndrome is the principal cause of acute kidney failure in children.

#### **E. Enteroaggregative *E. coli***

EAEC have been associated with acute, persistent, and bloody diarrhea disease in children and with a number of both nosocomial and community outbreaks worldwide (Cravioto et al., 1991; Fang et al., 1995; Cobeljic et al., 1996). In addition, the participation of EAEC as the causative agent of diarrheal disease in HIV-infected adults in the developed world has also been suggested (Durrer et al., 2000).

### **IV. EPIDEMIOLOGY**

Although regarded as part of the flora of the human intestinal tract, several highly adapted *E. coli* clones have evolved and developed the ability to cause disease in several areas of the human body.

Most of these diseases are related to mucosal surfaces. Sometimes, however, mucosal colonization of the intestine and urinary tract may be asymptomatic.

In developing countries, contaminated water is a principal vehicle for transmission of *E. coli* infection, either by direct ingestion or through contaminated foods that have been irrigated, washed, or prepared with contaminated water. This is less common in developed countries with higher standards of general hygiene. However, drinking water contaminated by sewage or animal waste has been implicated in outbreaks, and in one case water in a paddling pool was suspected as the means of spreading infection from an index case to other children. As food and person-to-person contact are concurrent alternative sources of exposure, the role of water may be difficult to prove.

The enteropathogenic *E. coli* group has been linked to infant diarrhea, principally in the developing world. EPEC infections are frequently spread from person-to-person.

Enterotoxigenic *E. coli* are a common cause of infectious diarrhea (Black, 1993), especially in tropical climates, where the water and food are frequently contaminated. Children living in developing countries and travelers to those countries form the main susceptible population. Diarrhea caused by ETEC has a lot in common with cholera; both result from ingestion of rather large inocula of bacteria, which then colonize the small intestine and produce toxins that cause net secretion into intestinal lumen. Most illness, in terms of both numbers of cases and severity of symptoms, occurs in infants and young children after weaning. In travelers, the diarrhea is not typically severe and resolves without treatment in about one week.

Epidemiological studies have shown the participation of enteroinvasive *E. coli* principally in foodborne and waterborne outbreaks, and it has been suggested that in sporadic cases, EIEC strains are probably misidentified as *Shigella* spp. or nonpathogenic *E. coli* strains (Faulder et al., 1988).

The EHEC strain most commonly found in the United States and in other developed countries is O157:H7. Other EHEC serotypes, including O26:H11 and O111:H8, have also been found in these countries (Griffin, 1995). Infection and most illness related to EHEC O157:H7 has been associated with eating undercooked, contaminated ground beef. Person-to-person contact in families and childcare centers is also an important mode of transmission. Infection can also occur after drinking raw milk and after swimming in or drinking sewage-contaminated water (Ackers et al., 1998).

Enterotoxigenic *E. coli* cause acute and/or chronic diarrhea, as well as diarrhea in HIV-infected patients. EAEC transmission may occur through food or water contaminated with human or animal feces. Person-to-person transmission may also occur, but this is likely to be less common.

Enterotoxigenic *E. coli* is the most common cause of travelers' diarrhea (TD) and has caused several foodborne outbreaks in the United States. There are an estimated 79,420 cases of ETEC in the United States each year. TD is characterized by a twofold or greater increase in the frequency of unformed bowel movements. Commonly associated symptoms include abdominal cramps, nausea, bloating, urgency, fever, and malaise. Episodes of TD usually begin abruptly, occur during travel or soon after returning home, and are generally self-limiting. The most important determinant of risk is the destination of the traveler. Attack rates of 20–50% are commonly reported. High-risk destinations include most of the developing countries of Central America, Africa (south, central, east, west, and north), the Middle East, and Asia (east, southeast, and the Indian subcontinent). Intermediate-risk destinations include most of the southern European countries (east and west) and a few Caribbean islands. Low-risk destinations include Canada, northern Europe, Australia, New Zealand, the United States, and a number of Caribbean islands (Siem and Bollini, 1992). TD is slightly more common in young adults than in older people. The reason for this difference is unclear but may include a lack of acquired immunity, more adventurous travel styles, and different eating habits. TD is acquired through ingestion of fecal-contaminated food or water. Both cooked and uncooked foods may be implicated if improperly handled. Risky foods include raw or undercooked meat, seafood, and raw fruits and vegetables. Tap water, ice, and unpasteurized milk and dairy products may be associated with an increased risk of TD; safe beverages include bottled carbonated beverages (especially flavored beverages), beer, wine, hot coffee or tea, or water boiled or appropriately treated with iodine or chlorine. The place in which food is prepared appears to be an important variable, with private homes, restaurants, and street vendors listed in order of increasing risk. TD

typically results in four to five loose or watery evacuations per day. The median duration of diarrhea is 3–4 days. In 10% of cases the duration can be longer than 1 week, approximately 2% last longer than 1 month, and fewer than 1% last longer than 3 months.

Persistent diarrhea is quite uncommon and may differ considerably from acute TD with respect to etiology and risk factors. Approximately 15% of those affected experience vomiting and 2–10% may have diarrhea accompanied by fever or bloody stools or both. Travelers may experience more than one attack of TD during a single trip. TD is rarely life-threatening.

## V. ISOLATION AND IDENTIFICATION

*E. coli* is easily recovered from different samples using general or selective media and incubating at 37°C in aerobic conditions. MacConkey or eosin methylene-blue agar are the media most often used to recover bacteria from the different samples. These media permit the differentiation of enteric organisms on the basis of their morphological characteristics. *E. coli* grows readily on simple culture media and synthetic media with glycerol or glucose as the sole source of carbon and energy. When grown on solid media, colonies are circular and smooth with an entire edge; some strains produce mucoid colonies. On media containing washed erythrocytes, a cell-associated  $\beta$ -hemolysin production can be demonstrated.

### A. Biochemical Characterization

*E. coli* identification can be performed using biochemical reactions that utilize individual culture tubes, test “strips,” or automated procedures. *E. coli* produces gas from glucose and is typically positive for indole, lysine, arabinose, mannitol, ONPG, trehalose, and xylose. Isolates are generally negative for DNase, H<sub>2</sub>S, phenylalanine deaminase, urease, Voges-Proskauer, inositol, and KCN. Other fermentation tests not included in the recommended routine batteries that may be useful to support the identification of the bacteria include cellobiose, arabitol, and mucate. *E. coli* is mannitol and mucate positive, but cellobiose and arabitol negative. These additional tests may be needed when dealing with isolates of EHEC strains that are typically sorbitol negative (Kelly et al., 1985).

### B. Serotyping

*E. coli* strains are serotyped according to their O (somatic), H (flagellar), and K (capsular) surface antigens (Lior, 1996). Each one of the 175 different antigens recognized in the *E. coli* strains determine the serogroup, and some of these have been associated with certain clinical syndromes. The specific combination of O (175) and H (56) antigens defines the serotype of each isolate of *E. coli*. Other specific structural antigens have been identified in the different pathogenic groups, but their use is not generally adopted for characterizing the bacteria.

### C. Other Typing Procedures

Bacteriophage and colicin typing are also employed to characterize *E. coli* strains, but they are not generally used.

### D. Virulence Assays

The virulence properties of diarrheagenic *E. coli* strains can be determined by the use of in vitro assays. One of the most commonly utilized is the HEp-2 cell adherence assay initially described by Cravioto et al. (1979). This procedure is considered the gold standard for the characterization of the three adherent groups of diarrheagenic *E. coli* (EPEC, EAEC, and DAEC) (Vial et al., 1990). HeLa cells have been used to detect invasive *E. coli* and *Shigella* strains (Sansone et al., 1986).



Other different lines of cultured cells have also been used for the detection of enterotoxins or cytotoxins; however, their use has diminished.

## **E. Animal Models**

Initially, the characterization of the virulence properties of diarrheagenic *E. coli* strains were determined using animal models, such as the rabbit ileal loops assay for enterotoxigenic *E. coli* strains that produce heat-labile enterotoxins (LT) or the Sereny test in guinea pigs for enteroinvasive *E. coli* (Sereny, 1955). However, the development of molecular methods contributed to reducing the use of these procedures.

## **F. Molecular Characterization**

Molecular methods allow for differentiation between diarrheagenic and nonpathogenic *E. coli* strains as well as the specific identification of strains that cause diarrhea. Specific nucleic acid probes are used to identify diarrheagenic *E. coli* groups. The colony blot procedure is one of the most commonly used methods for the identification of pathogenic strains. The method requires inoculation of 20–40 isolated colonies onto plates containing culture medium; the obtained cultures are then transferred to a solid support (nitrocellulose or Whatman filter paper), where the bacteria are lysed and the DNA is denatured and, finally, hybridized using the probe for each type of *E. coli*. Originally the probe techniques used radionucleotides for detection, but nowadays nonisotopic methods are more commonly used. PCR is another molecular method employed in the identification of pathogenic microorganisms. Over the last few years different PCR primers have been developed to identify diarrheagenic *E. coli* strains. This procedure can be used to detect one or several (PCR multiplex) virulence markers. Sensitivity and in situ detection of target templates are some of the advantages of PCR.

# **VI. PATHOGENICITY**

## **A. Enteropathogenic *E. coli***

Initially defined according to the differences in O and H antigens, EPEC is actually defined according to its pathogenic characteristics (Nataro and Kaper, 1998). Cravioto et al. (1979) proposed that adherence was a possible pathogenic mechanism of EPEC strains. Distinctive adherence patterns of *E. coli* to cells were proposed (Skaletzki et al., 1984) and then defined as the localized EPEC adherence pattern, this ability being associated with the presence of a 60 MDa plasmid (Baldini et al., 1983), named the EPEC adherence factor (EAF). The attaching and effacing histopathology on gut enterocytes has been referred to as the hallmark of EPEC infections, with these alterations having been observed in intestinal biopsy taken samples from naturally infected patients and from animals inoculated with EPEC strains (Frankel et al., 1998). The disturbance is characterized by localized destruction of brush border microvilli, intimate bacterial adhesion and gross cytoskeletal reorganization. To explain the attaching and effacing histopathology, Donnenberg and Kaper (1992) suggested a three-stage model consisting of localized adherence, a variety of signal transduction pathways in the eukaryotic cell and intimate adherence (Donnenberg et al., 1997).

## **B. Enterotoxigenic *E. coli***

Two enterotoxins are produced by ETEC strains: heat-stable toxin (ST) and heat-labile toxin (LT). ETEC may express one or both enterotoxins. LT enterotoxins found in ETEC are structurally and functionally related to the cholera enterotoxin (CT) produced by *Vibrio cholerae* (Sixma et al., 1993). Two immunological varieties of the LT enterotoxin have been recognized, and these have been defined as LT-I and LT-II. LT-I expression is associated with ETEC strains that cause disease

in both humans and animals. The LT-II enterotoxin has been identified principally in strains isolated from animals and occasionally from human strains.

The molecular weight of LT is ~86 kDa, constituted by one dimeric 28 kDa A subunit and five identical 11.5 kDa B subunits (Clemens et al., 1988). The A subunit expresses the enzymatic activity of the toxin, with the B subunits being arranged in a ring bound to the ganglioside GM1 receptor in the intestinal cells.

Two variants of LT, antigenically and functionally related, called LTpI (from pig strains) and LTpH (from human strains), are seen in ETEC strains (Finkelstein et al., 1987; Yamamoto et al., 1987). When the binding of the B subunits to the ganglioside GM1 on the host cell membranes is finished, the A subunit of the LT toxin is internalized by a trans-Golgi vesicular process. The A1 dimer of the A subunit induces an ADP-ribosylation of the Gs $\alpha$  subunit, causing the adenylate cyclase to be permanently activated, thereby increasing the levels of intracellular cyclic AMP (cAMP). The chloride channels in the apical epithelial cell membranes are activated, leading to Cl<sup>-</sup> secretion and inhibition of NaCl absorption from secretory crypt and villus tip cells, respectively (Nataro et al., 1998). Other mechanisms by which these toxins could act to cause diarrhea include prostaglandins (PGE1 and PGE2) and platelet-activating factor. The participation of a mild intestinal inflammatory response, related to the stimulation of the cytokine interleukin-6 (IL-6), which results in the activation of the enteric nervous system (ENS), has also been considered.

Two variants of the ST enterotoxin, St<sub>a</sub> and St<sub>b</sub>, contain multiple cysteine residues. The disulfide bonds of this amino acid contribute to the heat stability of these toxins (Chan and Giannella, 1981; Lazure et al., 1983). The genes that encode both St<sub>a</sub> and St<sub>b</sub> toxins are found on plasmids and transposons (Seriwatana et al., 1983). The St<sub>a</sub> toxin is a peptide made of 18 or 19 amino acids that exhibits two variants, STp or STI<sub>a</sub> (ST-porcine) and St<sub>h</sub> or STI<sub>b</sub> (ST-human). Guanylate cyclase C (GC-C), a membrane-spanning enzyme, is the only identified cellular receptor for St<sub>a</sub>. The St<sub>a</sub>-GC-C interaction stimulates guanylate cyclase activity, leading to the intracellular cGMP levels being increased, which in turn induces intestinal fluid secretion as a consequence of the stimulation of chloride secretion and/or the inhibition of sodium chloride absorption (Guarino et al., 1987; Crane et al., 1992).

The St<sub>b</sub> enterotoxin is principally related to ETEC porcine strains, but some ETEC strains isolated from humans have also been identified as St<sub>b</sub> producers. This enterotoxin does not increase the intracellular cAMP or cGMP concentrations directly, but stimulates increases in intracellular calcium levels from extracellular sources (Weikel et al., 1986; Dreyfus et al., 1993). Moreover, there have been suggestions that the ENS participates in the secretory response to this toxin (Hitotsubashi et al., 1992).

Adherence, mediated by surface fimbriae, is an important feature in the colonization of ETEC strains to the intestinal mucosa (Wolf, 1997). Although a large number of ETEC fimbrial antigens have been identified, the existence of many others has also been proposed. The fimbrial antigens of ETEC show species specificity. ETEC strains isolated from calves, lambs, and pigs express the K99 antigen, whereas the K88 fimbriae are specific for pathogenic strains of pigs (Moon and Whipp, 1976; Girardeau et al., 1988; Cassels and Wolf, 1995). Human ETEC strains exhibit colonization factor antigen (CFA) fimbriae with three morphological forms: rigid rods, bundle-forming flexible rods, and thin flexible wiry structures. Various CFAs have been identified, each with different morphological characteristics, and these have been categorized as CFA/I, CFA/II, CFA/III, and CFA/IV according to their description at the time (Wolf, 1997). The Longus fimbria, identified in an important number of human ETEC strains, was more recently described (Giron et al., 1994, 1995).

### C. Enteroinvasive *E. coli*

EIEC strains are probably misidentified as *Shigella* spp. or nonpathogenic *E. coli* strains (Faunder et al., 1988). EIEC is also related to *Shigella* in terms of diarrheal pathogenesis. Both bacteria invade the colonic epithelium and elaborate toxins (Day et al., 1981). Bacterial invasion includes

(a) epithelial cell penetration, (b) lysis of the endocytic vacuole, (c) intracellular multiplication, (d) directional movement through the cytoplasm, and (e) extension into adjacent epithelial cells.

#### **D. Enterohemorrhagic *E. coli***

*E. coli* O157:H7 infection often causes severe bloody diarrhea and abdominal cramps; sometimes the infection causes nonbloody diarrhea or no symptoms. Usually, little or no fever is present and the illness resolves in 5–10 days. In some cases, particularly in children under 5 years of age and the elderly, the infection can also cause a complication called hemolytic uremic syndrome, in which the red blood cells are destroyed and the kidneys fail. About 2–7% of infections lead to this complication. In the United States, hemolytic uremic syndrome is the principal cause of acute kidney failure in children, and most cases of hemolytic uremic syndrome are caused by *E. coli* O157:H7.

EHEC infections caused by the O157:H7 serotype, which can lead to death principally in young children and the elderly, are associated with severe symptoms including hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (Karmali et al., 1983; Grotorex and Thorne, 1994). Konowalchuk et al. (1977) reported that filtrated culture supernatants from *E. coli* strains caused a striking, irreversible cytopathic effect on Vero tissue culture cells and named it verotoxin (VT). O'Brien and Holmes (1996) showed that different *E. coli* strains were cytotoxic for HeLa cells and that this cytotoxic effect could be neutralized by antitoxin prepared against the Shiga toxin from *Shigella dysenteriae* 1. This toxin was termed as Shiga-like toxin (SLT). It was later shown that the verotoxin and Shiga-like toxin were in fact the same toxin and that it was produced by O157:H7 strains (Riley et al., 1983). Karmali et al. (1983) proposed that verocytotoxin/Shiga-like toxin (Stx) was the virulence factor of HC and HUS and was responsible for intestinal and renal tissue damage.

The participation of Stx in diarrhea and enterocolitis has been demonstrated in animal models. It was suggested that the selective killing of absorptive villus tips of intestinal epithelial cells by Stx was the possible mechanism that induced fluid intestinal secretion. Although Stx had been implicated in renal endothelial cell damage in HUS, several studies support the participation of cytokines in this process. Clinical studies showed elevated levels of IL-6 in serum and urine samples of HUS patients, and the levels of IL-6 correlated with the severity and outcome of disease (Tesh et al., 1994).

#### **E. Enteroaggregative *E. coli***

Enteroaggregative *E. coli* (EAEC) are a group of bacteria characterized by the ability to adhere to cultured cell monolayers in a “stacked-brick” pattern of adherence (Nataro 1987). The pathogenesis of EAEC infection is not completely understood despite histopathological alterations of intestinal epithelium from patients and animal models infected with EAEC having been reported (Tzipori et al., 1992; Hicks et al., 1996.). All these observations suggested that some of the alterations caused during EAEC infection were associated with the production of a cytotoxin. In relation to this cytotoxin, Eslava et al. (1993) identified two high molecular weight proteins, which, when tested in a rat ileal loop model, caused shortening of the villi, hemorrhagic and necrotic alterations, and ulceration of the upper epithelium.

### **VII. GENETIC FACTORS INVOLVED IN VIRULENCE**

#### **A. Enteropathogenic *E. coli***

The adherence of EPEC to epithelial cells is related to a 94 kDa outer membrane protein called intimin. The *eae* (*E. coli* attaching and effacing) gene that encodes intimin was initially reported by Jerse and Kaper (1991). This gene is present in all EPEC and EHEC strains and in other bacteria

capable of producing the attaching and effacing histopathology. Studies in volunteers demonstrated that the *eae* gene is an important virulence factor for EPEC pathogenesis

A 7-nm-diameter fimbriae named bundle-forming pilus (BFP) has been associated with localized adherence (Giron et al., 1991). The BFP fimbriae of EPEC strains provide for a bacterium-bacterium adherence, producing bacterial aggregates that attach to epithelium cells. BFP expression requires genes found on the EAF plasmid (Giron et al., 1993). For the global regulator element of EPEC, pathogenesis and *dsbA*, a chromosomal gene that encodes a periplasmic enzyme, are also necessary for the expression and assembly of BFP. The adherence of EPEC to epithelial cells induces a variety of signal transduction pathways in the eukaryotic cell (Donnenberg et al., 1997), which is located on a pathogenicity island called the locus of enterocyte effacement (LEE) where the bacterial genes responsible for this signal transduction are found. Infection by EPEC induces increases in intracellular calcium levels ( $\text{Ca}^{2+}$ ). It has been hypothesized that since increases in intracellular calcium can inhibit sodium and chloride absorption and stimulate chloride secretion in enterocytes, these changes may mediate the intestinal secretory response to EPEC. Other additional diarrhea mechanisms resulting from infection by EPEC strains have been proposed, and these relate to the activation of PKC and myosin light chain kinases. PKC activation induces changes in intestinal water and electrolyte secretion, while myosin light chain phosphorylation can lead to increased permeability of tight junctions (Crane and Oh, 1997).

## **B. Enterotoxigenic *E. coli***

The genes that encode LT (*elt* or *etx*) are found on plasmids that may also contain the ST and/or colonization factor antigens (CFAs). CFA genes are also encoded on plasmids, generally on those that encode the enterotoxins ST and/or LT (Echeverria et al., 1986).

## **C. Enteroinvasive *E. coli***

Bacterial invasion properties of EIEC are regulated by both plasmid (140 Mda) and chromosomal genes (Hale et al., 1985; Clerc and Sansonetti, 1987; Goldberg et al., 1993). In a study by Nataro, a plasmidborne gene (*sen*) from EIEC encoded a 63 kDa protein that elicited rises in Isc levels in the Ussing chambers model without having a significant effect on tissue conductance (Nataro et al., 1995).

## **D. Enterohemorrhagic *E. coli***

The term enterohemorrhagic *E. coli* was suggested for those strains that caused HC and HUS, secreted cytotoxin (VT/SLT), induced attaching and effacing histological alterations on epithelial cells, and possessed a ~60 Mda plasmid (Griffin, 1995). As seen in EPEC strains, *E. coli* O157:H7 contains the locus of enterocyte effacement (LEE), responsible for the attaching and effacing phenotype. The genes encoding intimin, the secreted proteins EspA and EspB, and the type III secretion pathway are all located in the LEE locus (Jarvis and Kaper, 1996). The major virulence component of EHEC is the Stx cytotoxin, the factor that leads to death in patients infected with EHEC strains. Stx belongs to a cytotoxin family that contains two immunologically non-cross-reactive groups called Stx1 and Stx2. While Stx1 is a highly conserved toxin, Stx2 shows sequence variation and consequently different variants exist, known as Stx2c, Stx2v, Stx2hb, and Stx2e. An EHEC strain may express Stx1 only, Stx2 only, or both or even some of the multiple forms of Stx2. The structural genes for Stx1 and Stx2 are found on lysogenic lambdaoid bacteriophages, and the genes for Stx2e are chromosomally encoded.

Another factor related to the virulence of EHEC strains is the 60 Mda plasmid, which contains genes that encode an hemolysin called enterohemolysin. These genes have a 60% identity with the *hlyA* gene that encodes hemolysin expressed by uropathogenic *E. coli* strains. However, the role of enterohemolysin in EHEC pathogenesis has not been determined. Intimin, a 94–97 kDa OMP en-

coded by the *eae* gene, is recognized as the only adherence factor in EHEC strains. Other adherence factors distinct from intimin have been suggested, but a specific candidate has not been identified. Other potential virulence factors such as the pO157 plasmid, different iron transport systems, and O157 LPS have also been proposed, but their role in the clinical disease of EHEC is unknown.

## E. Enteroaggregative *E. coli*

The genes for the expression of AAF/I and AAF/II fimbriae identified in EAEC strains are located on a 65 Mda plasmid named pAA (Nataro et al., 1998b). The AAF/I genes are organized as two separate gene clusters. In region 1 is the cluster that contains the genes necessary for fimbrial synthesis and assembly. Region 2 encodes a transcriptional activator for AAF/I expression. The AAF/II fimbria is morphologically and genetically distinct from AAF/I; the genes encoding this fimbria are organized as two unlinked regions separated by 15 kb from the required biogenesis gene cluster. An open reading frame that encodes EAST1, a 4100 dalton (38-amino-acid) protein, was also identified on pAA. This toxin (EAST1) tested in the rabbit mucosal Ussing chamber model yielded net increases of short-circuit current (Savarino et al., 1993), but its role in EAEC diarrhea pathogenesis has not been determined (Savarino et al., 1996). Different *in vitro* and *in vivo* procedures show that the virulence properties of EAEC are related to adherence and cytotoxin production (Hicks et al. 1996; Nataro, 1996). Eslava et al. (1998) identified two high molecular weight proteins in EAEC strains isolated from children with persistent diarrhea. One of these is located on 65 Mda EAEC pAA. This protein, named Pet (plasmid-encoded toxin), shows a high homology with type IV class autotransporter secreted proteins (Eslava et al., 1998). It was recently shown that Pet induced cytopathic effects on HEp-2 and HT29 C1 culture cells, characterized by a release of the cellular focal contact from the glass substratum, rounding and detachment of cells associated with contraction of the cytoskeleton, and loss of actin stress fibers (Navarro-Garcia et al., 1999). It was recently determined that these cellular alterations were related to spectrin and fodrin (spectrin analogs) degradation, causing disorders in the protein network of the membrane skeleton (Villaseca et al., 2000). Pic (for protein involved in intestinal colonization) is the other high molecular weight protein identified in EAEC strains. Pic is encoded on the chromosomes of EAEC and *Shigella flexneri* strains. The product of the *pic* gene is a 110 kDa protein secreted into the culture supernatant whose amino acid sequence also shows homology with the serine protease autotransporters of Enterobacteriaceae. The identified properties of Pic include mucinase activity, serum resistance, and hemagglutination, characteristics suggesting that Pic is a multifunctional protein involved in enteric pathogenesis (Henderson et al., 1999).

## VIII. CONTROL MEASURES

Bacteria in diarrheal stools can be passed from one person to another if hygiene or handwashing habits are inadequate. Several control measures can be put in place in order to reduce illness caused by *E. coli*:

*Sanitary:* As with other fecal-oral diseases, proper food handling and personal hygiene are the best means for preventing infection.

*Good cooking practices:* Consumers can prevent *E. coli* O157:H7 infection by thoroughly cooking ground beef, avoiding unpasteurized milk, and washing hands carefully. Since the organism lives in the intestines of healthy cattle, preventive measures on cattle farms and during meat processing are being investigated.

*Immunological:* New vaccines against fimbrial antigens are possible.

*Chemotherapeutic:* Antibiotic therapy is not generally recommended unless disease becomes life-threatening; oral rehydration is the best treatment.

Other measures include improving surveillance by transferring the techniques for identification and serotyping diarrheagenic *E. coli* to public health and clinical laboratories. Better understanding of the molecular genetics of the diverse virulence mechanisms of these organisms and developing tools to enable more rapid detection of emerging diarrheagenic *E. coli* strains are some of the challenges to the effective control of foodborne diseases.

A number of host factors also help prevent infection by *E. coli*. These include gastric acidity, intestinal motility, and normal intestinal flora. Some evidence suggests a possible genetic component due to the fact that bacterial fimbriae are used to attach to specific cellular receptors. In addition, breast milk contains neutralizing (nonimmunoglobulin) factors that help to prevent disease in nursing infants.

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# 9

## Nontyphoid *Salmonella*

Darcy Hanes

*U.S. Food and Drug Administration, Laurel, Maryland, U.S.A.*

### I. BACKGROUND

*Salmonella* continues to be a major cause of food poisoning throughout the world. Salmonellosis sickens as many as 3.84 million Americans and costs billions of dollars in lost productivity and medical costs per year (1,2). The number of reported cases of salmonella infection in both the United States and many European countries has also increased in recent years. Switzerland averaged a 51% increase per year since 1984 (3), and in Spain salmonellae are the principal agent of gastroenteritis (4). In many cases this increase has been due to a particular serotype, *Salmonella enterica* serotype Enteritidis, found primarily in poultry and poultry products. Italy reported a more than 6-fold increase in the number of Enteritidis outbreaks between 1990 and 1991 (3); the increase in Spain was 10-fold between 1983 and 1987 (3). In England, the number of cases increased 6-fold between 1982 and 1988 (3); France experienced a 7-fold increase between 1986 and 1990 (5). It appears, however, that the Enteritidis epidemic peaked in humans in 1992 in many European countries. The subsequent slight decline may set the stage for the reemergence of Typhimurium as the dominant serotype in human salmonellosis.

*S. enterica* serotype Typhimurium phage type DT104, a multiple drug-resistant variant, has increased in the United States, Canada, and the European continent. The disease-reporting system in England and Wales has revealed a 10-fold increase in the number of cases of DT104 between the years 1990 and 1996 (6). Typhimurium was second only to Enteritidis as the most common salmonella in humans in England and Wales in 1995, and more than 55% of cases were caused by DT104 (6). In Germany, DT104 accounted for up to 10% of all salmonellae species from human sources examined in 1995 and 10% of those examined in 1996 (6). Typhimurium DT104 has recently been reported in the United States, but few data are available on prevalence or mode of transmission. Preliminary evidence indicates that DT104 may have spread widely in the United States in the past 2–3 years, which would result in an increase in human illness in the near future. In general, over the past three decades most European countries and the United States have reported a sharp rise in the incidence of disease due to *Salmonella*. The same pattern is most likely occurring in a number of countries in the Middle East and Southeast Asia.

This increase in *Salmonella*, especially the emergence of more virulent isolates, emphasizes the need to understand the pathogenic mechanisms of this organism. It also underlines the need to control this pathogen in the food supply.

### II. CHARACTERISTICS

Salmonellae are facultative, gram-negative, motile bacilli that ferment glucose but not lactose or sucrose. Salmonellae are able to utilize citrate as a sole carbon source where other genera require a more complex source of this nutrient. All salmonellae, with the exception of *Salmonella typhi*,

produce gas during the fermentation process. Salmonellae are oxidase negative, reduce nitrates to nitrites, and do not require NaCl for growth. They grow at temperatures between 8 and 45°C in a pH range of 4–9 and require water activities ( $a_w$ ) above 0.94 (7). Salmonellae are sensitive to heat and are generally killed at temperatures of 70°C or above. They are also susceptible to pasteurization of milk at 72.1°C for 15 seconds (7). As a result, the bacteria is killed if food is cooked long enough to reach this temperature throughout. Salmonellae are resistant to drying and may survive for years in dust and dirt.

The genus *Salmonella* has been classically divided into three species: *S. typhi*, *S. cholerae-suis*, and *S. enterica*. These species are further cataloged by their antigenicity, as described by the Kauffmann-White scheme (8). Antigen formulas represent serotypes rather than species (*S. enterica* serotype Typhimurium), although designations such as *S. typhimurium* are still accepted. Serotypes are identified by highly specific O (somatic) and H (flagellar) antigens. A given serotype will contain a specific combination of multiple O and H antigens. Each serotype of salmonellae can be further subdivided into phage type depending upon their reactivity with a defined set of bacteriophages. Phage typing is generally used when origin and characteristics of an outbreak of infection must be determined. Phage typing is currently being used in the investigation of *S. enterica* serotype Enteritidis contamination of eggs and has played a key role in identifying certain phage types of Typhimurium that are highly antibiotic resistant. Both serotyping and phage typing are done only in major salmonellae typing centers, which have the collection of antisera and phages necessary for such work.

Salmonellae can also be broadly divided into groups on the basis of O antigen composition. The reagents for serogrouping are readily available commercially, and serogrouping can be done routinely in most standard microbiology laboratories. Most isolates from natural sources fall into five serogroups, A–E. *S. typhi* and *S. cholerae-suis* contain only one serotype each in groups D and C, respectively, while *S. enterica* contains over 2000 different serotypes in all groups including C and D. Considerable overlap in antigenic composition is responsible for the cross-reactivity commonly seen in serological tests with salmonellae.

Recently multilocus enzyme electrophoresis and comparative sequence analysis of housekeeping and rRNA genes have revealed that the genus *Salmonella* may actually contain only two lineages that have diverged considerably from each other during evolution (9,10). By using genetic distance determined by multilocus enzyme electrophoresis and results of DNA-DNA hybridization studies as criteria, it has been proposed that these lineages represent two distinct species, designated *Salmonella enterica* (11) and *Salmonella bongori* (12). *S. enterica* can be further subdivided into subspecies designated by Roman numerals (10). *S. enterica* subspecies I contains 1367 serotypes, is mainly isolated from warm-blooded animals (mammals and birds), and accounts for >99% of all clinical isolates. The remaining subspecies (II, IIIa, IIIb, IV, VI, and VII) are mainly isolated from cold-blooded animals and account for <1% of clinical isolates.

### III. DISEASE

*Salmonella* infections are among the most prevalent recognized communicable diseases caused by bacteria in the United States today. The vast majority of these infections are transmitted from animals to humans through food and occasionally from person to person through the fecal-oral route. Although gastroenteritis often occurs in large epidemics among individuals who have eaten contaminated food, family outbreaks and sporadic cases are even more common. Salmonellosis in the human occurs in a variety of forms, presenting a broad clinical spectrum.

#### A. Gastroenteritis

Typically, the incubation period for developing gastroenteritis is 6–72 hours following ingestion of contaminated food or water (7). There is sudden onset of abdominal pain and loose, watery

diarrhea, occasionally with mucous or blood. Nausea and vomiting are frequent but are rarely severe or protracted. Fever of 38–39°C is common, and there may be an initial chill. Abdominal pain is frequent and may cause mild to severe discomfort. In uncomplicated cases, the acute stage usually resolves within 48 hours. However, illness is occasionally more protracted, with persistent diarrhea and low-grade fever for 10–14 days. In severe cases, dehydration may lead to hypotension, cramps, oliguria, and uremia. Symptoms are likely to be more severe in infants and older adults (>60 years), although mild and subclinical cases have been seen in these age groups. *Salmonella* infections superimposed on other underlying diseases are usually more severe and can be fatal. Fatalities rarely exceed 1% of the affected population and are limited almost entirely to infants, the aged, and debilitated patients (13). One significant exception is infection with multidrug-resistant Typhimurium DT104. Infection with this organism has been associated with hospitalization rates twice that of other foodborne *Salmonella* infections and with 10 times higher case-fatality rates (6).

## **B. Bacteremia**

*Salmonella enterica* serotypes may produce a syndrome characterized by prolonged fever and a positive blood culture (14). Although symptoms of gastroenteritis can precede bacteremia, they are usually lacking. In many instances the only manifestations are prolonged fever, which is usually spiking and accompanied by rigors, sweats, aching, anorexia, and weight loss. The characteristic symptoms of typhoid fever, which include rose spots, leukopenia, and sustained fever, are absent. Stool cultures are normally negative. In contrast to the constant bacteremia seen with typhoid fever, discharge of the organisms into the bloodstream is intermittent, and repeated blood cultures may be necessary to identify the causative organism. At some time during the course of the disease, localizing signs of the infection appear in about one quarter of the cases. Bacteremia caused by salmonellae can be a very puzzling disorder and should be considered in cases of fever of unknown origin.

## **C. Enteric or Paratyphoid Fever**

Certain serotypes of *Salmonella* can produce an illness clinically indistinguishable from typhoid fever, with prolonged fever, rose spots, splenomegaly, leukopenia, gastrointestinal symptoms, and positive blood and stool cultures (14). The organisms most likely to produce this syndrome include *S. cholerae-suis* and *S. enterica* serotypes *paratyphi A* and *paratyphi B*. Occasionally a typical attack of food poisoning is followed in a few days by manifestations of paratyphoid fever. Generally, this tends to be milder than *S. typhi* infections, but differentiation on clinical grounds is not possible in the individual case. Recovery may be followed by continued excretion of the causative organism in the stools for several months, but the chronic carrier state is less frequent than in typhoid fever.

## **D. Local Infections**

*Salmonella* organisms can also produce abscesses in almost any anatomical site, and these can occur independently of symptoms of gastroenteritis or systemic illness. There is a strong tendency for salmonellae to localize in tissues that are the sites of preexisting disease. Meningeal localization of infections is common in newborns and infants, and occasional small outbreaks of *Salmonella* infections in nurseries have consisted almost entirely of meningitis. Suppurative joint disease and chronic aseptic polyarthritis has also been described.

## **E. Diagnosis**

Febrile gastroenteritis is differentiated from other bacterial and viral infections by appropriate stool cultures. The causative organism can often be isolated from the suspected food and from feces during the acute illness. Stool cultures usually become negative for salmonellae within 1–4 weeks,

but occasionally patients continue to excrete organisms for months. Organisms tend to persist in the stools of infants and young children for longer periods than in older children or adults.

Colonization of the gall bladder causes permanent shedding of a particular *Salmonella* serotype in the feces and makes the host a chronic carrier. A chronic carrier is potentially able to infect many individuals, which is of obvious benefit for the pathogen. Only 0.2–0.6% (10) of patients with nontyphoidal salmonellosis develop chronic carriage.

## F. Treatment

The treatment for salmonella gastroenteritis, if necessary, is generally supportive. Dehydration should be corrected by parenteral administration of fluids and electrolytes. Antimicrobial therapy does not appear to exert a beneficial effect on the clinical course of the gastrointestinal disease or decrease the duration of excretion of organisms in the stool. In fact, the period of excretion of *Salmonella* in stools during convalescence is actually longer in patients who have been treated with antimicrobial drugs during the acute illness than in patients who received no therapy (14). Unless there is documented bacteremia or a protracted febrile course suggesting the diagnosis of enteric fever, antibiotics are not indicated in uncomplicated *Salmonella* gastroenteritis.

## IV. EPIDEMIOLOGY

*Salmonella* can be isolated from the intestinal tracts of humans and many of the lower animals. The prevalence of asymptomatic excretors of these organisms in the general population is approximately 0.2% (10). The most important reservoir of *salmonella* is domestic and wild animals in which the infection rates vary from <1 to >20% (14). An incomplete list of animals from which salmonellae serotypes have been isolated includes chickens, turkeys, ducks, pigs, cows, dogs, cats, rats, parakeets, as well as certain cold-blooded animals and insects. Animals sold as pets, especially baby chicks, ducks, turtles, and other reptiles, may also harbor salmonellae and serve as a source of infection.

*Salmonella* infection is usually acquired by the oral route, normally by ingestion of contaminated food or drink. Any food product is a potential source of human infection. The source of contamination may be asymptomatic carriers or persons with active clinical disease, but the greatest single source of human infection in the United States is the vast reservoir of *Salmonella* in lower animals. The high incidence of infection in domestic feed, and the present methods of processing foods and food products in bulk, results in foods with a potentially high incidence of contamination with *Salmonella*. For example, a significant proportion varying from 1 to >50% of raw meats purchased in retail markets is contaminated with salmonella (14). The most common source of contamination is natural infection of animals raised for human consumption and contamination of the carcass during slaughter and processing. Eggs or egg products, including dried or frozen eggs, are also a very common source of salmonellae. Of the various animal species, domestic fowl, including chickens, ducks, and turkeys, constitute the largest reservoir of infection and the source most often responsible for human infection. Adequate cooking of food before consumption decreases the possibility of infection, but salmonellae may survive cooking at low temperatures (<70°C) and cooked food can be contaminated after cooking by organisms from kitchen utensils or individuals. Food or drink may also be contaminated by rats, mice, insects, or other vermin harboring these organisms. Cross-infection also occurs by the airborne route from dried foods such as egg whites or dust that contain viable *Salmonella*.

Salmonellae also can be transmitted directly or by fomites from humans to humans or from animals to humans without the presence of contaminated food or water, but this is not a common mode of transmission. However, cross-infection of this type is responsible for a number of outbreaks in hospitals and nurseries. Nosocomial salmonellosis is particularly devastating in newborns, immunocompromised individuals, patients in burn units, and those receiving multiple broad-spectrum antibiotics. Recent evidence suggests that individuals receiving antibiotics are more susceptible to

the newly emerging Typhimurium phage type DT104. Nursery outbreaks have been traced to newborn infants from mothers with recent *Salmonella* infections.

Fishmeal, meat meal, bone meal, and other by-products of the meatpacking industry are often contaminated with *Salmonella* organisms. These products are incorporated into animal and poultry feeds and apparently play an important role in the perpetuation of feed animal infections that can be spread to humans.

The true incidence of *Salmonella* infection is difficult to determine. Reported cases represent only a small proportion of the actual number. Normally only large outbreaks are investigated and documented; sporadic cases are underreported, mainly because only patients with protracted diarrhea report to a health care provider for microbiological evaluation. Although *Salmonella* infections occur throughout the year, the *Salmonella* Surveillance Unit of the National Communicable Disease Centers has observed a distinct seasonal pattern, with the greatest number of isolations reported from July through November of each year.

A close correlation exists between the *Salmonella* serotypes most often responsible for human infection and those isolated from animals in any one geographic location. These similarities document the importance of nonhuman reservoirs of *Salmonella* in the epidemiology of infection in humans.

## V. ISOLATION AND IDENTIFICATION

Most methods used to isolate and identify *Salmonella* are modifications of methods originally developed for clinical specimens. These modifications begin with preenrichment of test samples and follow through selective enrichments, selective plating, differential testing and plating, confirmatory biochemical determinations, and confirmatory serological testing (7,15).

A preenrichment culture is recommended for the recovery and identification of microorganisms that may have suffered injury or stress in some operational step of a process. The most common preenrichment broths are lactose and brilliant green, although lauryl tryptose broth, mannitol purple sugar broth, and nutrient broth are also used. These preenrichment broths allow for growth of most organisms present in the test sample. After this preenrichment step, selective enrichment is done to provide an opportunity for the small numbers of *Salmonella* to grow while the competing organisms are inhibited. Enrichment broths recommended for salmonellae include tetrathionate and selenite cystine broth. However, selenite cystine broth will quite likely be replaced with RV Rapport-Vassiliadis medium in the near future (15,16).

The use of selective enrichment broth, while allowing the growth of *Salmonella*, results in mixed cultures containing other organisms. Plating to highly selective media will allow the development of discrete salmonellae colonies while inhibiting the growth of other bacteria. Growth of discrete colonies on these media also permits recognition of colonies that are suspected to be *Salmonella* and transfer of these pure colonies to other media for confirmation of identity.

Selective and differential media used for salmonellae (Table 1) include *Salmonella-Shigella* (SS) agar, bismuth sulfite (BS) agar, and xylose lysine desoxycholate (XLD) agar. Others that are used include brilliant green agar and desoxycholate citrate agar. These media are highly selective and may inhibit growth of some salmonellae isolates. Several media that are less selective but primarily differential are MacConkey (MAC) agar, Hektoen Enteric (HE) agar, desoxycholate agar, and eosin-methylene blue (EMB) agar. The inclusion of the primarily differential media provides a better opportunity to obtain growth from all isolates, even those that may be inhibited by the highly selective media.

Suspected salmonellae isolates are subjected to a series of biochemical tests (Table 2) for further confirmation (7,15). These tests include glucose, lysine decarboxylase, hydrogen sulfide, urease, indole, Voges-Proskauer, citrate, methyl red, motility, failure to ferment sucrose or lactose, and growth in KCN broth. Once an isolate is confirmed as *Salmonella*, serological testing can be completed. Commercial preparations of *Salmonella* O, H, and Vi antibodies are available. Generally,

**TABLE 1** Typical Growth Characteristics of *Salmonella* on Some Commonly Used Selective and Differential Media

Media	Colony appearance
<i>Salmonella-Shigella</i> (SS) agar	Colorless colonies on a pink background
Bismuth sulfite (BS) agar	Black colonies surrounded by a brown to black zone that casts a metallic sheen
Brilliant green (BG) agar	Pink colonies surrounded by red zone
Xylose lysine desoxycholate (XLD) agar	Black-centered red colonies with H <sub>2</sub> S producers, red colonies with non-producers
MacConkey (MA) agar	Uncolored, transparent colonies
Hektoen Enteric (HE) agar	Blue to blue-green colonies, most with black centers (H <sub>2</sub> S producers)
Eosin-methylene Blue (EMB) agar	Translucent amber to colorless colonies

Source: From Ref. 7.

serological testing for *Salmonella* begins with the O antisera, and H and Vi are reserved for later use in specific identifications. *Salmonella* serotypes can be further differentiated based on reactivity with a defined set of bacteriophage. This is generally used for epidemiological purposes and is performed in highly specialized laboratories.

The conventional culture methods described above require 4 days to complete, and this does not include serotyping and, if necessary, phage typing. Several rapid methods are currently being developed to identify *Salmonella* in general, while specific tests to identify serotypes are also under development. Among the rapid assays (17) presently available are culture methods that use selective and differential media, enzyme immunoassays, latex agglutination, immunodiffusion techniques, DNA colony hybridization (17), and the polymerase chain reaction (PCR) (18,19). These methods

**TABLE 2** Biochemical Reactivity of *Salmonella*

Test or substrate	Positive reaction	Negative reaction	<i>Salmonella</i> reactivity <sup>a</sup>
Glucose	Yellow butt	Red butt	+
Lysine decarboxylase	Purple butt	Yellow butt	+
H <sub>2</sub> S	Blackening	No blackening	+
Urease	Purple-red color	No color change	-
Lysine decarboxylase broth	Purple color	Yellow color	+
Phenol red dulcitol broth	Yellow color and/or gas	No gas, no color change	+ <sup>b</sup>
KCN broth	Growth	No growth	-
Malonate broth	Blue color	No color change	- <sup>c</sup>
Indole test	Violet color at surface	Yellow color at surface	-
Phenol red lactose broth	Yellow color and/or gas	No gas, no color change	- <sup>c</sup>
Phenol red sucrose broth	Yellow color and/or gas	No gas, no color change	-
Voges-Proskauer test	Pink-to-red color	No color change	-
Methyl red test	Diffuse red color	Diffuse yellow color	+
Simmons citrate	Growth, blue color	No growth, no color change	v

<sup>a</sup> +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.

<sup>b</sup> Majority of *S. arizonae* cultures are negative.

<sup>c</sup> Majority of *S. arizonae* cultures are positive.

Source: From Refs. 7 and 15.

were designed to identify all *Salmonella* species. With the dramatic increase in outbreaks due to Enteritidis and the necessary surveillance of poultry and poultry products for this serotype, several methods have been developed for the rapid identification of Enteritidis. These assays include an enzyme-linked immunoabsorbent assay (ELISA) using two monoclonal antibodies (20), colony hybridization (21), and PCR (22). Although all of these methods are more rapid than traditional culture procedures, they currently are used only as a screening tool. Standard culture, serological, and biochemical methods are still used for definitive identification of *Salmonella*.

## VI. PATHOGENICITY

*Salmonella* infections are initiated when a pathogenic strain is ingested through contaminated food or water. *Salmonella* establish infection by sequentially surviving the acidic pH of the stomach, competing with the normal flora of the small bowel and passing through the epithelial barrier to proliferate in the Peyer's patch and the draining mesenteric lymph nodes (23). The specialized antigen-sampling M cells present in the follicle-associated epithelium overlying the Peyer's patches are the preferential site of *Salmonella* invasion in vivo, although the invasion of enterocytes has also been described.

Experiments using a murine model (23) demonstrate that invasive salmonellae are able to locate and invade M cells within the epithelium of the murine lymphoid follicles within 30 minutes of infection. The bacteria display a clear preference for M cells; no interactions can be found with enterocytes at these early time points. The entry of *Salmonella* into murine M cells occurs by a membrane-ruffling mechanism that appears identical to *Salmonella* entry into tissue culture cells. These ruffles occur on the membrane at the site of bacterial attachment and are presumed to occur in direct response to a bacterial signal. These membrane ruffles erupt from the host cell within seconds of bacterial attachment to the host cell, and subsequently columns of polymerized actin surround and enclose the organism. Within a short period of time the entry signal peaks, and as it dissipates the membrane ruffles return to the cell along with the enveloped bacterium. The formation of membrane ruffles is dependent upon actin monomer polymerization into microfilaments.

The pathogenic effect of invading salmonellae on M cells is seen within 60 minutes postinfection. Dying and disintegrating M cells that contain bacteria are observed detaching from the mucosal surface. This damage allows organisms that had been confined to the lumen of the small intestine access to underlying tissue. At later time points, significant damage to the intestinal epithelium is observed that includes invasion of enterocytes, sloughing of large sections of epithelial cells, and penetration of significant numbers of bacteria into deeper tissues. In salmonellosis, the diarrheal symptoms result from the inflammatory reaction that has been elicited in the small intestine due to the presence of invading bacteria. Some serotypes are also capable of producing enterotoxin(s), which are also important in the production of diarrhea (7).

Presence of the invading bacteria in the Peyer's patches causes a number of host responses (23). The total number of M cells increases in the follicle-associated epithelium as compared with uninfected mice, the average crypt lengthens, and the rate of enterocyte migration from the intestinal crypts increases. In addition, the numbers of CD4+ cells increase and CD8+ cells decrease. Although the pathogen causes significant damage to the epithelium of the lymphoid follicles at early points in the infection, data indicate that the host quickly replaces cells that have been damaged or destroyed. The humoral and cellular arms of the immune system are also activated by the invading salmonellae.

Following passage through the intestinal epithelium of the Peyer's patch, invading organisms quickly enter the lymphatic system, where interactions with professional killing cells determine the ultimate fate of the infection (23). These cells possess both oxygen-dependent and oxygen-independent mechanisms to kill internalized bacteria. Because the oxygen-dependent killing mechanisms of macrophages appear to have little effect on pathogenic *Salmonella*, the host is more dependent on oxygen-independent mechanisms. However, evidence suggests that salmonellae have evolved



mechanisms to circumvent or delay the killing activity of these mechanisms. For example, upon entry into macrophages Typhimurium delays the fusion of the phagosome to the lysosome, and acidification of vacuoles containing live *Salmonella* organisms is delayed by 4–5 hours compared to vacuoles containing dead organisms. The growth state of Typhimurium also changes rapidly after entry into macrophages; immediately after entry into phagocytic cells the bacteria begin growing rapidly and require protein synthesis for survival. However, after 2 hours within the phagocytic cell the bacteria switch from a rapidly growing state to a survival mode that does not require protein synthesis. Salmonellae that survive the hostile environment of the phagocytic cells subsequently drain through the lymphatics to the thoracic duct into the blood and ultimately to the liver and spleen.

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

The molecular and genetic basis for *Salmonella* adherence to and invasion of host cells is both complex and distinct. At least 60 genes are required for *Salmonella* virulence (24). Several of these genes reside as an operon on a large virulence plasmid common to most *Salmonella* serotypes. A majority of these virulence genes are found in several regions of the chromosome within pathogenicity islands, large clusters of virulence genes not found in related nonpathogenic species. Several operons containing fimbrial genes have also been localized to the bacterial chromosome and large virulence plasmid. Several small pathogenicity islets also have been identified on the chromosome that affect bacterial virulence.

### A. Pathogenicity Islands

#### 1. SPI-1

The best characterized of the pathogenicity islands is SPI-1, a 40 kb region harboring over 30 genes and mapping to the 63-min region of the Typhimurium chromosome (24). The SPI-1 island encodes two distinct regulatory proteins, InvF and HilA, as well as the components of a type III secretion system, termed Inv/Spa.

The Inv locus, present in essentially all virulent salmonellae, encodes genes for invasion into intestinal epithelial cells in vitro. Sequence analysis of this region identified 13 genes arranged in the following order (25): *invH*, *invF*, *invG*, *invE*, *invA*, *invB*, *invC*, *invI*, *invJ*, *spaO*, *spaP*, *spaR*, and *spaS*. Functional analysis of nonpolar mutants has shown that all of these genes except *invB* are required for Typhimurium entry into cultured epithelial cells. The *inv* locus is required not only for invasion into cultured cells, but also for salmonellae virulence. Typhimurium strains carrying a deletion in the *invA* gene are defective in colonizing the intestinal epithelium of Balb/c mice, resulting in a higher LD<sub>50</sub> for the Inv<sup>-</sup> mutants (26).

Sequence analysis of the predicted gene products from several Inv/Spa genes has revealed homology to the type III protein secretion systems of other plant and animal pathogens (25). Homologs of the Inv/Spa system occur in a broad range of bacterial pathogens including *Shigella*, *Yersinia*, *Erwinia*, *Xanthomonas*, and *Pseudomonas* (24). This phylogenetic distribution is attributable to the independent acquisition of these secretion genes by each taxon. Some of the proteins that make up this system are also homologous to components of the flagellar assembly apparatus of gram-positive and gram-negative bacteria.

Two genes coding for putative chaperone proteins have also been identified. *InvI* encodes a 17 kDa protein with a very high isoelectric point (9.6) and a high probability of forming coiled coils, both features consistent with chaperone activity (27). Mutations in *invI* render salmonellae deficient for entry into cultured epithelial cells. The other gene, *sicA*, encodes a protein homologous to the *Shigella* and *Yersinia* chaperones IpgC and LcrH. *sicA* is required for salmonellae entry, since a nonpolar mutation in this gene renders Typhimurium severely deficient for invasion (25).

The SPI-1 island has several characteristics indicating that it was acquired through horizontal

gene transfer (24). SPI-1 has a base composition of only 42% G+C, which is much lower than the 52% G+C found in the salmonellae genome. The size, order, and orientation of the *inv* and *spa* genes within SPI-1 are broadly similar to the invasion genes on the *Shigella* plasmid, suggesting that these sequences are transmissible. Unlike pathogenicity islands in pathogenic *E. coli*, SPI-1 is not inserted into a tRNA gene, and therefore its origin is unclear (10).

## 2. SPI-2

A second 40 kb pathogenicity island, SPI-2, has been mapped to 31-min on the *S. enterica* serotype Typhimurium chromosome (28,29). SPI-2 contains at least 17 genes that code for a two-component regulatory system and a type III secretion system designated Spi/Ssa. These systems are distinct in structure and function from the SPI-1 Inv/Spa system and the type III secretion system that mediates the export and assembly of flagellar components in other bacterial genera.

The role of the SPI-2 island has not been fully elucidated; data from mutants defective in SPI-2 genes suggest that SPI-2 is required for systemic disease. One of the hallmarks of systemic infection is the ability to spread from the intestinal tissue via the lymphatics into the bloodstream and to multiply within the macrophages of the liver and spleen. An important step during colonization of this intracellular niche was the acquisition of SPI-2. SPI-2 was identified using signature transposon mutagenesis to isolate Typhimurium virulence genes necessary for systemic infection in mice. Mutations in several SPI-2 genes result in strains with a very high median lethal dose (LD<sub>50</sub>) for mice when inoculated either orally or intraperitoneally (10).

Analogous to SPI-1, the SPI-2 island has a low base composition (45% G+C), which suggests that it was also acquired by horizontal gene transfer (10). The phylogenetic distribution of SPI-2 differs from that of SPI-1 in two respects: (a) the SPI-2 sequences are restricted to the genus *Salmonella*, whereas SPI-1-hybridizing sequences have been detected on other bacterial pathogens; (b) like the pathogenicity islands of *E. coli*, SPI-2 is inserted into the tRNA gene encoding tRNA<sup>val</sup> (30).

## 3. SPI-3

A new pathogenicity island, SPI-3, has recently been identified at 82-min on the Typhimurium chromosome. SPI-3 is located downstream of the insertion site of SPI-1 and LEE pathogenicity islands of uropathogenic and enteropathogenic strains of *E. coli*, respectively, which suggests a common mechanism for the acquisition of these sequences (24).

## B. The Large Virulence Plasmid

Since the initial description of the Typhimurium large virulence plasmid (31), genetically related plasmids have been identified in most of the serotypes of nontyphoidal *Salmonella* associated with systemic disease. A consensus has emerged that the large virulence plasmid enables salmonellae to cause progressive systemic infections of the reticuloendothelial organs in experimental animals (32). The virulence plasmid, however, does not affect the ability of salmonellae to cause enteric infection and damage (31). This observed pathogenesis suggests that the virulence plasmids encode resistance to phagocytosis and/or killing by macrophages and neutrophils (31).

The *spv* (salmonella plasmid virulence) genes were initially identified through deletion and insertion mutagenesis (31,33). Only 7.8 kb of the large virulence plasmid encoding five genes *spvRABCD* is necessary to confer virulence on plasmid-cured salmonellae. The *spv* genes are all transcribed in the same direction, beginning with *spvR*, followed by *spvABCD*. A sixth open reading frame encoding a protein not essential for virulence in mice (*orfE*) is encoded down stream of *spvD*.

Although the distribution of the *spv* operon has not been determined for all phylogenetic lineages of the genus *Salmonella*, its localization on the large virulence plasmid in *S. enterica* serotypes suggests that this operon was obtained by horizontal gene transfer. The G+C content of the *spv* genes, 46%, is lower than that reported for Typhimurium in general (51–54%), lending support to horizontal gene transfer (31).

## C. Pathogenicity Islets

### 1. Fimbrial Genes

To pave the way for invasion, salmonellae must adhere to the host intestinal epithelial cells. At least five (24) fimbrial operons, *fim*, *agfA*, *lpf*, *ser*, and *pef*, have been identified in *Salmonella*, the majority of which have not been found in other enteric species. The *Salmonella fim* operon encodes type I fimbriae that are distinct from those encoded on the *E. coli* chromosome. Phylogenetic analysis of these sequences indicates that the *fim* operon and the *agfA* gene, which specifies a salmonellae homolog of the *E. coli* gene encoding Curli, are ancestral to *Salmonella*. The adhesion encoded by the *lpf* operon is necessary for entry of Typhimurium into murine Peyer's patches and has no counterpart in the *E. coli* chromosome. Although the *fim* operon is present in all salmonellae, several serotypes lack the *lpf* operon. Sequence analysis has revealed that in these serotypes, the *lpf* operon was lost by a deletion event following its acquisition (10).

The sporadic distribution of fimbrial operons within the serotypes of *Salmonella* and the absence of hybridizing sequences from other enteric species suggest that these regions are subject to extensive transfer. Certain fimbrial operons have been detected on the virulence plasmid present in virtually all serovars, and genes encoding type I fimbriae map to different genomic locations in the *E. coli* and Typhimurium chromosomes, suggesting that fimbrial adhesions were acquired independently by enteric species. Moreover, many fimbrial systems are flanked by short repeats, and the movement or rearrangement of these genes is thought to regulate fimbrial phase variation (24).

The specific combination of fimbrial genes and the adhesive properties of the bacterial cell appear to be associated with the host range of the serovar. However, the mechanism by which the presence or absence of a fimbrial operon affects the colonization of a particular host is unknown.

### 2. The *sifA* Gene

At 27-min on the Typhimurium chromosome, a 1.6 kb segment contains the *sifA* gene, which is required for the formation of filamentous structures in the lysosomal vacuoles of infected epithelial cells (34). The *sifA* genes have no homologs in the sequence database and are apparently restricted to salmonellae. *SifA* mutants are attenuated for virulence.

### 3. The *pagC* and *msgA* Genes

Two virulence genes, *pagC* and *msgA*, have been localized to a low G+C content region at 25-min on the Typhimurium chromosome (35). The *pagC* gene encodes for an outer membrane protein that is similar in sequence to proteins found in other bacteria, including Ail from *Yersinia*. Although the *ail* locus of *Yersinia* has been implicated in the invasion of nonphagocytic cells, *pagC* mutants are invasive but cannot replicate in macrophages or cause lethal infections in mice when administered intraperitoneally, suggesting that the PagC protein plays a role in systemic disease (24).

The *msgA* gene is also required for intramacrophage survival and mouse virulence. Despite the similarity in their virulence phenotypes, the *pagC* and *msgA* genes differ in their regulation and phylogenetic distribution. Expression of *pagC*, but not *msgA*, is dependent on the PhoP regulatory protein, and *msgA*-hybridizing sequences are detected in enteric species that lack the *pagC* gene (21).

## VIII. CONTROL MEASURES

Breaking the cycle of salmonellae from the feed animal to the consumer has proven to be very difficult. The United States and many European countries have evaluated several approaches to eradicate or at the very least reduce the incidence of salmonellosis.

The first line of defense against salmonellae in the food supply is control on the farm. Large-scale and intensified farming practices that confine many animals or fowl in close quarters has significantly contributed to the increase in salmonellae seen in the food supply. *Salmonella* inadvertently introduced into a herd or flock quickly spreads since the confined conditions expose the stock

to contaminated feed and water through contact with infected animals and animal feces. Several methodologies are being evaluated to inhibit the spread of salmonellae on the farm, especially among broiler and laying flocks.

One technique currently being evaluated is competitive exclusion, a method that uses defined bacterial flora to compete with salmonellae for colonization of the ceca and other tissues. Several competitive exclusion products including the commercial BROILACT (36) have been evaluated using newly hatched chicks (36–39). The competitive exclusion cultures are administered to chicks using crop gavage, whole body spray, inclusion in drinking water, or encapsulation in alginate beads in feed, and chicks are then evaluated for colonization at various time points after exposure to salmonellae. Results of several studies indicate that competitive exclusion protects chicks from cecal colonization and deep tissue invasion by several *S. enterica* serotypes, including Enteritidis and Typhimurium (36,37). This technique even provided protection against deep tissue invasion by Enteritidis phage type 4 (36).

Vaccination is another alternative being assessed in several eradication programs. Several vaccine candidates including live, avirulent Typhimurium (40,41), inactivated Enteritidis phage type 4 (42), and genetically defined Enteritidis (43), to name just a few, have been evaluated for efficacy in preventing salmonellae colonization of chicks. Vaccination with avirulent Typhimurium induced protection against intestinal, visceral, reproductive tract, and egg colonization, and protection was shown to last at least 11 months (41). Reports also indicate that vaccination also prevented transmission of both Typhimurium and Enteritidis into eggs without affecting egg production (41). Chicks immunized with the genetically defined Enteritidis vaccine (43) demonstrated a significant reduction in colonization of spleen, liver, ovaries, and ceca. There was also a marked decrease in fecal shedding of salmonellae in the vaccine group.

Results from the competitive exclusion and vaccine trials indicate that salmonellae control on the farm may be feasible. However, complete eradication may prove difficult. Therefore, control of bacterial contamination during slaughter and processing is also necessary to decrease salmonellae present in the food supply.

The Hazard Analysis Critical Control Point (HACCP) system was designed to identify potential sources of contamination during slaughter and processing (7). This program consists of (a) identification and assessment of hazards associated with all stages of food processing, (b) determination of critical points at which identifiable hazards can be controlled, and (c) establishment of procedures to monitor the identified control points to determine whether or not a hazard does occur. HACCP is a systematic approach to food safety where a procedure is set up to both establish food safety and monitor and quickly correct problems.

A pathogen reduction and HACCP plan for salmonellae has recently been implemented in several large meat and poultry plants in the United States (1,2). Preliminary results indicate that *Salmonella* prevalence in broiler carcasses dropped from 20% before the program to 10.4% after implementation. For swine carcasses, prevalence dropped from 8.7 to 5.5%. Although these are preliminary data, they suggest that HACCP programs can reduce salmonellae in the food supply.

Education of the consumer is also critical for the control of salmonellosis. Making the consumer aware that *Salmonella* is present in foods such as fresh poultry and eggs is critical in the control of disease. More important, consumers must be made aware of potential cross-contamination from cooking utensils such as cutting boards and knives to foods that will not be cooked. Finally, simple hygiene such as hand washing before food preparation will greatly reduce the number of cases of *Salmonella* food poisoning seen each year.

Continuous surveillance and careful reporting of *Salmonella* isolates also contributes to control of disease. This surveillance improves awareness of new serotypes, common sources, antibiotic resistance, and carrier state.

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# 10

## Typhoid *Salmonella*

Lan Hu and Dennis J. Kopecko

*U.S. Food and Drug Administration, Bethesda, Maryland, U.S.A.*

### I. BACKGROUND

*Salmonella* is a genus of the family Enterobacteriaceae (1). This genus is named in honor of D. E. Salmon, an American bacteriologist and veterinarian who studied animal-pathogenic salmonellae. These enteric (i.e., associated with the intestine) bacteria are gram-negative, usually motile, facultatively anaerobic, and non-spore-forming bacilli. *Salmonella* infections of humans and animals continue to be a major public health problem worldwide and also have a large negative economic impact on food production. Enteric fever (i.e., typhoid or paratyphoid fever) is a more serious form of salmonellosis in which specific salmonellae produce invasive disease in humans. The incidence of human typhoid fever in developing countries was recently estimated to be 17–20 million cases annually, with 600,000 deaths, most of which occur in Asia (2). However, the incidence and severity of enteric fever vary geographically, but disease is infrequent in developed nations. Variably high levels of endemic enteric fever exist in developing regions of the world, and imported human cases or contaminated food from endemic regions continue to cause problems in developed nations (3).

In most cases of enteric fever, the etiological agent is *S. typhi* (new terminology = *S. enterica*, subgroup enterica, serovar Typhi) and the disease is termed “typhoid fever.” Other salmonellae, specifically serovars Paratyphi A, B, C, can also cause enteric fever (termed “paratyphoid fever”), but the symptoms are milder and the mortality is lower. Typhoid fever is typically acquired by ingesting food or water that has been contaminated by typhoid-infected individuals. Food handlers who happen to be asymptomatic typhoid carriers shedding large numbers of fecal *S. typhi* are a common source of contaminated food. Typhoid fever can be fatal if untreated and generally causes mortality in 5–30% of typhoid-infected individuals in the developing world.

Foodborne outbreaks of intestinal disease and enteric fever have been common throughout the development of civilization, but the causes only came to light with the development of the science of bacteriology (propelled by the germ theory of disease) in the latter half of the 1800s. Despite the absence of an identified cause of typhoid fever and based upon careful observation of his patients in England, William Budd (4) demonstrated that bowel discharges were the main source of infection, which could contaminate food, water, or linens that could serve as a secondary sources of disease transmission. He proposed that typhoid could be controlled by proper disposal of patient feces.

The causative agent of typhoid fever was observed microscopically by Eberth in 1880 (5) and was first pure cultured by Gaffky in 1884 (3,6) from the spleens of infected patients. Following identification of the causative agent and additional studies of typhoid epidemiology, Robert Koch in 1903 outlined three logical methods of typhoid control, which are still important today: disinfect the excreta at its source, improve sewage handling, and isolate convalescent patients until they become *S. Typhi*-free (3). Though these control measures were useful, case-fatality rates continued to range up to 30% until Theodore Woodward (7) initiated the use of antibiotic therapy for typhoid

fever showing that chloromycetin eliminated *S. Typhi* from the blood of 10 acutely ill Malaysian patients. Since that time, antibiotic usage for the treatment of enteric fevers has markedly lowered the mortality rate from this disease. The first whole-cell, killed *S. Typhi* vaccines were employed by the British military around 1900 (8) and, despite lack of evidence of effectiveness, quickly gained widespread use by other militaries. During the past 30 years an improved, killed, whole-cell injectable vaccine and an attenuated, live, oral vaccine have been widely used. These newer vaccines have undoubtedly lowered disease incidence and benefited society. However, despite these important medical treatment and prophylactic advances, enteric fever continues to be a tremendous disease burden worldwide due largely to continued poor sanitation and the scarcity of appropriate antibiotics/vaccines in the developing world.

This short chapter is meant to introduce the reader to the causative agents of enteric fever (i.e., formerly termed *S. typhi* and *S. paratyphi*) as well as to the disease, its diagnosis, and prevention. Here we review the classical characteristics of these pathogenic organisms, the epidemiology of disease, common procedures used in enteric fever diagnosis and treatment, the latest molecular understanding of disease pathogenesis, and practical control measures.

## II. CHARACTERISTICS

### A. Nomenclature and Classification

There are many methods for the nomenclature and classification of the numerous *Salmonella* types encountered in nature, which are all gram-negative, typically motile, and facultatively anaerobic bacilli. These include determining key diagnostic biochemical traits (e.g., salmonellae do not typically utilize lactose as a carbon source, but all utilize glucose and are oxidase negative) or susceptibility to various bacteriophages (i.e., phage typing). However, the Kauffmann-White antigenic scheme (9,10) constitutes the principal diagnostic tool for the differentiation of more than 2000 *Salmonella* serotypes. Serotype identification, derived from agglutination reactions with specific antisera, is based upon the organism's complement of somatic (O-antigen), capsular, and flagella (H antigen) antigens. The O antigens are the lipopolysaccharides (LPS) of the outer membrane, similar to the O antigens of other Enterobacteriaceae. The H antigens are the proteins that make up the peritrichous flagella of these bacteria, but they can be expressed in one of two forms (termed phases). The phase 1 H antigen is specific and is associated with the immunological identity of that particular serovar. However, *Salmonella* strains can alter flagellar antigens to phase 2 (containing a different antigenic subunit protein), which can be shared by many serovars. Certain salmonellae express a surface-bound polysaccharide capsular antigen, which typically blankets the O antigen and blocks O-agglutination; however, the capsule can be selectively removed by heat treatment prior to O-agglutination assay. The virulence (Vi) capsular antigen is expressed by all *S. Typhi* and certain Paratyphi serovars upon primary isolation. Because each isolate can be identified relatively easily and quickly by agglutination with group-specific antisera, serotyping is very useful in studying the epidemiology of *Salmonella* outbreaks.

Based upon new technologies that measure DNA relatedness, all serotypes of *Salmonella* have been found to contain genomic DNA of 50–53% G+C average content and to belong to one related DNA hybridization group, within which seven subgroups (in numerical order: enterica, salmae, arizonae, diarizonae, houtenae, bongori, and indica) were identified (11–13). The name *Salmonella enterica* is now officially recognized as the only genospecies for all salmonellae (14). Most clinically important salmonellae (>99% of isolates) belong to subgroup 1. Despite these advances in understanding, which have led to a refined nomenclature system, practical identification of *Salmonella* isolates still depends on standard bacteriological tests and serotyping. In the past, different *Salmonella* serotypes were given “species” designations depending upon place of first isolation (e.g., *S. montevideo* or *S. minnesota*) or type of disease produced (e.g., *S. typhi*, *S. paratyphi*, or *S. choleraesuis*), and these names are still used today. However, in keeping with current nomenclature, the accepted serovar designation has been utilized in this chapter (i.e., *S. Typhi* and *S. Paratyphi*).



## B. Growth Characteristics

The optimum growth temperature of *S. Typhi* is 35–37°C, but they can grow between 5 and 47°C (15). The optimal pH for salmonellae growth lies between 6.5 and 7.5, with possibilities for growth at pH values ranging from 4.5 to 9.0 (15). Below pH 4.0 and above pH 9.0, the organism will die slowly. All salmonellae grow well on ordinary bacteriological media under both aerobic and anaerobic conditions. Salmonellae have simple nutritional requirements (essential salts plus glucose), but their growth rate increases in complex media to a doubling time of approximately 30 minutes. These bacteria grow well in liquid or semi-liquid media with high water concentration [i.e., water activities ( $A_w$ ) between 0.999 and 0.94; pure water  $A_w = 1.0$ ] (15). At lower  $A_w$  levels between 0.20 and 0.9, the *Salmonella* death rate increases as the  $A_w$  increases. However, *Salmonella* can survive for long periods of time, even under adverse storage conditions of temperature and acidity, at  $A_w$  levels of <0.2 (e.g., in dried foods). These organisms generally grow well in the presence of 0.4–4% NaCl. However, salmonellae are sensitive to heat and are killed at temperatures of 70°C or above. So, ordinary cooking is sufficient to destroy *Salmonella* if applied long enough to reach 70°C throughout the food being cooked. Salmonellae are resistant to drying and may survive in dust for long periods of time, even years, and may tolerate as much as a 20% salt environment for weeks. Salmonellae are, however, sensitive to many of the treatments commonly used to preserve foods. Thus, salmonellae (including serovars Typhi and Paratyphi) have the ability to survive in contaminated water, to multiply in very moist food, and to persist for long periods in dried foods or under dry conditions. Foods prepared without adequate sanitation or treatment (e.g., heat) pose a very real health hazard.

## C. Infectious Dose

The infectious dose of *Salmonella* depends upon the serovar, the bacterial strain, bacterial growth conditions, and host susceptibility. For example, *S. Typhi* was found to cause typhoid fever in 50% of adult volunteers who ingested  $10^7$  virulent bacteria in nonbuffered skim milk, but onset of disease occurred earlier on average in 95% of adult volunteers who ingested  $10^9$  bacteria in nonbuffered skim milk (16,17). However, Blaser and Newman (18) reported that infectious doses in 10–12 natural disease outbreaks occurring over the period 1908–1980 were estimated at  $<10^3$  cells of *S. Typhi*, whereas in 2 outbreaks the estimate was about  $10^5$ – $10^9$  CFU per infectious dose. The host factors controlling susceptibility to infection include the condition of the intestinal tract (e.g., the level of gastric acidity, use of antacids, or prior gastric surgery), age (the very young and the very old are most susceptible), and other underlying illnesses or immune deficiencies. The food matrix in which *S. Typhi* resides may also affect relative levels of pathogenicity [recent studies have shown that growth in NaCl concentrations above ~1.75% induces increased production of secreted proteins needed for virulence (19,20)].

## III. DISEASE

*Salmonella* spp., in general, cause one or more of four broad clinical syndromes in humans: gastroenteritis (inflammatory diarrhea), enteric fever, septicemia with associated focal lesions, and asymptomatic long-term carriage (the latter three being due to tissue-invasive *Salmonella* serovars). The human-specific serovars Typhi and Paratyphi A, B, or C produce enteric fever, a rather distinct clinical disease. Levine et al. (21) defined “enteric fever” as a generalized infection of the intestinal lymphoid tissue and the reticuloendothelial system (RES), accompanied by sustained fever and bacteremia. Infection can range from asymptomatic to mild (typical of paratyphoid) or severe (typical of typhoid) disease, and typhoid is much more common (~10-fold) globally than paratyphoid fever. During infection, these serovars readily penetrate and cross the human small intestinal mucosa, then invade and survive within the mononuclear phagocytic system, from which they disseminate

via the bloodstream, causing intermittent low-level bacteremia, colonization of the RES, and hepatosplenomegaly.

## A. Incubation Period and Onset

From the time of ingestion, symptoms typically begin within 7–21 days but can take as long as 5–6 weeks to appear. In classical enteric fever the onset of malaise, weakness, headache, and fever is slow and insidious. The fever rises gradually, is usually higher at night, until it reaches 39–40°C. In some geographic areas, diarrhea lasting several days may precede systemic illness. Respiratory symptoms (e.g., dry cough), constipation, abdominal pain, and anorexia are often encountered early in disease. Relative bradycardia and mild splenomegaly are common, and, as disease progresses, patient confusion can turn to stupor and delirium. During the second week of disease, about half of all cases exhibit rose spots on the abdomen, chest, and back due to dissemination of the organisms to peripheral skin foci (22,23). Enteric fever can be difficult to diagnose because any of these symptoms, including fever, can be absent. Recovery can be observed typically by the third week of illness, but in the absence of antimicrobial therapy approximately 10% of patients may relapse, die, or undergo complications such as intestinal hemorrhaging or perforation. Weight loss and weakness often persist for weeks or months following untreated illness.

During the first week of acute infection, typhoid bacteria can be cultured from stool in 20% of cases and from blood in ~75% of cases. By the third week of illness, the causative agent can be isolated from feces in 85% of cases and the urine of 25% of patients, but detection from blood is already declining. The highest rate of detection of enteric fever organisms is obtained from culture of bone marrow aspirates (23).

## B. Complications

### 1. Relapse

Relapse is the most common sequela of typhoid fever, and the rate of relapse increases to ~20% following certain antimicrobial therapies (e.g., chloramphenicol), possibly due to premature termination of therapy, inadequate tissue penetration by the antibiotic, or the bacteriostatic nature of chloramphenicol. In relapse, after an afebrile period of 7–10 days, fever and malaise return and can serially recur following similar cycles until immunity develops or successful antimicrobial therapy is instituted.

### 2. Intestinal Hemorrhage and Perforation

Though less frequent in occurrence due to the common use of antibiotics, hemorrhaging from bowel ulceration can be serious. Intestinal perforation is a dreaded complication of typhoid that is difficult to diagnose and requires a long convalescence (24).

### 3. Carrier State

Fecal excretion of *S. Typhi* following acute illness is common and may continue for weeks to months. About 3% of typhoid cases (even asymptomatic infections) become long-term intestinal shedders. The chronic carrier state (shedding > 1 year) is associated, although not invariably, with gall bladder disease and is more common in women and the elderly. Evidence now suggests that chronic carriers have an elevated risk of hepatobiliary cancer (25). Short-term excretion of *S. Typhi* in the urine also occurs during acute illness.

### 4. Other Involved Sites

Hepatic involvement with associated jaundice occurs more frequently in typhoid-endemic regions. Though rare, cystitis caused by *S. Typhi* can occur. Various neurological sequelae, including psychosis, ataxia, polyneuritis, and seizures, have been reported, but the exact molecular bases are

unknown (24). The enteric fever salmonellae may settle at many internal foci, only to reappear later as lesions/abscesses at various sites.

## **IV. EPIDEMIOLOGY**

*Salmonella* serovars Typhi and Paratyphi A, B, and C only colonize humans, and, therefore, disease spread is always associated with a person who has enteric fever or is an asymptomatic carrier. Food or water contaminated by the feces or urine of food handler-carriers is considered the most common source of enteric fever bacteria. Direct person-to-person transmission is infrequent, but disease transfer from acute cases to care providers and direct anal-oral transmission of *S. typhi* has been documented (26). These facts have led to the routine quarantine of hospitalized, acutely ill typhoid patients. Laboratory accidents, via contaminated hand-to-mouth transmission, have also resulted in typhoid fever in research workers (27).

### **A. Factors Influencing Incidence**

The distribution and prevalence of typhoid fever can be directly associated with population density and the availability of modern sanitary methods for sewage treatment and water purification. Economic and military disruptions of modern sanitation facilities can quickly reverse a reduced incidence of enteric fever.

Typhoid cases tend to peak locally in the warmer months. However, increased international air travel by individuals coming from endemic regions and the widespread availability of international foods (i.e., the global economy) has created a year-round occurrence of disease. In outbreaks of typhoid, the age incidence is heavily dependent upon the source of the outbreak (e.g., ice cream or salad) and the nature of the outbreak (e.g., family picnic or store-distributed, contaminated food). However, children appear to be at increased risk of disease in highly endemic areas of the world. For developed countries, unimmunized individuals of all ages may be susceptible to typhoid fever, with more severe disease occurring in the very young and the very old. Immunocompromised individuals have increased susceptibility to typhoid (28).

### **B. Incidence**

A recent report estimates that 17–20 million cases of typhoid fever occur worldwide annually, with 600,000 associated deaths (2). In developed countries with a high level of sanitation, the incidence is expectedly low. For example, during the last decade fewer than 500 annual cases of typhoid were reported in the United States (29). The annual incidence ranges from 0.24 to 3.7 cases per 100,000 population in Western Europe, Japan, and the United States (3). However, the incidence in the Far East, where typhoid is highly endemic and the fifth leading cause of death, averages 1000 cases per 100,000 population.

For endemic countries, the high typhoid incidence most likely results from both the use of raw foods and water contaminated with sewage as well as commercial food preparation by the many existing asymptomatic typhoid carriers in those populations. In contrast, the low disease burden in developed nations results mainly from a few immigrant food worker carriers and from importation of contaminated international foods, both of which generally cause small outbreaks.

### **C. Foodborne Outbreaks**

Many outbreaks of enteric fever due to contaminated foods have been documented over the past century. Vehicles have included milk, ice cream, butter, cheese, shellfish, watercress, dried coconut, orange juice, canned corned beef, and chicken salad (15). *S. Typhi* can also contaminate shellfish or vegetables grown in water, or crops simply washed in water, when the water is polluted by sewage. Though a larger population exposure typically occurs from a waterborne outbreak, the

causative organisms may be concentrated following growth in food items and may cause a higher incidence of or more severe disease.

Food contaminated by a typhoid carrier during preparation may result in a series of intermittent typhoid cases over time or in a more dramatic, contained outbreak. Typhoid Mary, the now infamous typhoid carrier, was a transient, hired household cook in New York State during the early 1900s. This chronic typhoid carrier succeeded in initiating at least 10 typhoid outbreaks, which were ultimately responsible for at least 53 cases of typhoid fever and 3 deaths (30). She refused cholecystectomy and evaded authorities for many years until she was eventually quarantined for the remainder of her life. Older women are three times as likely to become carriers as men, due to a higher prevalence of gallbladder disease. Women over age 40 have a 16% chance of becoming a chronic carrier after infection by *S. Typhi* if not given appropriate antibiotic therapy. Foodborne disease outbreaks caused by chronic typhoid carriers are still common today due to frequent international travel. For example, in a 1981 San Antonio, Texas outbreak, 80 persons were infected over time by one female employee who was involved in food preparation. She had gallstones, and a culture of the stones and her gallbladder grew the causative organism (31). In 1986, 10 clustered cases of typhoid fever occurred in Maryland as a result of salad contaminated by a female carrier food handler (32). Larger-scale foodborne outbreaks of typhoid have also occasionally occurred. Two recent major occurrences of typhoid in the United States followed from contamination of orange juice during preparation (33). In 1964, an outbreak causing 505 cases appeared in Aberdeen, Scotland, due to contaminated corned beef (34), and one in Germany due to contaminated potato salad caused 344 cases.

#### **D. Waterborne Outbreaks**

In highly endemic developing countries, various factors, including poor personal hygiene, poor sanitation, improper disposal of sewage, and lack of modern, clean water systems, lead to an increased rate of contamination of existing local water sources with the causative organisms. In these regions, water represents the major vehicle of typhoid transmission. Sewage-contaminated rivers, streams, and ponds serve as direct and indirect infection sources [e.g., *S. Typhi* has been isolated from vegetables grown in fields irrigated by sewage-contaminated water (35)]. In Baramullah in the Kashmir Valley, 230 cases of typhoid fever occurred during an outbreak, apparently caused by ingestion of contaminated water (36). Consumption of unboiled water during a 1997 typhoid outbreak in Dushanbe, Tajikistan, caused 2200 cases of illness and 95 deaths (37).

In developed countries with modern sanitary systems, waterborne spread of typhoid is rare, but it has occurred (38). Effective sewage management systems and surveillance/treatment of known carriers have reduced the number of typhoid fever cases in the United States from more than 5000 annually in 1942 to fewer than 500 annually in the 1990s. Seventy percent of all U.S. cases are now acquired during travel to endemic regions (39).

### **V. ISOLATION AND IDENTIFICATION**

The clinical diagnosis of enteric fever can be difficult because early symptoms are usually vague and could have multiple potential causes, and disease onset is insidious. Clinical suspicion must be verified by laboratory isolation of the causative agent to ensure optimal antibiotic therapy and initiation of relevant public health measures to prevent disease spread. Similarly, isolation of the responsible agents from asymptomatic food handler-carriers or from contaminated foods/water can also be difficult. Damaged bacteria in dried foodstuffs or low numbers of *S. Typhi* in heavily contaminated water may require preenrichment/enrichment culturing, as described below.

#### **A. Isolation**

Typically, *S. Typhi* is maximally isolated from the blood in the first 2 weeks of acute illness, from feces in the second and following weeks, and from urine in the third to fourth weeks. Culture of

bone marrow aspirate samples have yielded the highest rate (75–90%) of causative agent isolation (23), but this procedure is not routine because it is invasive and causes moderate discomfort. Multiple stool, blood, or urine samples should be taken over several days from an acutely ill typhoid case to optimize detection. Several stool cultures taken over several days should be assessed for suspected chronic carriers.

Some samples (e.g., feces) can be plated directly onto highly selective and differential agars. However, many types of specimens, such as “suspect” food, blood, vomitus, and tissue, may contain only a very low concentration of salmonellae, sometimes in the presence of large numbers of other bacteria, and may have to be preenriched and enriched. Preenrichment involves culturing the sample in a nonselective broth such as peptone water or tryptic soy broth to allow the growth of all organisms present. Enrichment involves culturing the sample in growth media containing selective inhibitors (e.g., tetrathionate broth, selenite broth, or Rappaport-Vassiliadis broth), which prevent/reduce the growth of other organisms and effectively increase the relative number of salmonellae. Enriched samples are then cultured on selective and differential media to identify salmonellae. Media widely used as selective and/or differential include bismuth sulfite agar, brilliant green agar, xylose lysine desoxycholate agar, *Salmonella-Shigella* agar, and MacConkey’s agar.

## B. Identification

After primary isolation, selected colonies are usually tested in *Salmonella* polyvalent O antisera or inoculated into triple sugar iron agar (TSI), lysine iron agar, urease test broth, indole nitrate medium, and others. Like other members of the family Enterobacteriaceae, Salmonellae produce colonies on agar media that cannot be differentiated by colonial characteristics but can be differentiated based upon key biochemical traits and serological properties. Salmonellae are typically non-lactose fermenting and are negative for indole production, the Voges-Proskauer test, phenylalanine deaminase, and urease. They typically produce hydrogen sulfide on triple sugar iron agar and do not grow in potassium cyanide. *S. Typhi* differs from almost all other salmonellae in that this serovar never produces gas from glucose and does not utilize citrate, decarboxylate ornithine, or ferment rhamnose. They do not produce DNase or lipase. Like *S. Typhi*, *S. Paratyphi C* also synthesizes an identical Vi capsular antigen. The results of standard biochemical tests are utilized in conjunction with data from serological assays to define the serovar identity of a *Salmonella* isolate, which is indicative of its pathogenic potential. Most clinical laboratories use commercially available polyvalent antisera to determine the O-antigen serogroups of an isolate. *Salmonella* subgroup 1 can be serotyped according to O antigens into serogroup A, B, C1, C2, D, E, or F (1). More than 99% of *Salmonella* strains causing human infections belong to these serogroups. For example, *S. Paratyphi A, B, C*, and *S. Typhi* are members of serogroups A, B, C1, and D, respectively. This typing is rapid and very valuable in clinical practice for preliminary identification to initiate therapy. For epidemiological purposes, further biochemical and serological determinations are necessary to define the specific *Salmonella* serovar. These methods might include the use of biochemical assays, phage typing assays (40), DNA probes, PCR, and serological assays for other antigens.

While the identification of serovars Typhi or Paratyphi in blood specimens obtained from patients with enteric fever symptoms is sufficient for diagnosis, the mere identification of salmonellae in foodstuffs or contaminated water that has been epidemiologically associated with diarrheal disease is not, in itself, sufficient to establish cause and effect. More advanced molecular epidemiological identification methods (e.g., chromosomal restriction fragment length polymorphism studies, polymerase chain reaction assays) may aid in assessing direct association of the contaminant organism with that found in diseased subjects.

## VI. PATHOGENICITY

Although the causative agents of human enteric fever have been known for about 100 years, investigations of the pathogenesis of disease have been limited by lack of suitable experimental animal

models of disease (enteric fevers are specific to humans). As a result, our understanding of typhoid pathogenesis comes mainly from pathological observations of patients, from limited studies in volunteers, and from in vitro studies in cultured cells (e.g., Refs. 3, 41). A systemic disease of mice caused by *S. Typhimurium* has provided some potential insight into human enteric fever disease but is sufficiently different to avoid direct comparisons (42,43).

## A. General Understanding of Typhoid Pathogenesis

Data from patient observation and volunteer studies have led to the following general concept of enteric fever pathogenesis. Following ingestion, *S. Typhi* proceed to the ileum of the small intestine, where they gain access to the submucosa by invasion of microfold (M) cells of Peyer's patches or by direct translocation across absorptive epithelial cells. In the subepithelial spaces, *S. typhi* enter cells of the monocyte lineage and transit, via these vehicles, through the thoracic duct into the bloodstream. Widespread dissemination occurs early, leading to transient symptoms (e.g., rose spots, dry cough) and then sustained infection of the RES (i.e., spleen, liver, and bone marrow).

During the second week of illness, *S. Typhi* establishes an infection of the gall bladder, and replicating organisms are shed via the bile duct into the small intestine resulting in fecal shedding and, sometimes, intestinal ulceration. Disease clearance follows effective antibiotic therapy or the development of appropriate humoral and cellular immune responses. Below, we briefly discuss data from clinical studies and from in vitro experiments, which have enhanced our knowledge of various stages of this disease.

## B. The Acid Barrier of the Stomach

*Salmonella* must transverse the acid barrier of the stomach—an early line of defense against enteric infections. Although *Salmonella* survive poorly at normal gastric pH (1.5), the organisms survive well at pH 4.0 and have an adaptive acid tolerance response that likely promotes survival at pH < 4.0 (44). A higher infectious dose (>10<sup>6</sup> bacteria) or protection of lower bacterial doses in a buffered food matrix is thought to overcome the normal gastric acid defense mechanism. People who take antacids or drugs that lower gastric acidity or who have undergone gastrectomy are susceptible to low infective doses of salmonellae.

## C. Translocation Across the Intestinal Mucosa

Human feeding studies (45) and histopathological findings from typhoid cases (3,46) demonstrated that invasion of the ileal intestinal mucosa is an essential early step in disease. After crossing the protective mucus layer covering the ileal epithelium, *S. Typhi* apparently adhere to and invade both absorptive epithelial cells and microfold (M) cells (45–47). M cells are specialized epithelial cells that overlie the lymphoid follicles or Peyer's patches, which are likely a key target of *Salmonella* infection. One set of host cell surface receptors required for *S. Typhi* invasion has been defined as the CFTR protein (48), but other receptors are likely necessary, as host cells lacking CFTR can still efficiently internalize *S. Typhi*. In vitro studies in cultured human intestinal cells have shown that *S. Typhi* encode virulence properties that trigger endocytic entry into host cells via a process of macropinocytosis (41,49–52). This entry process involves specific rearrangement of the host microfilament-dependent cytoskeleton. Extrapolated from studies mainly of *S. Typhimurium*, upon contact with host epithelial cells invasive salmonellae secrete special effector proteins, which diffuse into the host cell membrane, triggering a signal transduction cascade. This *Salmonella*-induced signaling occurs via host protein phosphorylation (i.e., functional activation) events and leads to an increase in intracellular free Ca<sup>2+</sup> and reorganization of the microfilaments, which causes membrane ruffling and engulfment by macropinocytosis of the adjacent *Salmonella* (20,53). In vitro studies reveal that *S. Typhi* entry into intestinal epithelial cells occurs at a high efficiency relative to other *Salmonella* serovars (41,54).

Following internalization into cultured epithelial cells, the organisms are released basolaterally via an exocytosis process (55,56) without causing cellular damage. This finding correlates with the silent translocation of *S. Typhi* across the ileal epithelium during this early disease phase, which occurs typically without symptoms.

#### **D. Bacterial Survival and Intra-host Dissemination Within Phagocytes**

Case histopathology revealed that *S. Typhi* invasion induces primarily a mononuclear response in the human gut. *S. Typhi* enter and survive within subepithelial monocytes and can be isolated most easily from the mononuclear fraction of blood (57). In vitro findings have supported the concept that *S. Typhi* survival within monocytes is essential to pathogenesis and allows for dissemination of the pathogen within the host. Recently, Schwan et al. (43) found that serovar Typhi, as opposed to *S. Typhimurium*, can specifically survive and multiply in elutriated human monocytes for at least 21 days.

Studies, mainly of *S. Typhimurium*, have revealed a series of bacterial genes (58–60) that control the organism's ability to survive and replicate within murine macrophages. A similar set of intramacrophage survival/multiplication functions are thought to exist for serovar Typhi.

#### **E. Mediators of Disease Manifestation**

*S. Typhi* are apparently disseminated via monocytes to various host sites (e.g., spleen, liver, bone marrow, gall bladder) from which disease symptoms are manifest. The organisms typically cause a very low level bacteremia, and circulating endotoxin has not been found to play a major role in typical disease (22). The molecular involvement of specific proinflammatory cytokines in disease is not well understood, but serovar Typhi was recently shown to stimulate IL-6 secretion from cultured intestinal cells (61). Separate studies of typhoid in Nepal (62) and in Indonesia (63) have demonstrated that increased levels of TNF receptor and lower production of proinflammatory cytokines are markers of severe, complicated illness.

### **VII. GENETIC FACTORS INVOLVED IN VIRULENCE**

The fact that *S. Typhi* causes typhoid fever but that serovar Typhimurium typically causes gastroenteritis in humans argues that these related organisms have unique pathogenicity genes and different gene regulatory systems that affect their abilities to cause different disease syndromes. In recent years, a large number of bacterial genes have been implicated in *Salmonella* virulence. In addition, some host factors have been noted that affect susceptibility to disease. A brief description of some of these factors is given below.

#### **A. Host Factors**

Host specificity is a hallmark of serovars Typhi and Paratyphi; these organisms only infect humans. The molecular bases for this strong host specificity are unknown but may partially involve bacterial ability to survive and multiply in human monocytes (43,47). Limited evidence has suggested a potential role of host genetic factors in typhoid susceptibility. These include the predisposition of nonsecretors of ABO blood group substance to become chronic typhoid carriers (64) and the proposed enhanced susceptibility to typhoid of certain individuals expressing wild-type homozygous intestinal CFTR protein (48).

#### **B. Bacterial Factors**

Lack of an experimental animal system has limited the direct study of *S. Typhi* virulence functions. However, a large number of *S. Typhimurium* genes affecting its ability to cause a typhoid-like

disease in mice have been defined in the past 10–15 years (20,24,53), and some of these genes have been shown to exist in *S. Typhi*. Many *Salmonella* virulence genes have been located in chromosomal gene clusters, termed pathogenicity islands. There are at least five pathogenicity islands in *S. Typhi* (65). *Salmonella* pathogenicity island one (SPI-1) encodes some effectors of host cell invasion, gene expression regulators, and a Type III secretion system to translocate these effectors into the host cell (24,53). A separate set of *S. Typhi* invasion genes, defined by cloning and mutational analysis, were reported by Elsinghorst et al. (51). A third locus affecting invasion was reported by Behlau and Miller (66). Whether these multiple invasion systems are complementary or show differential tissue specificity and are involved at different stages of disease is yet undefined.

A large composite of genes located at SPI-II and -III, at least some of which are coordinately regulated by the PhoP/PhoQ gene regulatory system, have been defined as being essential for bacterial survival within macrophages (20,24,59,60,67). These bacterial products help resist the action of cationic antimicrobial peptides and the acidic pH of macrophage/monocytes.

Mutants in a variety of *Salmonella* metabolic genes have been found that attenuate virulence. These include, among many, the *crp/cya* genes, which regulate catabolite repression, the *ompR* gene, which regulates transcription of membrane porins, the *katF* gene, which controls transcription of stress proteins (e.g., catalase), several genes controlling biosynthesis of purines and aromatic amino acids, and the genes controlling synthesis of the Vi capsular antigen. Some of these mutations (e.g., *aroA*, *purB*) have been incorporated into candidate, live, oral vaccine strains for testing in human volunteer studies (24).

## VIII. CONTROL MEASURES

Prior to the availability of chemotherapy, typhoid fever patients were treated symptomatically with adequate bed rest, sustaining food and fluids, and lukewarm baths to control fever. By 1900, the value of patient isolation and proper handling of typhoid feces was also appreciated. Since that time the measures for patient treatment, immunization, and typhoid control have undergone many advances.

### A. Antibiotic Treatment

Appropriate antibiotic treatment will block typhoid progression at all stages of disease. However, not all antibiotics to which *S. Typhi* shows in vitro sensitivity (e.g., the aminoglycosides, the tetracyclines, the early cephalosporins) have proven to be useful clinically. Still used in some areas of the world, chloramphenicol therapy results in rapid clinical improvement and lower mortality, but is associated with an increased incidence of relapse and serum toxicity. Trimethoprim-sulfamethoxazole has been employed successfully as a second-line therapy, but only the trimethoprim component is effective. Ampicillin was not found to be highly effective in treating typhoid, but amoxicillin given orally, which has increased absorption, was very effective in clinical trials (68), providing fast disease clearance plus reduced incidences of relapse and carrier formation compared to chloramphenicol. Finally, newer generation cephalosporins (i.e., cephmandole, cefoperazone, ceftriaxone) have been shown to be effective for typhoid therapy (69).

*S. Typhi* resistance to chloramphenicol was first reported prior to 1972 (70) and was initially sporadic in nature. By 1990 a high percentage of isolates in many areas of the world had become resistant to chloramphenicol, ampicillin, amoxicillin, and trimethoprim (71). Thus, multiply resistant *S. Typhi* strains make chemotherapeutic selection very problematic. Fortunately, the fluoroquinolones (e.g., ciprofloxacin and ofloxacin), which penetrate tissues well and concentrate in the phagocytes and bile, have been found to be highly effective in typhoid treatment. Most *S. Typhi* strains remain sensitive, and fluoroquinolones are the drugs of choice for treating enteric fever (72).

Eradication of the carrier state, which previously required cholecystectomy, has recently been achieved following therapy with ampicillin or amoxicillin (64) or with fluoroquinolones (73,74).



## B. Immunity and Vaccines

*S. typhi* infection induces both cell-mediated and humoral immunity (75,76). Most people acquire immunity to reinfection after acute typhoid illness, but some can be reinfected if exposed to a higher infectious dose. The mechanism of specific protective immunity is still poorly understood. The O polysaccharide seems to be an important antigen, since a single monoclonal IgA antibody against O antigen provided measurable protection against *Salmonella* infection in animal studies (77) and the titers of IgA and IgG anti-O antibodies increase in adult volunteers following oral administration with the live *S. typhi* vaccine, Ty21a (78). The Vi polysaccharide antigen is also important in protection, since a vaccine containing only this antigen triggered increased protection in endemic regions (79). The development of cell-mediated immune responses in individuals living in a typhoid-endemic region has also been found to correlate with protection against disease (80). Finally, immunocompromised individuals are more susceptible to typhoid and may experience more severe symptoms and higher recurrent bacteremia. Together, these data suggest that both humoral- and cell-mediated immune responses are important in protection from typhoid fever.

Typhoid fever can be prevented by immunization with one of three available vaccines. A parenterally administered, heat- and phenol- inactivated, whole-cell *S. Typhi* vaccine has been widely used for many years and provides immunity for at least 3 years. However, immunization with this vaccine or related whole-cell, inactivated vaccines has been associated with high rates of moderate to severe, local and systemic adverse reactions, which have become unacceptable with the development of new, safer vaccines. The live, attenuated *S. Typhi* Ty21a vaccine is administered in four enteric-coated capsules, one given every other day. This vaccine provides long-lasting protection (5 years), is associated with few and minor side effects, but is not recommended for children under 6 years old or the immune compromised. The third vaccine is comprised of the purified Vi polysaccharide, is given in a single injectable dose, causes relatively few adverse effects, and can be given to children as young as 2 years of age. The response to this purified polysaccharide vaccine is T-cell independent, and booster doses are recommended every 2 years. Also, this latter vaccine has only been tested in endemic regions, where it has augmented preexisting immunity.

The identification of specific genes in pathogenicity studies that attenuate *Salmonella* virulence has stimulated the continued search for an improved live, attenuated typhoid vaccine (76). In addition, the use of safe, attenuated *Salmonella* strains as live, oral vaccine carriers of foreign antigen genes, first demonstrated by Formal et al. (81), offers the promise of developing multivalent vaccines to protect against multiple disease agents (e.g., HIV, malaria, *Shigella*, and typhoid).

## C. Prevention

Enteric fever can be prevented by blocking the routes by which *S. Typhi* or *S. Paratyphi* transfer from the intestine or urinary tract of an infected person to a susceptible person. Breaking the chain of spread of infection depends upon adequate sewage disposal and clean water supplies, good food hygiene, good personal hygiene, and the administration of an affordable vaccine to the high-risk population. In addition to typhoid patient isolation and improved chemotherapy of disease, the following measures/practices have dramatically reduced the incidence of enteric fever (15):

1. Maintaining an adequate sewage disposal system and an uncompromised clean water supply.
2. Practicing good personal hygiene—adequate hand washing after using the bathroom.
3. Practicing good food hygiene: exclusion of typhoid carriers from employment involving food handling; establishing regulations controlling the limits of fecal coliform contamination of water from which shellfish and water vegetables are harvested (ensuring compliance); pasteurization of milk and protection of pasteurized milk from subsequent contamination; establishing commercial standards for prepared foods, cooking temperature and time, storage of food, and cleanliness of work area.

4. Surveillance for and treatment to eradicate the chronic carrier state.
5. Provide prophylactic protection for persons at high risk of infection, e.g., persons residing in endemic regions, travelers to regions of high endemicity, laboratory/health care workers.

## IX. SUMMARY

Typhoid exacts a tremendous disease burden worldwide and remains a major public health problem. The worldwide incidence of human typhoid is estimated at about 20 million cases with 600,000 associated deaths annually. The causes of enteric fever and methods for disease control have been known for ~100 years. Despite advances in disease diagnosis, chemotherapy, and immunoprophylaxis, and a better understanding of typhoid epidemiology, we still have large, typhoid-endemic regions in the world. Why?

Control measures are well characterized and have been very effective in reducing disease in developed nations. Establishment of modern sanitation systems, by themselves, have been shown to be a key factor in disease eradication. In the absence of controlling sewage and supplying clean water, developing country populations are left with using limited available methods to identify and treat typhoid-infected individuals. The cycle of disease has not been broken in these countries; and this guarantees the maintenance of low-level disease in developed countries via increased importation of contaminated food and immigration of infected people. Evidence of the development of herd immunity following the widespread use of the live, oral Ty21a typhoid vaccine (75) suggests that an extensive immunization strategy in highly endemic areas might bring this disease under much better control.

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# 11

## ***Shigella* Species**

**Keith A. Lampel**

*U.S. Food and Drug Administration, Washington, D.C., U.S.A.*

**Anthony T. Maurelli**

*Uniformed Services University of the Health Sciences, Bethesda, Maryland, U.S.A.*

### **I. BACKGROUND**

Bacillary dysentery, or shigellosis, is caused by infection with bacteria of the genus *Shigella*. Evidence of dysentery in human populations can be traced back to the writings of Hippocrates, who used the term to describe a condition marked by the passage of bloody and mucoid stools. *Shigella* are frank pathogens and readily cause disease in humans even when low doses are ingested. This feature of shigellosis gives the pathogen epidemic outbreak potential and can rapidly be disseminated through contaminated food and water. For example, dysentery has played a pivotal role in the outcome of many military operations over the centuries. Prolonged conflicts such as the American Civil War and the Napoleonic campaigns saw more casualties among soldiers and civilians due to disease than to battle. In the twentieth century, shigellosis has been controlled by the introduction of modern methods of wastewater treatment and the availability of potable drinking water in most developed countries. Nevertheless, shigellosis remains endemic in underdeveloped areas where food and water supplies are frequently contaminated by human excrement. Even in developed regions with safe water supplies, a breakdown in the water treatment and distribution infrastructure due to war or natural disasters can place whole populations at risk of infection with a waterborne pathogen such as *Shigella*.

While often considered primarily a waterborne pathogen, epidemiological data suggest that foodborne *Shigella* infection is a real and persistent danger (1). In this chapter we will review the characteristics of the pathogen and the disease it produces and examine its epidemiology. We will also discuss some of the most recent scientific literature on the pathogenic mechanisms of *Shigella* and state-of-the-art methods for detection of the organism in food and water sources. As this chapter does not attempt to be a comprehensive review on the topic of *Shigella*, the reader is referred to several excellent recent reviews for additional information (2–5).

### **II. CHARACTERISTICS**

Bacteria of the genus *SHIGELLA* are gram-negative, non-spore-forming, nonmotile, rod-shaped bacteria. *Shigella* are classified into four major groups based on their O-antigen type and biochemical

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**TABLE 1** Characteristics of *Shigella* spp.

Species	Serogroup	Serotypes	Geographic distribution	Distinguishing characteristics
<i>S. dysenteriae</i>	A	15	Indian subcontinent, Africa, Asia, Central America	Produce shiga toxin, causes most severe dysentery, high mortality rate if untreated
<i>S. flexneri</i>	B	6	Most common isolate in developing countries	Less severe dysentery
<i>S. boydii</i>	C	19	Indian subcontinent, rarely isolated in developed countries	Biochemically identical to <i>S. flexneri</i> , distinguished by serology
<i>S. sonnei</i>	D	1 <sup>a</sup>	Most common isolate in developed countries	Mildest form of shigellosis

<sup>a</sup> Forms I and II are serotypically distinguishable.

characteristics: *S. dysenteriae* (A), *S. flexneri* (B), *S. boydii* (C), and *S. sonnei* (D). Each group, except *S. sonnei*, is further subdivided into serotypes (see Table 1). Shigellae are facultative anaerobes and are not particularly fastidious in their nutritional requirements for growth. Important biochemical characteristics of *Shigella* species include (6):

- Inability to ferment lactose
- Lysine decarboxylase negative
- Oxidase negative
- Unable to utilize citrate as sole carbon source
- do not produce H<sub>2</sub>S
- do not produce gas from glucose except for *S. flexneri* 6
- do not grow on KCN agar plates

*Shigella* are members of the family Enterobacteriaceae. Genetically, shigellae are nearly identical to *Escherichia coli* and are also closely related to *Salmonella* and *Citrobacter* species (7). Gene transfer by conjugation occurs between *Shigella* spp. and *E. coli*, and the transconjugants recovered demonstrate the extensive genetic homology between the two species (8,9). DNA-DNA hybridization experiments also confirm the high degree of similarity at the genomic level between *Shigella* and *E. coli* (10). According to the evolutionary biology community (11), *Shigella* species are grouped as *E. coli* strains and are placed within groups A or B1 of the ECOR *E. coli* reference collection. *E. coli* O157:H7, a strain often compared to *S. dysenteriae* because both bacteria produce a similar toxin that is believed to be responsible for hemolytic uremic syndrome (HUS; see below), is not classified within either ECOR group.

*Shigella* can be distinguished from most *E. coli* strains, but one strain, enteroinvasive *E. coli* (EIEC), is even more closely related to *Shigella*. This bacterium causes the same disease as shigellae, has the same genetic properties for pathogenesis, and some serogroups of EIEC have O-antigens that cross-react serologically with those found on some *Shigella* species.

### III. DISEASES

Unlike disease caused by most of the other foodborne pathogens discussed in this volume, infection by *Shigella* spp. leads to an inflammatory diarrhea (12). Shigellosis is transmitted orally through contaminated food and water. As a rule of thumb, the mechanisms for transmission of shigellae

within a population include fingers, flies, food, and feces. Disease can progress from ingestion of a very low infectious dose, and studies in volunteers have demonstrated that ingestion of as few as 200 organisms is sufficient to cause dysentery (13). The incubation period is from 1 to 7 days, with symptoms commonly manifesting on day 3. Shigellosis often begins with a watery diarrhea, which precedes the characteristic dysentery symptoms. The diarrhea phase probably results from production of enterotoxins by the bacteria as they transit through the small intestine. Fever, severe abdominal pain, and cramping follow and are accompanied by the passage of small-volume bloody and mucoid stools. This phase correlates with extensive sloughing and denuding of the colonic mucosa. *Shigella* penetrate into the epithelial cells lining the colon, multiply within these cells, and spread from cell to cell through the mucosa. The foci of infected cells coalesce to form abscesses. Dead cells detach and are expelled in the feces along with mucus and large numbers of bacteria. The presence of polymorphonuclear leukocytes in the stools is also a feature of the inflammatory nature of dysentery.

The clinical signs of shigellosis can range from a mild diarrhea to severe dysentery. The most severe forms of shigellosis are caused by strains of *S. dysenteriae* 1. *S. sonnei* cause milder forms of the disease, while *S. flexneri* and *S. boydii* can cause either severe or mild illness. Despite the severity of the symptoms, dysentery is a self-limiting disease. The bacteria do not spread beyond the underlying mucosa of the colon, and systemic spread leading to septicemia is rare. Untreated shigellosis lasts from 1 to 2 weeks, and, barring any conditions affecting host immunity, the patient recovers fully. Shigellosis is usually not life-threatening, but complications may arise in very young and very old patient populations as well as in individuals who are malnourished or otherwise medically compromised (14). Some of these complications can be fatal, such as severe dehydration, intestinal perforation, toxic megacolon, septicemia, seizures, and HUS (15). HUS is a rare complication associated with infection by *S. dysenteriae* 1 (16). Shiga toxin produced by this species of *Shigella* has been implicated as the cause of HUS. Reactive arthritis, a triad of symptoms comprising urethritis, conjunctivitis, and arthritis, is a postinfection sequelae of shigellosis that is associated with patients of the HLA-B27 histocompatibility group (17). Other invasive bacterial pathogens such as *Salmonella*, *Yersinia*, and *Campylobacter* also cause reactive arthritis postinfection (18).

#### IV. EPIDEMIOLOGY

Diarrheal diseases are the leading worldwide cause of death among children. The World Health Organization estimates that 5 million deaths occur annually from diarrheal disease, and shigellae are responsible for 10% of these mortalities (19). Epidemic dysentery, caused by *S. dysenteriae* type I (the only *Shigella* to produce shiga toxins), is a recurrent problem in many of the poorest areas of the world, notably in Africa, Central America, and parts of Asia. Many of these outbreaks are caused by multiple antibiotic-resistant strains; the fatality rate of these infections can be as high as 20%. Children 1–6 years of age are most susceptible to infections due to shigellae. This phenomenon is compounded in poorer developing nations because of the high numbers of malnourished children. These children face an increase in attack and relapse rates and greater mortality. *S. sonnei* is predominantly found in industrialized countries and has become a significant problem in day care centers and preschools. The bacteria can easily spread among children as a result of poorly developed personal hygiene or by the day care or preschool personnel whose duties include attending to toddler's sanitary needs and preparing food.

*S. dysenteriae* is the predominant species in the tropics and causes the most severe form of dysentery, whereas *S. flexneri* is found in more economically advanced countries and also in significant numbers in the tropics. *S. boydii* is rarely isolated except in the Indian subcontinent, and some cases have been reported recently from Europe.

Humans are the only known reservoir and host of shigellae, although there have been several reports of *Shigella* being isolated from higher primates, particularly at zoos and primate centers (20). During the acute phase of infection, individuals can shed  $10^3$ – $10^9$  colony-forming units (cfu)



per gram of stool, and in convalescing patients,  $10^2$ – $10^3$  cfu can be recovered. Adults who have lived in one habitat for many years may become asymptomatic carriers of shigellae. This is often a reflection of living conditions and socioeconomic factors that affect sanitary conditions. In many situations shigellosis outbreaks are seasonal, with summer months showing the highest incidences. Asymptomatic carriers may have a significant role in carrying this pathogen through the colder months. In warmer months, people's interactions increase; this augments the likelihood of asymptomatic carriers coming into contact with the uninfected and susceptible population.

## V. ISOLATION AND IDENTIFICATION

### A. Biochemical Techniques

Foods are not routinely examined for the presence of *Shigella* unless epidemiological data suggest they may be a source of an outbreak. Vegetables and salads are commonly contaminated sources (21). Foods can be contaminated in several ways, including fecally contaminated irrigation water and, more likely, an infected food handler with poor personal hygiene. Unlike clinical samples of stool with high numbers of shigellae and uniform composition, foods pose significant obstacles to successful isolation and identification of this bacterium. It may take well over a week before suspected foods are analyzed for the presence of *Shigella*. In this time period the food, if any still remains, may be further adulterated and the bacterial population may also be compromised to the point where recovery of viable organisms is virtually impossible.

The *Bacteriological Analytical Manual* (22) details one method to isolate shigellae from foods. Twenty-five g samples are added to 225 mL of *Shigella* broth supplemented with 3 µg/mL novobiocin (0.5 µg/mL is used for *S. sonnei*). After 10 minutes at room temperature, the mixture is incubated anaerobically overnight at 44°C. Alternatively, other enrichment broths can be used, e.g., gram-negative broth. Samples are processed in a sterile stomacher bag, decanted into a flask, and incubated at 37°C with shaking for 16–20 hours. Gram-negative broth is less inhibitory for *Shigella* because of the low amount of desoxycholate present in the media, and added mannitol encourages the growth of *Shigella*.

Because the analysis of suspected contaminated foods may not commence for a period of 7–10 days, the presence of injured *Shigella* cells is probable (23). Selective media without bile salts and desoxycholate are recommended since the presence of these compounds may adversely affect the growth of impaired cells (24). Food samples are added to 100 mL of tryptic soy broth, the pH is adjusted to 7.0, and then blended. After 8 hours of incubation at 37°C, 125 mL of an enrichment broth is added, and the incubation is extended for 16–20 hours.

A range of selective agar media can be used to plate the overnight cultures. Two to three different selective media should be used to increase the chance of recovering *Shigella*. Growth of *Shigella* on MacConkey agar, a low selectivity medium, produces colonies that are translucent, slightly pink (*Shigella* are lactose negative), and that may possess rough edges. Eosin methylene blue (EMB) and Tergitol-7 agar are alternative low selectivity agars containing lactose. Nonpigmented, semitranslucent colonies on EMB plates or bluish colonies on the yellowish-green Tergitol-7 agar are indicative of *Shigella*. Desoxycholate and xylose-lysine-desoxycholate (XLD) agars are intermediate selective media and are preferred media to isolate *Shigella* spp. *Shigella* colonies on XLD agar medium are translucent and red (alkaline). Although most *Shigella* do not ferment xylose, some species, e.g., *S. boydii* (variable), may be missed, and therefore plating on XLD and MacConkey agar plates is recommended. *Shigella* spp. form reddish colonies on desoxycholate agar. Highly selective medium include *Salmonella-Shigella* and Hektoen agars. Some *Shigella* spp., such as *S. dysenteriae* type I, are unable to grow on highly selective *Salmonella-Shigella* medium. *Shigella* produce colorless, translucent colonies on this agar medium. Colonies on Hektoen agar appear to be green, as do colonies from *Salmonella* spp; *E. coli* strains form yellow colonies. All presumptive colonies are inoculated into semisolid (motility) test agar. *Shigella* spp. are nonmotile.

Biochemical tests are used to further identify *Shigella* spp. Suspected colonies that are gram-

**TABLE 2** Tests to Differentiate *Shigella* spp. from *E. coli*

Test	Reaction	
	<i>Shigella</i>	<i>E. coli</i>
Motility	—	+ <sup>a</sup>
Gas from glucose	— <sup>b</sup>	+ <sup>c</sup>
Lysine decarboxylase	—	+ <sup>d</sup>
Christensen's citrate	—	+
Acetate	—	+
Mucate	—	+

<sup>a</sup> Most positive, some negative.

<sup>b</sup> Some strains of *S. flexneri* 6 produce small amounts of gas from glucose.

<sup>c</sup> Some exceptions.

<sup>d</sup> Enteroinvasive *E. coli* are also negative.

negative, nonmotile rods are inoculated onto lysine iron or Kligler iron agar. *Shigella* produce alkaline slants, acid butt, and no gas on these agars. Similar to other enteric bacteria, *Shigella* are oxidase-negative, ferment glucose, and, except for *S. dysenteriae* type 1, catalase positive. Further biochemical characterizations show that *Shigella* spp. are negative for H<sub>2</sub>S production, phenylalanine deaminase, sucrose and lactose (*S. sonnei* may after long incubation) fermentation, do not utilize citrate, acetate, KCN, malonate, inositol, adonitol, and salicin, and lack lysine decarboxylate. Shigellae are negative for the Voges-Proskauer test (*S. sonnei* and *S. boydii* serotype 13 are positive); however, all shigellae are methyl red positive and are unable to produce acid from glucose and other carbohydrates (acid and gas production occur with *S. flexneri* serotype 6, *S. boydii* serotypes 13 and 14, and *S. dysenteriae* 3). One *Shigella* spp., *S. dysenteriae*, is catalase negative and has ornithine decarboxylase activity.

Tables 2 and 3 show key biochemical reactions to differentiate *Shigella* from *E. coli* and also to distinguish *Shigella* spp. from each other. Growth on Christensen citrate, sodium mucate, or acetate agar is one characteristic that discriminates between *E. coli* and *Shigella*; shigellae are unable to utilize citrate, acetate, or mucate as the sole carbon source.

Other biochemical tests are used to identify the serotypes of *Shigella*. The ability to utilize mannitol, dulcitol, xylose, rhamnose, raffinose, glycerol, and indole and the presence of ornithine decarboxylase have been used to physiologically discriminate between *Shigella* spp.

Serological testing using polyvalent antiserum is used to identify the *Shigella* groups A–D. A note of caution should be addressed. EIEC causes the same disease, bacillary dysentery, as do the shigellae. Some EIEC strains share homology with O-antigen structures of some *Shigella* serotypes (25). Several serotypes of *S. dysenteriae*, *S. flexneri*, and *S. boydii* have reciprocal cross-reactivity with *E. coli* O antigens of the Alkalescens-Dispar bioserogroup or EIEC.

## B. DNA and Antibody-Based Assays

Several types of DNA-based assays have been used to detect *Shigella* spp. in foods. DNA probes, either DNA from virulence genes or short oligonucleotides that are specific for a particular genetic marker, can be used in a colony hybridization format (26). The limitation of this method is similar to isolating shigellae from foods using conventional bacteriological techniques. The physical state of the *Shigella* within the food and each food matrix can reduce the chance of isolating the pathogen.

Initially, DNA probes and the polymerase chain reaction (PCR) were developed to detect *Shigella* from clinical samples. These techniques have been applied to foods. PCR primers are selected that specifically target virulence genes and have been successfully used in PCR-based assays to

**TABLE 3** Biochemical Differentiation of *Shigella* Species

Text	<i>S. dysenteriae</i>	<i>S. flexneri</i>	<i>S. boydii</i>	<i>S. sonnei</i>
β-Galactosidase	— <sup>a</sup>	—	—	+
Ornithine decarboxylase	—	—	—	+
Indole production	+/- <sup>b,c</sup>	+/- <sup>c,d</sup>	+/- <sup>c</sup>	—
Acid from:				
Dulcitol	— <sup>e</sup>	— <sup>f</sup>	—	—
Lactose	—	—	— <sup>g</sup>	+ <sup>h</sup>
Mannitol	—	+	+	+
Raffinose	—	+/- <sup>c</sup>	—	+ <sup>h</sup>
Sucrose	—	—	—	+ <sup>h</sup>
Xylose	—	—	+/- <sup>c</sup>	—

<sup>a</sup> *S. dysenteriae* type 1 is positive.

<sup>b</sup> *S. dysenteriae* type 1 is negative; *S. dysenteriae* type 2 is positive.

<sup>c</sup> Reaction is variable.

<sup>d</sup> *S. flexneri* 6 is negative.

<sup>e</sup> *S. dysenteriae* type 1 may be positive.

<sup>f</sup> *S. flexneri* 6 may be positive.

<sup>g</sup> *S. boydii* may be positive.

<sup>h</sup> Positive reactions may take 24 hours or longer.

+/-, variable reaction.

detect the presence of *Shigella* in foods (27,28). Unlike other methods, which may take several days to draw a conclusion regarding the presence of *Shigella* in foods, PCR assays can yield a result in less than one day.

## VI. PATHOGENICITY

The hallmarks of *Shigella* pathogenicity are induction of diarrhea, the ability to invade eukaryotic cells, multiplication inside these cells, and spread from cell to cell. In vitro and in vivo model systems that measure pathogenic characteristics of *Shigella* demonstrate that the clinical presentation of dysentery can be related to each one of these hallmarks.

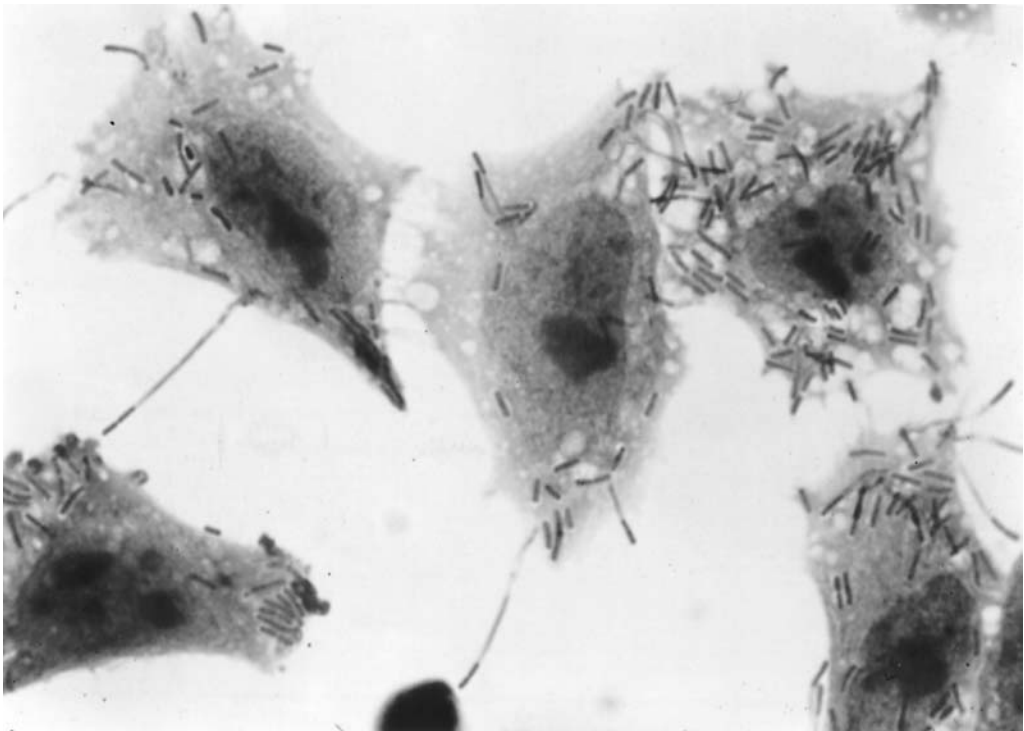
The first use of animal and tissue culture models to demonstrate the importance of the invasive capacity of *Shigella* to pathogenesis were the classic studies of Formal and coworkers (29). They demonstrated that spontaneous variants of wild-type *S. flexneri* did not penetrate into tissue culture cells and were also unable to cause disease in monkeys. The monkey model also was used to show that *Shigella* colonize the small intestine only transiently with little tissue damage (30). However, the diarrhea that generally precedes onset of dysentery probably results from the production of enterotoxins by the bacteria while they are in the small bowel (31,32). The jejunal secretions elicited by these toxins may serve to facilitate passage of the bacteria through the small intestine and into the colon, where they colonize and invade the epithelium.

Once in the large intestine, *Shigella* probably enter the epithelium through specialized lymphoid cells called M cells (33). These cells are general antigen sampling cells scattered throughout the intestinal mucosa, and entry by *Shigella* into these cells is probably rather nonspecific. In vitro invasion assays on polarized cells in tissue culture, however, demonstrate that *Shigella* preferentially penetrate these cells via the basolateral surfaces (34). Thus, the initial entry through M cells may provide the bacteria with access to the underlying mucosal surface where *Shigella* can more efficiently invade the basolateral surface of target epithelial cells. The molecular details of the entry

process are beyond the scope of this review, but *Shigella*, like other invasive pathogens of the intestinal mucosal surface, invade via a pathogen-directed phagocytic process that actively involves elements of the host cytoskeleton apparatus (2,35–37). Unlike most other intracellular bacterial pathogens, however, *Shigella* rapidly lyse the endocytic vacuole upon entry and are released into the host cell cytoplasm, where they replicate.

During growth inside the host cell, *Shigella* display an unusual motility phenotype shared with *Listeria monocytogenes*, another intracellular bacterial pathogen (Chapter 5). The bacteria catalyze the polymerization of actin filaments in a unipolar fashion at one end of the bacteria (3). Formation of these actin tracks literally propels the bacteria through the cytoplasm as actin monomers polymerize into filaments. When the bacteria are visualized microscopically inside invaded tissue culture cells, they appear to form “fireworks” or “comet tails.” These structures are actin filaments forming a tail at one end of the bacterium (Fig. 1).

The ability to polymerize actin molecules suggested a role for intracellular motility in intercellular spread of the bacteria. This hypothesis was confirmed in *in vitro* assays for cell-to-cell spread and electron microscopy that showed “fireworks” containing bacteria being taken up by adjacent cells in confluent tissue culture monolayers (38,39). The bacteria, now surrounded by the plasma membrane of two host cells, lyse the membranes and are again free to multiply in the cytoplasm of the new host cell. This model emphasizes how a *Shigella* infection can rapidly spread through mucosal tissues without any need for the bacteria to be released into the intestinal lumen before

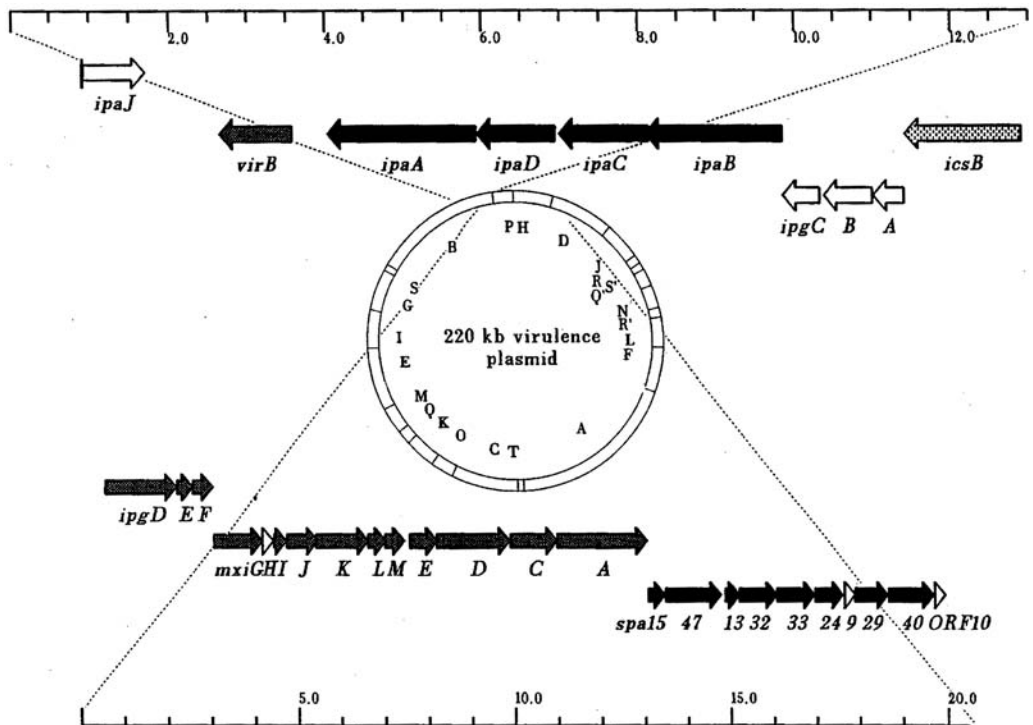


**FIGURE 1** CaCo-2 cells infected with *Shigella flexneri* 2a. A semiconfluent monolayer of CaCo-2 cells were infected with *Shigella flexneri* 2a. Bacteria form long projections of polymerized actin (“fireworks” or “tails”) extending from one end of the bacterium after 2 hours postinfection. (Photo kindly provided by Robin C. Sandlin.)

re-infecting a new host cell. Studies on mutant strains of *Shigella* that fail to display this intracellular motility confirm this phenotype as a critical hallmark of *Shigella* pathogenesis as these mutants produce a greatly attenuated form of disease in the monkey model (40).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

Virulence in *Shigella* is subject to regulation by environmental signals, the most influential of which is temperature. Virulent *Shigella* grown at 37°C display all the attributes of virulence, whereas the same strains grown at 30°C are avirulent in the guinea pig conjunctivitis model for virulence and fail to invade tissue culture cells (41). The loss of virulence is reversible and the organisms regain full virulence after the growth temperature is shifted to 37°C. This form of global regulation by temperature is consistent with the fact that virulence in *Shigella* is multigenic, with genes on both the chromosome and a large virulence-associated plasmid contributing to pathogenicity. A role for the large plasmid in *Shigella* virulence was first demonstrated in *S. flexneri* and *S. sonnei* (42,43). Subsequently, all virulent species of *Shigella* and enteroinvasive strains of *Escherichia coli* were shown to carry a 180–220 kilobase (kb) plasmid that shares substantial DNA homology with these prototype virulence plasmids (44). Introduction of these plasmids into plasmid-cured strains of *Shigella* or laboratory strains of *E. coli* K-12 imparts on these strains the ability to invade mammalian cells in tissue culture. Thus, the *Shigella* virulence plasmid encodes all the genes required for the invasive phenotype of *Shigella* (Fig. 2). A 37 kb region of the plasmid that contains the minimal



**FIGURE 2** Map of the virulence plasmid of *Shigella flexneri* 2a. A *Sall* restriction map of the 220 kb plasmid is shown (center). Sections of *Sall* fragments B and P (upper map) and fragments P, H, and D (lower map) are expanded to illustrate the 32 virulence loci encoded in these regions. The direction of transcription are marked by arrows. *icsB* and *ipgD* are separated by 314 bp. The complete sequence of the virulence plasmid has been determined (78), and the GenBank accession number is AL391753.

sequence needed for invasion was identified by cosmid cloning; transposon mutagenesis revealed the presence of at least five unlinked loci essential for invasion (45,46). The nucleotide sequence of this part of the virulence plasmid of *S. flexneri* and *S. sonnei* has been determined and are available under GenBank accession numbers AL391753 and D50601, respectively. The region encodes about 33 virulence genes that can be classified as encoding transcriptional regulators, invasion effector molecules, and a specialized secretion apparatus to export the effector molecules. While a rigorous transcription map of the region has yet to be defined, the genes are roughly grouped into two clusters transcribed from opposite strands.

The *ipa* genes (invasion plasmid antigens; *ipaBCDA*) encode the secreted effector molecules that mediate the bacteria-induced phagocytic event. Expression of these genes is temperature-regulated, and they produce the immunodominant antigens recognized by convalescent sera from shigellosis patients and challenged monkeys (45,47). IpaB is a multifunctional protein that plays a role in invasion, lysis of the endosomal vacuole, and induction of apoptosis of infected macrophages (48,49). Together with IpaC, and probably IpaA, IpaB forms a secreted complex that likely mediates invasion. In fact, IpaB and IpaC can form a complex that, when coated onto latex beads, promotes uptake of the beads by HeLa cells (50). This complex of Ipa proteins has also been shown to bind to cell surface receptors such as  $\beta$ 1 integrins (51). On permeabilized cells, purified IpaC induces significant cytoskeletal reorganization, including formation of filopodia and lamellipodial extensions (52). IpaA also modulates bacterium-cytoskeletal interactions by associating with vinculin (53). IpaD appears to form an antisecretion complex with IpaB since *ipaD* mutants are hypersecretors of the Ipa products (54). IpgC, encoded immediately upstream of the *ipa* genes (and probably part of the same transcriptional unit), is a cytoplasmic chaperone that keeps IpaB and IpaC from forming a complex in the bacterial cytoplasm before secretion. In the absence of IpgC, IpaB and IpaC are rapidly degraded (55).

The Ipa proteins are actively secreted into the extracellular medium despite the fact that they lack signal sequences commonly found on proteins that are secreted by gram-negative bacteria (56). A dedicated secretion apparatus comprised of products of the *mxi/spa* (membrane expression of invasion plasmid antigens/surface presentation of Ipa antigens) genes include lipoproteins (MxiJ and MxiM), a transmembrane protein (MxiA), a possible transcriptional regulator (MxiE), and inner and outer membrane proteins. The *spa* locus mediates secretion of the Ipas. This secretion machinery contains homologs to proteins of secretion systems in other bacterial pathogens (both plant and animal pathogens), all of which are classified as type III secretion systems (see Ref. 57 for review). Expression of the type III secretion machinery is essential for *Shigella* virulence. Nonpolar null mutations in all of the *mxi/spa* genes so far tested result in loss of the ability to secrete the Ipas and loss of invasive capacity for cultured cells. Products of the *mxi* locus include *spa47*, which encodes a protein that has sequence similarities with ATPases of the flagellar assembly machinery of other bacteria and probably provides energy for the secretion apparatus.

Secretion of the Ipa proteins via the Mxi/Spa apparatus is induced when the bacterium contacts the host cell (58,59). Other compounds that mimic this signal and induce Ipa secretion are fibronectin, laminin, collagen type IV, Congo red, bile salts, and fetal bovine serum (58,60). A similar phenomenon of contact-induced secretion is observed in other bacterial pathogens that utilize a type III secretion pathway for extracellular transport of virulence effectors. For example, both *Yersinia* spp. and *Salmonella typhimurium* induce secretion of their virulence effector proteins upon contact with host cells (61,62). However, the virulence effector proteins of *Yersinia* and *Salmonella* are secreted directly into the host cell, unlike *Shigella*, which secretes its proteins into the extracellular medium (see [Chapters 7](#) and [15](#)).

The intracellular motility of *Shigella* is due to its ability to catalyze polymerization of F-actin and is attributed to the product of the *icsA* gene (38,63). This gene encodes a 120 kDa protein that is processed at both amino- and carboxyl-terminal ends. IcsA is anchored in the outer membrane and is localized in an asymmetrical pattern to one pole of the bacteria (65). This unipolar localization of IcsA is dependent on components of the lipopolysaccharide and is absolutely critical for full virulence of the bacteria (66). Mutants in the LPS pathway express IcsA circumferentially and retain

the ability to polymerize actin. Yet these mutants remain immobile within the host cell cytoplasm and are unable to form the cytoplasmic protrusions necessary to allow the bacteria to spread to adjacent cells.

In sharp contrast to the genes of the virulence plasmid, which are involved in invasion and postinvasion steps, the chromosomal genes associated with virulence appear to be largely modulators of overall virulence (Table 4). Several chromosomal loci that regulate expression of virulence genes on the plasmid have been identified, including activators and a repressor (67–70). Perhaps of greater interest are the genes that are present in the closely related nonpathogen *E. coli* but that are missing from the chromosome of *Shigella*. *ompT* encodes an outer membrane protease and is part of a cryptic prophage in the *E. coli* chromosome. This prophage is missing in *Shigella* spp. When the *ompT* gene is introduced into *Shigella* by conjugation, the strain loses the ability to spread from cell to

**TABLE 4** Virulence-Associated Loci of *Shigella*

Locus	Product	Role in virulence
Plasmid-encoded genes		
<i>ipgC</i>	17 kDa protein	Chaperon for IpaB and IpaC
<i>ipaB</i>	62 kDa protein	Invasion; lysis of vacuole; induction of apoptosis
<i>ipaC</i>	43 kDa protein	Invasion; induces formation of filopodia and lamellipodial extensions
<i>ipaD</i>	38 kDa protein	Invasion; forms antisecretion complex with IpaB
<i>ipaA</i>	70 kDa protein	Invasion; associates with vinculin
<i>mxi/spa</i>	20 proteins	Secretion of Ipa and other virulence proteins
<i>icsA (virG)</i>	120 kDa cell-bound and secreted protein	Actin polymerization for intracellular motility and intercellular spread
<i>virB</i>	Transcriptional activator	Temperature regulation of virulence genes
<i>virF</i>	Transcriptional activator	Temperature regulation of virulence genes
<i>sen</i>	ShET2	Enterotoxin
Chromosomally encoded genes		
<i>rfa; rfb</i>	Enzymes for LPS core and O-antigen synthesis	Unipolar localization of IcsA biosynthesis
<i>stx<sup>a</sup></i>	Shiga toxin	Destruction of vascular tissue
<i>virR (hns)</i>	Histone-like protein	Repressor of virulence gene expression
<i>vacB (rnr)</i>	Exoribonuclease RNase R	Posttranscriptional regulation of virulence gene expression
<i>cpxR</i>	Response regulator of CpxA-CpxR two-component system	Activator of <i>virF</i>
<i>iuc</i>	Aerobactin and receptor	Acquisition of iron in the host
<i>sobB</i>	Superoxide dismutase	Inactivation of superoxide radicals; defense against oxygen-dependent killing in host
<i>set<sup>b</sup></i>	ShET1	Enterotoxin

<sup>a</sup> The *stx* locus and production of Shiga toxin is observed only in *S. dysenteriae* 1.

<sup>b</sup> The *set* locus and production of ShET1 is observed almost exclusively in *S. flexneri*.

cell and is attenuated. This phenotype is due to degradation of IcsA by the *ompT* protease (71). Another example of a missing genetic locus or “black hole” in the *Shigella* genome is *cadA*, the gene for lysine decarboxylase. While present in >85% of *E. coli* strains, lysine decarboxylase activity is missing in all strains of *Shigella* spp. and enteroinvasive *E. coli*. When *cadA* is reintroduced back into *S. flexneri*, the generation of cadaverine (by the decarboxylation of lysine) acts to inhibit the action of the *Shigella* enterotoxins (72). Thus, these examples of “antivirulence genes” illustrate another way that pathogens evolve from their nonpathogenic commensal relatives by both acquiring genes (e.g., the virulence plasmid) that contribute to virulence and deleting genes that are incompatible with expression of these new virulence traits.

## VIII. CONTROL MEASURES

As noted earlier, shigellosis is generally a self-limiting disease, and patients can recover even in the absence of antimicrobial therapy. The incidence of multiple antibiotic-resistant strains of *Shigella* is increasing throughout the world. Isolates resistant to sulfonamides, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, and streptomycin have been reported (73).

Antimicrobial therapy shortens the duration of the illness and reduces the excretion of infectious organisms in the feces (74). However, it contributes only minimally to control of epidemic outbreaks. The single most effective means of preventing secondary transmission of shigellosis is hand washing. Food handling and preparation are important processes that deserve attention, and persons with diarrhea should not handle food. Unfortunately, despite many years of intensive effort, an effective vaccine against shigellosis still has not been developed. One such candidate, SC602, is an attenuated, oral vaccine strain of *S. flexneri* that is undergoing testing. This strain has mutations that block iron uptake (*iuc*) and abolish the intracellular and intercellular motility phenotypes (*icsA*) (75). While a limited challenge study showed protection among vaccines, one major drawback still to be resolved is the transient fever and mild diarrhea associated with administration of the vaccine (76). This study highlights one of the persistent problems faced by investigators attempting to develop a safe *Shigella* vaccine: designing a strain that is capable of inducing a protective immune response without producing unacceptable side effects.

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# 12

## ***Campylobacter* Species**

**Lan Hu and Dennis J. Kopecko**

*U.S. Food and Drug Administration, Bethesda, Maryland, U.S.A.*

*Campylobacter* is a leading bacterial cause of diarrhea in humans in all parts of the world. In the United States *Campylobacter* cause approximately 2.5 million illnesses per year (or 12.4% of all defined foodborne illnesses) and are responsible for 124 deaths each year. About 80% of *Campylobacter* illnesses are thought to be foodborne (Mead et al., 1999). In developing countries, infection is hyperendemic among young children <5 years of age (Oberhelman and Taylor, 2000). Infection of domesticated animals is widespread (including poultry, pigs, sheep, cattle, dogs, cats, and birds). This pathogen can be transmitted to human populations through consumption of undercooked poultry, pork, and beef, unpasteurized milk, contaminated drinking water, and the feces of infected pets (Aho et al., 1989; Shane, 1992; Stern et al., 2001).

*Campylobacter jejuni* and *Campylobacter coli* are the most important human pathogens in the genus, with the former usually responsible for 80–90% and the latter responsible for ~5–10% of *Campylobacter* enteric infections (Skirrow, 1994). Most other *Campylobacter* spp. are also associated with a range of diseases in humans and/or animals, including diarrhea, pancreatitis, meningitis, bacteriemia, septicemia, abortion, and periodontitis (On, 1996; Skirrow, 1994). *Campylobacter* organisms can also cause postinfectious complications, the most important of which is Guillain-Barré syndrome (GBS).

In this chapter we will focus on *C. jejuni* and *C. coli*, which are the main causes of *Campylobacter* enteritis in humans. We will also mention some other diseases caused by *Campylobacter* organisms.

### **I. BACKGROUND**

In 1886 Theodor Escherich described spiral organisms that may have been *Campylobacter* in the colonic mucus of infants who had died of “cholera infantum,” but they could not be cultured (Kist, 1986). McFadyean and Stockman in 1909 first isolated *Campylobacter fetus* from aborted sheep fetuses (McFadyean and Stockman, 1913). They were initially called *Vibrio fetusoid* because of the curved shape. The name was shortened to *Vibrio fetus* when Smith and Taylor (1919) reported that the same organisms also caused septic abortion in cattle. In 1931 Jones et al. found that a bacterium they called *Vibrio jejuni* led to winter dysentery outbreaks in calves. In 1957 King isolated two groups of organisms from blood cultures of patients suffering from bloody diarrhea. One group corresponded closely to *V. fetus* and the other group was named “related *Vibrios*.” Sebald and Veron (1963) found that these two groups differed from the classical *Vibrio* species in carbohydrate

fermentation and DNA G+C contents. As a result, these species were given the new genus name *Campylobacter*, meaning curved rod in Greek. In 1972, *Campylobacter* organisms were first isolated from stool samples of patients with diarrhea (Butzler et al., 1973; Dekeyser et al., 1972). The development of filtration methods combined with selective growth media led to crucial breakthroughs in the identification of *Campylobacter* species in more laboratories (Butzler et al., 1973; Skirrow, 1977). Soon, *Campylobacter* spp. were established as common human pathogens worldwide (Griffiths and Park, 1990; Penner, 1988). Now *Campylobacter* is recognized as the leading cause of bacterial diarrheas, causing  $\geq 50\%$  of acute bacterial diarrhea illnesses.

## II. CHARACTERISTICS

*Campylobacter* species are small, non-spore-forming, curved, or spiral gram-negative rods. Dimensions range from 0.2 to 0.5  $\mu\text{m}$  wide and from 0.5 to 5  $\mu\text{m}$  long. They are motile via a single polar flagellum at one or both ends, and they exhibit a rapid darting motion.

### A. Nomenclature and Typing

Since the “related Vibrios” were reclassified into the new genus *Campylobacter* in the mid-1960s, the taxonomy of *Campylobacter* and related organisms has been extensively revised (e.g., the separate genera *Helicobacter* and *Arcobacter* have been created). Based upon 16 S rRNA sequencing, *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Wolinella* form a distinct group, rRNA superfamily VI (Vandamme et al., 1991). Now *Campylobacter* and *Arcobacter* are included in the family Campylobacteraceae (Logan et al., 2000). Sixteen species and 6 subspecies are recognized in the genus *Campylobacter* (On, 1996; Skirrow, 1994), 12 of which are associated with human disease. The human *Campylobacter* pathogens can be divided into two major groups: those that primarily cause diarrheal disease (including *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari*, and *C. fetus*) and those that can cause extraintestinal infections (including *C. fetus* and others).

*Campylobacter* have heat-stable lipooligosaccharide or capsular antigens and heat-labile surface and flagella proteins. Surface polysaccharide antigens form the basis of the Penner heat-stable serogrouping scheme ( $>65$  serotypes) (Penner and Hennessy, 1980). Surface and flagella proteins form the basis of the Lior heat-sensitive antigen serogrouping scheme (160 serogroups) (Lior et al., 1982). Phage typing and molecular typing methods can give extremely fine discrimination within serotypes. The molecular typing methods include pulsed-field gel electrophoresis (PFGE) (Chang and Taylor, 1990), *fla* typing, random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism fingerprinting (AFLP) (Newell et al., 2000), to mention the most common. Strain typing is not routinely used in clinical practice, but is useful for epidemiological studies.

### B. Growth Characteristics

*Campylobacter* species are typically microaerophilic, requiring 3–15% oxygen and 3–5%  $\text{CO}_2$  for growth. All *Campylobacter* grow at 37°, but thermophilic *Campylobacter* spp. (*C. jejuni*, *C. coli*, and *C. lari*) grow best at 42°C.

*Campylobacter* organisms do not ferment or oxidize carbohydrates and do not need blood components for growth. Gelatin is not hydrolyzed. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates. Menaquinone-6 and methyl-substituted menaquinone-6 are major respiratory quinones. Hydrogen may be required for growth by some species. The thermophilic campylobacters reduce selenite, are oxidase and catalase positive, and are indole negative. They can be differentiated on the basis of nalidixic acid sensitivity and hippurate hydrolysis (Skirrow, 1990). The G+C content of their DNA ranges from 29 to 46 mol%.

Campylobacters are more sensitive to adverse conditions (e.g., drying, heat, acidity, disinfectants,  $\gamma$ -irradiation) than most other enteric pathogenic bacteria (Jacobs-Reitsma, 2000). This suggests that they are best adapted for existence in vivo. Campylobacters can survive in water at 4°C

for many weeks, but typically survive only a few days at temperatures above 15°C. Freezing and thawing causes a 1–2 log<sub>10</sub> fall in viable count, but they can survive for many months at consistent temperatures of –20°C or below (Blaser et al., 1980). *Campylobacter* are sensitive to low pH. They are rapidly killed by gastric acids unless protected by food matrices. *Campylobacter* can survive in 6.5% NaCl for 3 weeks at 4°C, so they may survive in salted, uncooked meat if the initial level of contamination is high (Abram and Potter, 1984).

*Campylobacter* in old cultures or exposed to air may have spherical forms. They may also survive in a “viable but nonculturable” form for longer times (Rollins and Colwell, 1986).

### C. Infectious Dose

The infection dose of *C. jejuni* is low. In volunteer studies, infection has been established with as few as 50–500 organisms (Black et al., 1988; Robinson and Jones, 1981). Milk and foods (or medical therapies) that neutralize gastric acids effectively decrease the infectious dose.

## III. DISEASE

*Campylobacter* can cause either gastrointestinal or extraintestinal infections.

### A. Enteritis

Infections typically occur in healthy individuals and follow ingestion of *Campylobacter*-contaminated food or water or occupational exposure to *Campylobacter*-infected animals. The average incubation period is about 3 days, with a range of 1–7 days from ingestion of the *Campylobacter*. The illness may start with cramping abdominal pain and diarrhea. Patients may experience fever, chills, headache, myalgia, and occasionally delirium. Diarrhea and abdominal pain are the major symptoms. Nausea is common, but vomiting is rare. As is common with many infectious agents, up to 25% of exposed individuals are asymptomatic. In most patients, the illness is self-limited, and recovery occurs in 2–6 days. Fecal leukocytes are found in 75% of cases; gross or occult blood is seen in ~50% of cases in developed countries. In developing countries *Campylobacter* tend to cause a watery, noninflammatory diarrhea. Untreated patients may shed bacteria intestinally for several weeks to several months. Only patients with an immune deficiency will become long-term carriers. The proportion of patients admitted to the hospital is 5–10%. A fatal outcome is rare and is usually confined to elderly patients or those already suffering from another serious disease (Skirrow et al., 1993; Smith and Blaser, 1985).

The clinical symptoms of *Campylobacter* enteritis are difficult to distinguish from those of *Salmonella*, *Shigella*, or other causes of bacterial enteritis. However, abdominal pain tends to be more severe in *Campylobacter* infection and sometimes mimics acute appendicitis, which can result in unnecessary surgery. Occasionally, illness starts with symptoms of colitis without a preceding ileitis, which can make it difficult to distinguish from acute ulcerative colitis.

### B. Extraintestinal Infection

In addition to typical bacterial enteritis, *Campylobacter* can cause extraintestinal infections. Extraintestinal infections are usually the result of systemic spread.

#### 1. Bacteremia

*C. jejuni* bacteriemia occurs in approximately 1.5 per 1000 intestinal infections (Skirrow et al., 1993). The rate is higher in elderly individuals and in immunodeficient persons (Ladron de Guevara et al., 1994) and nearly twice as high in males as in females. Transient bacteremia might occur in many cases of *C. jejuni* enteritis but is not detected because blood cultures are not routinely conducted. Clinically significant bacteremia is infrequent because most *C. jejuni* strains are susceptible

to killing by normal human serum. In contrast, *C. fetus* is serum-resistant because it is covered with a capsule-like protein (i.e., S layer) that prevents complement-mediated killing in serum. *C. fetus* infections easily induce bacteremia and systemic spread. However, *C. jejuni* still accounts for most (89%) *Campylobacter* isolated from blood. *C. jejuni* Penner serotypes O4 and O18 are more frequently isolated from blood than feces (Skirrow et al., 1993).

## 2. Other Infections

Secondary complications resulting from primary *Campylobacter* enteritis are relatively rare. These complications include cholecystitis, pancreatitis, peridontitis, gastrointestinal hemorrhage, meningitis, endocarditis, arthritis, peritonitis, cellulitis, hepatitis, and septic abortion. With improved methods for identification of *Campylobacter*, more species are now recognized to be important in causing human infections, especially when they infect immunocompromised persons. For example, *C. fetus* subsp. *fetus* is primarily associated with infectious abortion in cattle and sheep and is an infrequent cause of human infections. *C. fetus* can cause fetal death or septicemia in debilitated persons with chronic disease or in immunosuppressed patients (Blaser, 2000). *C. jejuni* and *C. coli* can also cause human abortion. Other *Campylobacter* spp., *C. concisus*, *C. rectus*, and *C. curvus* are known to be implicated in human periodontal disease (Tanner et al., 1987).

## C. Postinfectious Complications

### 1. Guillain-Barré Syndrome

GBS is an acute inflammatory polyneuropathy resulting in neuromuscular paralysis. Approximately 5% of patients with GBS die, and 20% of patients are left with some chronic disability (Altekruse et al., 1999). Approximately 1 in 1000 *C. jejuni* infections may be complicated by GBS (Nachamkin et al., 2000; Allos and Blaser, 1995b). Recent studies suggest that antibody cross-reactivity occurs between *C. jejuni* surface polysaccharides and GM1 or other peripheral nerve gangliosides (Jacobs et al., 1996, 1998; Moran, 1997). Most GBS-related *C. jejuni* strains belong to the specific Penner serotypes O:19 and O:41 (Fujimoto et al., 1992; Kuroki et al., 1993; Lastovica et al., 1997).

### 2. Reiter's Syndrome

This syndrome is a reactive arthropathy involving multiple joints and affects approximately 1% of *Campylobacter*-infected patients. Pain and swelling of joints appear 2 weeks postinfection; joint incapability can remain for months or can become chronic. Most patients with Reiter's syndrome carry the human leukocyte antigen B27 phenotype (Peterson, 1994).

## IV. EPIDEMIOLOGY

The epidemiological and symptomatic patterns of disease differ greatly between developed and developing countries (Blaser et al., 1983a,b; Skirrow, 1991; Oberhelman and Taylor, 2000). The vast majority of *Campylobacter* infections are sporadic individual infections. Large outbreaks are uncommon but do occur.

The routes of transmission of *Campylobacter* infection include direct contact by exposure to infected animals and the handling of infected pets, such as dogs, cats, and birds. Indirectly, infection can occur by the consumption of contaminated food and water. Person-to-person transmission rarely occurs. Some *Campylobacter* species also are sexually transmitted.

## A. Incidence

In the United States *Campylobacter* spp. cause approximately 2.5 million illnesses and 124 deaths each year. About 80% of these illnesses are thought to be foodborne (Mead et al., 1999). In developing countries including Bangladesh, Indonesia, Gambia, and Mexico, *C. jejuni* infection is most

frequently diagnosed in young children (Oberhelman and Taylor, 2000). Children with *Campylobacter* enteritis in developing countries tend to have watery diarrhea and are infected with multiple pathogens, including multiple *Campylobacter* strains. Travelers to developing countries are at risk for developing *Campylobacter* infection, with isolation rates ranging from 0 to 39% (Oberhelman and Taylor, 2000).

*C. fetus* infections are relatively uncommon, with fewer than 250 cases reported annually in the United States. Unlike *C. jejuni*, *C. fetus* infects the immunocompromised and the elderly.

## B. Factors Influencing Incidence

### 1. Age

As opposed to the prevalence of cases in children <5 years of age in developing countries, *Campylobacter* affect all age groups in developed nations. Infants and young children have the highest rate of infections, and a second surge occurs in young adulthood, between 15 and 29 years old (Tauxe et al., 1988).

In developing nations, infection is hyperendemic. Children are repeatedly exposed to *Campylobacter* infection from an early age. By the time they are 2–5 years old they have developed substantial immunity that persists into adulthood. In adults and in older children, infections are usually asymptomatic (Glass et al., 1983; Taylor et al., 1988).

### 2. Sex

Males have a higher morbidity than females (male:female ratio = 1.7:1) (Skirrow, 1987a,b).

### 3. Season

Campylobacteriosis shows a seasonal peak in the summer and early fall in most developed countries (Friedman et al., 2000). In the United States, there is also an increased incidence in December (Tauxe et al., 1988). This pattern is attributed to seasonal consumption of food at barbecues and catered social events.

## C. Reservoirs

*Campylobacter* spp. are widely distributed in nature and are commonly found as commensals of the gastrointestinal tract in wild or domesticated animals. Primary acquisition of *Campylobacter* by animals often occurs early in life and may lead to morbidity or mortality, but a lifelong carriage develops in most colonized animals. The vast reservoir in animals is probably the ultimate source for most enteric *Campylobacter* infections in humans. Domestic animals include cattle, sheep, poultry, dogs, and cats. Domestic poultry are a major source of *C. jejuni* infections in humans. Pigs are a primary host for *C. coli*. *C. fetus* has been isolated from sheep, cattle, poultry, reptiles, and swine (Smibert, 1984).

Natural water surfaces are frequently contaminated with *C. jejuni*, *C. coli*, and *C. lari* throughout the year. Infection can be caused by drinking unchlorinated, contaminated water or the consumption of food prepared with untreated/improperly treated water.

## D. Foodborne Outbreaks

### 1. Food

*C. jejuni* and *C. coli* colonize domestic poultry with particular ease. At least 60% of chickens sold in stores are contaminated with campylobacters, and broiler chickens are thought to cause about 50–70% of human infections in developed countries. Eating undercooked chicken appears to be the most common cause of *C. jejuni* infection. However, red meat can also be contaminated (Blaser, 2000). Eating barbecued pork or sausages also can cause infection (Kapperud et al., 1992). Raw or poorly cooked fish, shellfish, and mushrooms can also lead to infection (Harris et al., 1986;



Hopkins et al., 1984). *Campylobacter* do not typically multiply in foods left at ambient temperatures due to their microaerophilic nature, so they generally do not cause food poisoning. However, their low infectious dose allows this organism to exhibit high infection rates.

## 2. Milk

Raw and unpasteurized milk were, in the recent past, a common source of *Campylobacter* and accounted for 55% of the foodborne *Campylobacter* outbreaks in the United States between 1978 and 1987 and 42% of the outbreaks in England and Wales between 1984 and 1990. A typical example of these outbreaks is a school field trip to a dairy farm where drinking raw milk was part of the experience (Wood et al., 1992). However, outbreaks due to raw milk have markedly decreased with routine pasteurization of milk in developed countries and increased public awareness.

## E. Waterborne Outbreaks

Drinking unchlorinated water is also an important source of *Campylobacter* infection. The first waterborne outbreak in the United States occurred in Burlington, Vermont, when a contaminated community water supply affected an estimated 3000 people (Vogt et al., 1982).

## V. ISOLATION AND IDENTIFICATION

### A. Direct Examination

It is possible to detect *Campylobacter* infection by observing directly the characteristic gram-negative nature, curved or spiral morphology and the characteristic darting motility in wet mounts of fresh stools detected by phase-contrast microscopy or darkfield microscopy.

### B. Isolation

Campylobacters multiply more slowly than the other enteric flora and therefore cannot be isolated from fecal specimens unless selective media are used. The most common isolation methods used in the past include blood and antibiotic media. Skirrow's, Butzler's, and Campy-BAP media or variations of these have been widely used. Butzler's and Campy-BAP media contain cephalothin, which inhibits *C. fetus* and several *Campylobacter* subspecies, and are best suited for isolating *C. jejuni*. Because people infected by *Campylobacter* usually excrete  $10^6$ – $10^9$  *C. jejuni* colony-forming units per gram stool, enrichment usually is not necessary in stool samples. However, enrichment methods are essential for food, environmental samples, and old stool samples where the number of organisms is low due to delayed transport to the laboratory or the acute stage of disease has passed (Nachamkin, 1999; Scotter et al., 1993). Organisms that have been sublethally damaged by freezing or heat should be preincubated for a few hours in nonselective broth at 37°C.

Campylobacters are very small and thin, so they can pass through 0.45–0.65 µm filters that generally retard other enteric flora. Filtration methods permit isolation without using antibiotic-containing media. The use of filtration techniques and nonselective media at 37°C improves the growth of both *C. jejuni* and the atypical enteric campylobacters from stool samples and is now recommended for primary isolation of campylobacters from fecal specimens or swabs.

### C. Identification

*Campylobacter* colonies usually appear on the plate within 24–48 hours but occasionally require 72 hours. The campylobacters can be distinguished from other bacteria by the characteristics listed below.

## 1. Specific Characteristics

1. *Campylobacter* spp. usually produce gray, flat, irregular, spreading colonies in freshly prepared media. They do not induce hemolysis on blood agar.
2. They are gram-negative curved (spiral) rods.
3. They are oxidase and catalase positive. Primary isolates should first be tested for hippurate hydrolysis. It is the major test for distinguishing *C. jejuni* from other *Campylobacter* spp.
4. They exhibit characteristic darting motility (Allos, 1998).

## 2. Serotyping

A retrospective serological diagnosis for the causative strain is sometimes important for patients exhibiting severe, postdiarrhea complications and for which bacteriological examination was not conducted during the original illness.

## VI. PATHOGENICITY

Bacterial diseases are typically the result of a complex set of interactions between the offending bacteria and the host. In the process of evolution, humans have developed a variety of ways to protect themselves from pathogenic organisms (e.g., mucin barrier of gut, organized monolayer of the absorptive epithelial mucosa, nonspecific and specific immune defenses, gut peristalsis). At the same time, bacteria have evolved ways to circumvent these host defenses (e.g., motility to penetrate and navigate within the mucin barrier, gut mucosal adherence mechanisms, and epithelial cell internalization processes). It is now well accepted that bacterial virulence is multifactorial. The pathogenesis of *C. jejuni* gastrointestinal disease is not completely understood. Proposed components of *C. jejuni* virulence include toxins (such as enterotoxin, various cytotoxins), adherence, motility, iron acquisition ability, and bacterial invasion (Kopecko et al., 2001; Ketley, 1997; Walker et al., 1986).

Following ingestion, campylobacters have to pass the gastric acid barrier of the stomach. Reduction of gastric acidity (e.g., by antacid use) increases the risk of *Campylobacter* infection. Campylobacters apparently first colonize the jejunum and ileum, and then affect the colon and rectum. Mucosal damage and inflammation is observed in both the small and large bowels. Histopathologically, the mucosal surface is ulcerated, with crypt abscesses in the colon and infiltration of the lamina propria with neutrophils and mononuclear cells. In developed countries, the symptoms of infection suggest an invasive, inflammatory disease (Healing et al., 1992), which is consistent with the above histopathology. However, in developing countries the symptoms of *Campylobacter* infection suggest a toxigenic, but noninflammatory, disease.

### A. Toxins

Enterotoxin, cytopathic toxins, and endotoxic activity have been associated with *C. jejuni* isolates. However, infected persons do not develop neutralizing antibodies to these toxins. Strains lacking apparent enterotoxin activity are still fully virulent. A cytolethal distending toxin (CDT) has been found in all *C. jejuni* strains (Pickett et al., 1996). CDT locks target eukaryotic cells in the G2 phase and eventually leads to apoptosis over several days (Whitehouse et al., 1998). The genome of *C. jejuni* strain NCTC 11168 contains a gene encoding the cytolethal distending toxin but does not contain a sequence homologous to the cholera-like enterotoxin gene (Parkhill et al., 2000). Thus, the cause of the noninflammatory, watery diarrhea induced by *Campylobacter* in developing countries is likely not due to a classical enterotoxin.

### B. Adherence

*C. jejuni* does adhere to enteric epithelial cells. A superficial antigen (PEB1) that appears to be a major surface adhesin is conserved among *C. jejuni* strains and is a target of the normal humoral

immune response (Kervella et al., 1993; Pei et al., 1991). Flagella and motility also appear to be important adherence determinants (Grant et al., 1993; Guerry et al., 1990). Konkel and coworkers (1997) have reported the presence of a fibronectin-binding protein of *C. jejuni*, but its involvement in virulence is yet uncharacterized.

### C. Invasion

Penetration of the epithelial mucosa is now considered to be an essential virulence mechanism of several pathogenic enteric bacteria including *Salmonella* and *Shigella*. *C. jejuni* infection can result in penetration of the gut epithelial mucosa, suggesting that, at least for a subgroup of strains, tissue invasion represents an important component of pathogenesis (Ketley, 1997; Hu and Kopecko, 2000).

Invasive bacterial pathogens interact with host cells via a biochemical crosstalk and stimulate signal transduction pathways that result in host cell cytoskeletal rearrangements and bacterial internalization into host cells. Only a few *C. jejuni* strains have been studied in any detail for molecular mechanisms of invasion, but the results suggest that *Campylobacter* may encode separate microtubule-dependent and microfilament-dependent pathways for host invasion; some strains may encode both mechanisms, while others may exhibit only a single uptake mechanism (Hu and Kopecko, 1999; Oelschlaeger et al., 1993). *C. jejuni* internalization requires intact caveolae and involves both host protein phosphorylation and dephosphorylation events (Kopecko et al., 2001). Bacterially induced release of host intracellular  $\text{Ca}^{2+}$  is likely to be required for the cytoskeletal rearrangements that result in *Campylobacter* internalization (Hu and Kopecko, 2000; Kopecko et al., 2001; Wooldridge et al., 1996).

### D. Translocation Across the Intestinal Mucosa

Bacterial translocation entails the movement of viable bacteria across the gastrointestinal barrier, where further extraintestinal dissemination can occur. *Campylobacter*s have been observed to translocate across a tight epithelial cell monolayer (Everest et al., 1992; Grant et al., 1993; Konkel et al., 1992). *C. jejuni* can penetrate from the apical to the basolateral surface of polarized Caco-2 cells without disrupting transepithelial electrical resistance. Bacteria were found within endosomal vacuoles inside Caco-2 cells and presumably pass through the monolayer while remaining within the endosome. Electron microscopic studies also indicate that *Campylobacter* pass between cells (Konkel et al., 1992), but some isolates appear to transcytose without host cell invasion (Everest et al., 1992). Thus, it appears that *Campylobacter* may cross polarized epithelial cells via transcellular and paracellular routes.

Grant et al. (1993) and Yao et al. (1994) reported that either *C. jejuni* motility or the product of the *flaA* gene is essential for the bacterium to cross polarized monolayers, since *flaA*<sup>-</sup> *flaB*<sup>-</sup> Mot<sup>-</sup> and *flaA*<sup>-</sup> *flaB*<sup>+</sup> mot<sup>+</sup> mutants were unable to cross the cell barrier. The bacterial protein synthesis inhibitor chloramphenicol also reduced monolayer translocation of *C. jejuni* (Konkel et al., 1992), suggesting that nascent bacterial protein synthesis is also required for mucosal translocation.

### E. Bacterial Survival and Interaction with Phagocytes

Following invasion into host cells, pathogens must have the ability to survive within and/or replicate intracellularly. The bacterial and host factors that determine the fate of internalized *Campylobacter*s are not well understood. Superoxide dismutase (SOD) catalyzes the breakdown of superoxide radicals and is one of the bacterial cell's major defense mechanisms against oxidative damage. Mutant *sodB*<sup>-</sup> *C. jejuni* 81-176 are significantly decreased in their survival within INT407 cells relative to the parent strain, indicating that SodB is important in intracellular survival (Pesci et al., 1994). Day et al. (2000) reported that catalase provides resistance to hydrogen peroxide in vitro. Though catalase

does not appear to play a role in intraepithelial cell survival, catalase is apparently essential for *C. jejuni* intramacrophage survival.

Internalized *C. jejuni* changed from the spiral to coccal form within 4–8 hours and survived for 6–7 days in human mononuclear phagocytes (Kiehlbauch et al., 1985). Monocytes, therefore, could play an important role in dissemination of *C. jejuni* following intestinal translocation by protecting *C. jejuni* during transit to secondary sites.

During the course of human *Campylobacter* infections, infecting organisms elicit phagocytic cells into the intestinal lumen (Blaser, 1997; Duffy et al., 1980) and encounter them systemically during bacteremic conditions (Field et al., 1986; Longfield et al., 1979). Intestinal infection with *C. jejuni* is typically associated in developed countries with an inflammatory response and the presence of fecal leukocytes (Black et al., 1988; Duffy et al., 1980). Recent studies have shown that interleukin-8 (IL-8) secretion by epithelial cells may be an early signal for the acute inflammatory response following various enteric bacterial infections (Eckmann et al., 1993; Fierer et al., 1993; Crowe et al., 1995). Many strains of *Campylobacter* spp. can induce secretion of IL-8 by INT407 cells. Induction of IL-8 secretion requires live cells of *C. jejuni* strain 81–176 and is dependent on de novo bacterial protein synthesis (Hickey et al., 1999).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

The identification and characterization of *Campylobacter* spp. is severely hampered by the fact that most diagnostic techniques developed for use with *Escherichia coli*, *Shigella* spp. or *Salmonella* do not work with *Campylobacter* (Van Vliet et al., 1998), which has special atmospheric growth requirements.

*C. jejuni*, *C. coli*, and *C. fetus* have genomes of approximately 1700 kb (Chang and Taylor, 1990; Nuijten et al., 1990), which is only about one-third the size of the *Escherichia coli* genome. This is consistent with the limited biochemical repertoire. They also have an unusually high A+T content of 70%.

Recently, Parkhill et al. (2000) reported the genome sequence of *C. jejuni* NCTC 11168. *C. jejuni* has a circular chromosome of 1641 kilobase pairs (30.6% G+C) and is predicted to encode 1654 proteins and 54 stable RNA species. The genome of *C. jejuni* reveals hypervariable regions that encode the biosynthesis or modification of surface structures, or comprise uncharacterized genes.

### A. Flagella

Flagella have been implicated as adhesins or transporters of adhesins because (1) nonflagellated mutant strains adhere much less efficiently to host cells than the flagellated parent strain and (2) isolated flagellar preparations bind to monolayers of INT407 cells (McSweegan et al., 1987). In *C. jejuni*, the flagellum is composed of two closely related proteins, the major subunit FlaA and the minor subunit FlaB. The *flaA* and *flaB* genes have been cloned, individually mutated, and extensively characterized (Grant et al., 1993; Guerry et al., 1990). Studies have shown that *C. jejuni* mutants in which wild-type flagella are present but paralyzed and nonfunctional due to mutation at *pflA* can bind to but are greatly impaired in ability to invade INT407 cells (Yao et al., 1997). Nonflagellated mutants are impaired in adherence to host cells, which is enhanced by bacterial centrifugation onto the monolayer (Yao et al., 1994). Also, antibodies directed against flagella have been shown not to inhibit *Campylobacter* attachment to INT407 cells (Wassenaar et al., 1991) and sheared flagella were not effective in blocking attachment of *C. jejuni* to INT407 cells (McSweegan et al., 1987). These results suggest that motility is required for optimal *Campylobacter* adherence to host cells and for invasion ability, but do not reveal a definitive role for flagella in adherence. Nevertheless, the polar flagellum and unique characteristics of the spiral shape of campylobacters confer a distinctive motility that is particularly effective in a viscous matrix. This motility, aided by chemotactic

signaling (Hugdahl et al., 1988; Takata et al., 1992), may allow *Campylobacter* to penetrate the mucus layer and seek host cell receptors involved in colonization or invasion of the intestinal mucosa.

## B. Pili and Outer Membrane Proteins

Doig et al. (1996) reported that growth in the bile salt deoxycholate induces the synthesis of pili in six strains of *C. coli* and *C. jejuni*. A nonpiliated mutant of *C. jejuni* 81–176 showed no reduction in adherence to or invasion of INT407 cells in vitro. Further, this mutant could colonize, but caused significantly reduced disease symptoms in infected ferrets. De Melo and Pechere (1990) identified four *C. jejuni* proteins with apparent molecular masses of 28, 32, 36, and 42 kDa that bind to Hep-2 cells. A *C. jejuni* gene encoding a 28 kDa protein, termed PEB1, has been identified as a conserved antigen in *C. jejuni* and *C. coli* strains and is proposed to be an adhesin (Pei and Blaser, 1993; Pei et al., 1991). PEB1 shares homology with a periplasmic binding protein involved in nutrient acquisition (Garvis et al., 1996).

## C. LPS-LOS

Lipopolysaccharides (e.g., O-antigen) (LPS) and lipooligosaccharides (LOS) are the major surface antigens of gram-negative bacteria and play an important role in the interaction of these bacteria with their host and/or the environment. The endotoxic properties of *Campylobacter* LPS/LOS are comparable with those of other enterobacterial LPS (Moran, 1995). Chemical analyses of many of the >50 thermostable antigen serotypes representing predominant *C. jejuni* isolates have suggested that ~40% of the serotypes express classical core-linked, high molecular weight O-antigen (Penner and Aspinall, 1997). The remainder of the serotypes have low molecular weight LOS molecules or capsular polysaccharide polymers unlinked to the core (Karlyshev and Wren, 2001; Moran, 1995; Penner and Aspinall, 1997). In some *C. jejuni* serotypes, sialylation of the terminal core oligosaccharide sugar has been shown to create structures that mimic human gangliosides (e.g., GM<sub>1</sub>, GD<sub>1a</sub>, GD<sub>3</sub>, GT<sub>1a</sub>). Antibodies raised against these mimetic molecules are suspected to play an autoimmune role in the development of post-diarrheal Guillain-Barré syndrome (Allos and Blaser, 1995a,b; Blaser et al., 1997; Saida et al., 1997). The suggestion has been made that LPS may be an important adhesin of *C. jejuni* for cellular and mucus substrates, but no direct evidence has yet been obtained. Nevertheless, the important role played by LPS or other surface polysaccharides in the virulence of other enterobacteria suggests the likelihood for similar roles in *C. jejuni* virulence.

## D. Nascent Protein Synthesis

To provoke disease, invasive microorganisms must express products that bind host cell receptors and facilitate subsequent internalization as well as survival in this changed environment. New proteins are induced in *C. jejuni* upon contact with both viable and nonviable host cells, and synthesis of a subset of these bacterial proteins is upregulated by released host cell components (Konkel et al., 1993). One recently identified, host cell–induced *C. jejuni* gene (termed *ciaB* for *Campylobacter* invasion antigen B) encodes a protein of ~73 kDa. Mutants in *ciaB* are noninvasive for INT407 cells and somehow are blocked in the secretion of at least eight *C. jejuni* F38011 proteins when in the presence of INT407 cells. *CiaB* shares 40–45% amino acid similarity to the invasion ligands of *Salmonella* (i.e., SipB) and *Shigella* (i.e., IpaB) and to the *Yersinia* YopB virulence protein (Konkel et al., 1999). How these *Campylobacter* proteins are secreted or interact with the host cell remains uncharacterized. No type III secretion systems other than the flagellin export apparatus were identified in the genome-sequenced strain *C. jejuni* NCTC 11168 (Parkhill et al., 2000). However, the *CiaB* protein might be secreted by the flagellin export apparatus (Young et al., 1999). Bacon et al. (2000) reported that plasmid pVir is involved in the virulence of *C. jejuni* strain 81–176. There

are four open reading frames in pVir that encode proteins with significant sequence similarity to *Helicobacter pylori* proteins. All four of these plasmid-encoded proteins show significant homology to components of type IV secretion systems. Thus, *C. jejuni* upregulate the synthesis of and secrete proteins upon host cell contact, and some of these proteins are probably involved in interactions with the host that lead to bacterial internalization and IL-8 synthesis. However, the molecular details of these processes remain undefined.

## E. Iron and Ferritin

The strict limitation of free iron within mammalian hosts embodies a nonspecific, host-protective mechanism, since bacteria require  $10^{-6}$ – $10^{-7}$  M  $\text{Fe}^{2+}$  for survival. Bacterial pathogens have evolved mechanisms to sequester iron within the host. These bacterial pathways are maximally expressed under iron-restricted conditions (Payne, 1993). Unlike other invasive enteric bacteria, *C. jejuni* have not been found to produce siderophores to sequester iron. However, *Campylobacter* can bind exogenous siderophores (Field et al., 1986), possess an enterochelin uptake system (Richardson and Park, 1995), and synthesize bacterial ferritin, which is involved in both iron storage and bacterial protection from oxidative stress (Wai et al., 1996).

## VIII. CONTROL MEASURES

Supportive measures, particularly fluid and electrolyte replacement, are usually sufficient treatment for most patients with campylobacteriosis. These include proper oral intake of liquids and intravenous administration of fluids when necessary.

Some studies have reported that antibiotic therapy can shorten the duration of symptoms and of fecal shedding of *Campylobacter*. However, most patients do not seek medical care during the primary stage of infection, and antibiotics do not effectively shorten the duration of symptoms (Goodman et al., 1990; Petruccioli et al., 1992).

### A. Treatment

Patients with marked high fever, bloody diarrhea, and severe or persistent diarrhea, infants, or elderly and immunosuppressed patients should be treated with antibiotics. In vitro, *C. jejuni* is susceptible to a wide variety of antimicrobial agents, including erythromycin, the tetracyclines, the aminoglycosides, chloramphenicol, quinolones, nitrofurans, and clindamycin. Erythromycin remains a priority treatment choice for *Campylobacter* infection because it has few side effects, is inexpensive, and causes less interference with the enteric bacterial flora than other antibiotics. Clarithromycin is also effective. Ciprofloxacin is an alternative therapeutic agent, but resistance to it is increasing. Other alternatives include tetracycline, norfloxacin, and furazolidone. *C. jejuni* strains are almost universally resistant to cephalosporins, vancomycin, and rifampin; 25% are now resistant to tetracycline (Allos and Blaser, 1995a).

### B. Immunity and Vaccines

*Campylobacter* induce both specific humoral immune responses and cellular immune responses. People from areas of highly endemic *Campylobacter* disease develop measurable levels of specific serum and secretory antibodies and have less severe disease. Serum antibody IgG, IgM, and IgA levels rise in the serum 5 days postinfection with *Campylobacter*, show a peak within 2–4 weeks, after which serum and fecal IgA levels fall rapidly. IgG persists for several weeks or months (Black et al., 1988; Blaser and Duncan, 1984; Blaser et al., 1985). Volunteer studies show that *Campylobacter* infection induces immunity to the homologous strain (Black et al., 1988), but it is not known how long immunity lasts or whether any cross-serotype immunity develops. Patients with hypogam-

maglobulinemia have prolonged, severe disease with *C. jejuni* (Kerstens et al., 1992). No effective *Campylobacter* vaccine is available.

### C. Prevention

The prevention of *Campylobacter* infection is based upon interruption of transmission routes, either from animal reservoirs to humans or from consumption of contaminated foods and water. Major focal points for control include:

1. Control of *Campylobacter* contamination on the farm and with pets.
2. Practicing good food hygiene. This includes adequate refrigeration of poultry and meat from processing to preparation, separation of raw and cooked foods, thorough cooking of poultry, red meat, and seafood to obtain a core temperature of 72°C for  $\geq 1$  minute before eating (Butzler and Oosterom, 1991; Shane, 1994).
3. Effective pasteurization of milk.
4. Maintaining an uncompromised clean water supply.
5. Individuals who have diarrheal symptoms should not work as food handlers or day care employees until they have recovered from acute symptoms and should practice good hygiene (e.g., handwashing).
6. Additional precautions and supervision should be exercised in kitchens preparing food for persons at high risk of infection, e.g., infants, the elderly, and the immunosuppressed (Shane, 1994).

## IX. SUMMARY

The U.S. Centers for Disease Control estimate that there are 76 million foodborne cases of diarrhea annually in the United States, which result in 5,000 deaths, >60,000 hospitalizations, and cost an estimated \$5–25 billion in direct treatment costs and worktime loss (Mead et al., 1999). *Campylobacter* are the leading bacterial cause of foodborne illnesses, despite the likelihood that *Campylobacter* infections are greatly underdiagnosed because the organism is microaerophilic (i.e., difficult to culture) and gastrointestinal symptoms may be limited. In addition, a large proportion of GBS cases in U.S. neurology clinics have been associated with a prior *Campylobacter* gastroenteritis (Allos and Blaser, 1995b; Nachamkin et al., 2000). Thus, *Campylobacter* is an important pathogen that causes considerable morbidity and mortality.

Though campylobacters have only been recognized as human gastrointestinal pathogens for the past 30 years, many advances have been made in their diagnosis and disease treatment. Data obtained from clinical infections, experimental infections in humans and animals, and in vitro analyses of adherence and invasion in cultured human cells have now revealed that cell invasiveness is a necessary step in *Campylobacter*-induced inflammatory diarrhea. Much progress has been made over the past decade in characterizing the *C. jejuni*- and host-cell requirements for mucosal invasion (Kopecko et al., 2001). We now have a solid conceptual understanding of how *C. jejuni* 81–176 are internalized into host cells. Despite these advances, many important questions await further study. Are all human-pathogenic strains of *C. jejuni* and *C. coli* invasive, or are some strains toxigenic and adherent? Identifying and cloning the responsible *C. jejuni* invasion determinants will provide appropriate probes to answer this question. The current understanding of the host cell signaling events and structural rearrangements triggered by *C. jejuni* now provides the molecular foundations on which to build a detailed molecular cell–biological model for bacterial internalization, transcytosis, and disease pathogenesis. What are the bacterial ligand(s) and host receptor(s) for *Campylobacter* uptake? Are these receptors present only on certain host cell types? Do all *C. jejuni* secrete effector proteins that trigger host cell signal transduction events? How do these signal transduction events lead to internalization of *C. jejuni*, intracellular bacterial movement, stimulation of

IL-8 production, or eventual host cell death? Do *C. jejuni* encode unique mechanisms for entry and survival in human monocytes that allow for local dissemination of *Campylobacter*? Finally, volunteer and animal model studies involving some defined mutants defective in, for example, mucosal adherence, invasion, or translocation will help determine the relative importance of these different factors in disease pathogenesis. Improved understanding of the molecular mechanisms of *C. jejuni*-host cell interactions will improve our knowledge of disease pathogenesis and aid in the development of new chemotherapeutic, diagnostic, and prophylactic tools. Finally, enhanced efforts to control *Campylobacter* contamination in animals, improved methods to detect and decontaminate food products (e.g., poultry, red meats, milk, and dairy products), and increased awareness of public health measures to reduce the risk of infection will all lead to a healthier environment for humans and animals.

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## *Helicobacter pylori*

**Audun E. Berstad and Per Brandtzaeg**

*University of Oslo, Rikshospitalet, Oslo, Norway*

### I. BACKGROUND

Spiral organisms were described for the first time in the human stomach in 1906 (1). This finding was viewed with skepticism because most samples of spiral organisms were obtained postmortem and contamination could therefore not be ruled out. Physicians and microbiologists believed that the stomach was sterile because of its acidic environment.

In 1975, Steer (2) noted that spiral bacteria were in close proximity to the mucus-secreting cells of the gastric mucosa and that the bacteria possessed at least one flagellum. He emphasized that polymorphonuclear leukocytes migrated through the gastric mucosa, presumably in response to the bacteria. Endoscopic biopsy specimens were cultured but yielded only *Pseudomonas aeruginosa* (3). Some years later, Robin Warren and Barry Marshall at the Royal Perth Hospital, Australia, attempted to culture bacteria from gastric mucosal tissue specimens several times without success. The first culture of *Campylobacter pyloridis* was achieved in April 1982. Normally, the blood or chocolate agar plates were left in a microaerophilic environment for only 2 days, which was the standard technique for culture of *Campylobacter jejuni*. During the Easter weekend they had been left for 6 days and microbial colonies appeared. An article based on clinical observations, histological examination, and culture of antral specimens of 100 consecutive patients subjected to gastroscopy was published in the *Lancet* in 1984 (4). Interestingly, the authors predicted that the bacterium was an important factor in the etiology of chronic antral gastritis and, probably, also peptic ulceration. However, this suggestion was met with almost universal skepticism. The described bacterium was regarded as an opportunistic pathogen attracted by changes in the gastric mucosa; ulcers were believed to be caused by acid, and they would heal if the acid could be suppressed.

The new bacterium was referred to as a “campylobacter-like organism,” and as a result its first official name was *C. pyloridis*. Nevertheless, the organism was considered too different taxonomically from other *Campylobacter* species to be included in the same genus; therefore, the new genus *Helicobacter* was created (5). The name *Helicobacter* refers to the helical morphology of the organisms in vivo. Currently this genus contains about 18 species, most of which are of nonhuman origin (6). *Helicobacter pylori* that causes human disease will be the focus of this chapter.

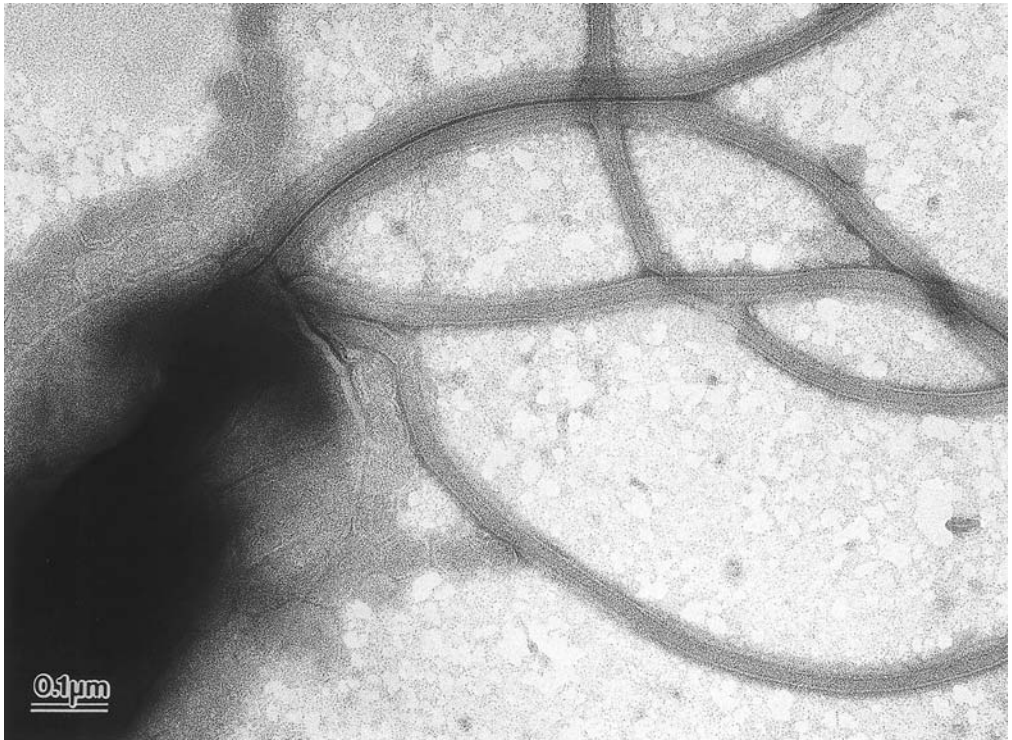
Sufficient data regarding this bacterium as an important human pathogen have gradually accumulated to convince even the most skeptical gastroenterologist. Although *H. pylori* is a relative newcomer to the scientific community, it is already one of the most intensively studied microorganisms. The first complete genome of a *H. pylori* strain was reported in 1997 (7), but it remains uncertain how the infection is transmitted and why the immune response is ineffective in clearing the infection, and to what extent immunological mechanisms explain the associated mucosal pathology.

## II. CHARACTERISTICS

*H. pylori* is a gram-negative rod with a spiral or slightly curved shape and four to six characteristic unipolar (lophotrichate) flagella (Fig. 1). The bacterium has bluntly rounded ends and measures 2.5–4.0  $\mu\text{m}$  in length and 0.5–1.0  $\mu\text{m}$  in width. The flagella measure 2.5  $\mu\text{m}$  in length, with a diameter of  $\sim 30$  nm, and have a distinct terminal bulb. Each flagellum consists of a central filament enveloped by a flagellar sheath. The filament consists mainly of a polymer of a 53 kDa flagellin protein (8). Flagella combined with spiral morphology enable *H. pylori* to efficiently penetrate the gastric mucus layer and to colonize the underlying gastric epithelium (9).

The complete genome of *H. pylori* strain 26695 is a circular chromosome with 1.67 M base pairs and approximately 1600 open reading frames or predicted coding sequences (7). The genome size is only about one-third that of *Escherichia coli*. The fairly low number of genes is consistent with its restricted niche, few regulatory networks, and limited metabolic repertoire and biosynthetic capacity. In contrast to *E. coli* and *Salmonella* spp., there is a high level of interstrain genetic diversity among *H. pylori* isolates (10).

The presence of homopolymeric tracts (polyC or polyG) and dinucleotide repeats (e.g., CT or AG) present in the genome of strain 26695 could provide a mechanism for generating genotypic variation by slipped strand mispairing within such repeats. This might provide one means of en-



**FIGURE 1** Electron micrograph of *H. pylori* in suspended culture subjected to negative staining. Note characteristic flagella to the right. (Courtesy of Prof. T. Hovig, Institute of Pathology, Rikshospitalet, Oslo, Norway.)

abling the bacterium to adapt its key surface structures (such as lipopolysaccharide) for host interactions (7).

### III. DISEASES

#### A. Acute Infection

The clinical and histopathological manifestations of acute *H. pylori* gastritis have been difficult to document. Infection in volunteers ingesting the bacterium produces acute dyspeptic illness that lasts for 1–2 weeks with nausea and abdominal pain (11,12). Gastritis and hypochlorhydria develop within 8 days following the infection. The histopathology is characterized as neutrophilic gastritis (11,13). After a couple of weeks, the infection becomes asymptomatic but histological gastritis persists (14).

#### B. Peptic Ulcer Disease

*H. pylori* plays an important role in the pathogenesis of “idiopathic” peptic ulcer disease. *H. pylori* infection can be diagnosed in 90–100% of duodenal ulcer patients and in 60–100% of gastric ulcer patients (15). The estimated risk of developing peptic ulcer disease during long-term follow-up of *H. pylori*-infected subjects varies between 10 and 20% (15). However, it remains unclear how or why ulcers develop, although variability among bacterial strain virulence and host defense, as well as environmental and dietary factors, may influence the pathogenesis. Eradication of *H. pylori* results in a marked decrease in the recurrence rates of duodenal and gastric ulceration (16,17) and improved quality of life of the patients (18). Excluding those taking aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs), recurrence of these diseases is completely prevented for at least 9 years after successful *H. pylori* eradication (19).

Both in apparently healthy subjects and patients with duodenal ulcer, *H. pylori* infection results in modest elevation of serum gastrin concentrations in the fasting state and quite substantial elevation after a meal or gastrin-releasing peptide stimulation. Acid secretion in response to a gastrin-releasing peptide is threefold higher in *H. pylori*-infected healthy subjects and sixfold higher in duodenal ulcer patients than in noninfected controls (20). Antral levels of somatostatin, a potent inhibitor of gastrin secretion, are decreased in *H. pylori*-infected subjects, which probably contributes to the gastrin increase (21). The elevated acid response to gastrin stimulation by the oxyntic mucosa in duodenal ulcer patients is due to the combination of a large parietal cell mass and the fact that these cells are not functionally impaired as long as the gastritis is virtually confined to the antral mucosa. The increased acid secretion results in an increased duodenal acid load with subsequent development of gastric metaplasia within the duodenal bulb, allowing *H. pylori* colonization there (22). The resulting duodenitis weakens the mucosa and predisposes to duodenal ulceration. The extent of gastric metaplasia in the duodenum is reduced after *H. pylori* eradication but not after duodenal ulcer healing alone (23).

A physiological decrease in gastric mucosal surface hydrophobicity with aging may contribute to the risk of ulcer development in the elderly population and may act synergistically with *H. pylori* and/or NSAIDs on gastric mucosal defense (24). The enzyme phospholipase A<sub>2</sub>, which is synthesized by both *H. pylori* and inflammatory cells such as neutrophils, may damage the phospholipid-rich zone of the gastric mucus, which has a protective barrier function. Phospholipase A<sub>2</sub> activity in gastric juice is substantially higher in duodenal ulcer patients than in those with healed ulcers after *H. pylori* eradication (25). *H. pylori* phospholipase A<sub>2</sub> activity is highest in the pH range of 6.5–7.0 (26). This observation suggests that the enzyme activity in gastric juice is low in the stomach of patients with normal or high acid production, but increases exponentially when the enzyme meets the neutral environment of the duodenal bulb.

Active gastritis of the body mucosa with glandular atrophy is frequently detected in gastric



ulcer patients. Intestinal metaplasia, regarded as a precursor lesion for gastric adenocarcinoma, is more common in the antrum of these individuals than in duodenal ulcer patients (27).

### C. Atrophic Gastritis and Gastric Carcinoma

Persistent gastric inflammation can lead to loss of the normal mucosal architecture with gradual disappearance of glands accompanied by increased interglandular connective tissue and reduced mucosal thickness. Chronic *H. pylori* gastritis predisposes significantly to the development of gastric atrophy and intestinal metaplasia (28,29). However, the natural progression of *H. pylori* atrophic gastritis appears to be slow, showing only mild or no progression, and sometimes even regression, over a 12-year follow-up (30). Thirty to 40 years of continuous inflammation may be necessary before moderate or severe atrophic gastritis develops.

Importantly, atrophic body gastritis or multifocal atrophic gastritis has been reported to be associated with increased incidence of gastric adenocarcinoma (31,32). Multiple studies have demonstrated that *H. pylori* infection is more common among patients with such malignancies than among matched noncancer patients (33–36). These studies are so compelling that the World Health Organization has classified *H. pylori* as a Group I (definite) human carcinogen (36a). The fact that long-term infection with *H. pylori* induces adenocarcinoma in Mongolian gerbils strongly supports an involvement of *H. pylori* infection in human gastric carcinogenesis (37). In a prospective Japanese study, gastric cancer developed in approximately 3% of *H. pylori* infected patients, but not at all in age-matched uninfected control patients, during a mean follow-up period of almost 8 years (38). Among the infected patients, those with severe atrophy accompanying intestinal metaplasia, corpus-predominant gastritis, or both were at particularly high risk. While *H. pylori* infection increases the risk of adenocarcinomas outside the cardia, it decreases the risk of this type of cancer within the cardia (36a). Remarkably, infection with *H. pylori* strains positive for the cytotoxin-associated gene (*cag*) A (see below) is inversely related to the occurrence of adenocarcinoma in both the cardia and esophagus (39).

Epidemiological and experimental data suggest that *H. pylori* does not cause gastric adenocarcinoma directly but, instead, predisposes to its development in a stepwise series of events from gastritis, atrophic gastritis, intestinal metaplasia, and epithelial dysplasia. When chronic atrophic gastritis becomes severe and extensive, the resulting hypochlorhydria favors bacterial overgrowth with the appearance of nitrites and *N*-nitroso compounds in the gastric lumen. Such compounds may induce epithelial dysplasia because of their mutagenic and carcinogenic properties (40). Interestingly, duodenal ulcer disease protects against, whereas gastric ulcer increases the risk of, subsequent gastric carcinoma (38,41). This disparity suggests that normal or high acid output associated with duodenal ulcer disease represents a defense factor against a malignant development and/or that the low acid output associated with gastric ulcer favors oncogenesis.

Environmental factors appear to affect the outcome of long-term *H. pylori* infection. Concurrent enteric helminth infestation attenuates *Helicobacter*-associated gastric atrophy in mice (42), which suggests that a concurrent parasitic burden may alter the immune response to *H. pylori* in humans. High salt intake increases the access of carcinogens to proliferating epithelial stem cells in rat stomachs (42a) and may likewise increase the risk of gastric carcinoma in humans (43). Tobacco smoking and *H. pylori* are independent risk factors for the development of gastric carcinoma, and there is a significant dose effect with increasing tobacco consumption (44).

Release of reactive oxygen metabolites from leukocytes in inflamed gastric mucosa may also lead to DNA damage and therefore represents a putative important mechanism by which inflammation promotes gastric oncogenesis (45). Ascorbic acid (vitamin C) scavenges free oxygen radicals, and high dietary intake of this vitamin apparently protects against gastric carcinoma (46).

### D. Gastric Lymphoma

Patients with *H. pylori* infection show increased risk of development of low-grade malignant gastric B-cell neoplasia, so-called gastric mucosa-associated lymphoid tissue (MALT) lymphoma (47,48).

The infection causes induction of reactive lymphoid follicles (49), which represent precursor lesions of gastric lymphomas. This progressive B-cell response to the bacteria depends on growth factors secreted by *H. pylori*-specific T cells (50). Such an indirect antigen-driven nature of primary gastric MALT lymphomas is supported by the observation that at an early stage they can completely regress after *H. pylori* eradication (51).

#### IV. EPIDEMIOLOGY

Persistent gastric *H. pylori* colonization is found in at least one third of the world's population, and it is thus one of the most common human bacterial infections. In developing countries, 70–90% of the population carries *H. pylori*. In westernized countries the prevalence of infection is considerably lower, especially in early childhood, but the infection rate increases with age (52). Because acquisition of new infections among adults in highly industrialized countries is quite low (<1% per year) (53,54), *H. pylori* apparently colonizes mainly throughout childhood and early adulthood.

The mechanisms by which *H. pylori* is transmitted are not well understood. Intrafamilial clustering of *H. pylori* infection (55), the lack of a well-defined nonhuman reservoir, and strong linkage to conditions associated with residential crowding during childhood (56) suggest that most transmissions take place between individuals. People from industrialized countries who live in a developing country for a short time appear to be at particularly high risk of infection (57). Positive associations between *H. pylori* seropositivity, birth order, and the number of children in Taiwanese families indicate that early childhood spread among siblings is important (58). Both oral-oral and fecal-oral transmissions are potential routes of spread. *H. pylori* has been isolated from the feces of 9 of 23 randomly selected African children (59) but has not as yet been commonly cultured from the feces of persons in westernized countries. Iatrogenic transmission of *H. pylori* has been reported to occur via fiberoptic gastroduodenoscopy and suggests that instruments may be contaminated with gastric secretions (60).

*H. pylori* infection was reported to be independent of sex but highly correlated with socioeconomic status in Peruvian children; prevalence of infection was higher among children from low-income families than among those from high-income families (61). Furthermore, municipal water supply has been claimed to be an important source of infection among children from families of both low and high socioeconomic status (61). *H. pylori* DNA has been detected in samples of drinking water collected from high-risk areas in Peru (62), which provides additional evidence for waterborne transmission of *H. pylori* in some environments.

Gastric carcinoma is the second most common fatal malignancy in the world and is the cause of more than 750,000 deaths annually (63). Prospective serological studies consistently show that there is roughly a threefold increased risk of gastric carcinoma in *H. pylori*-positive subjects, which can be translated into a worldwide burden of at least 300,000 new cancer cases annually attributable to this infection (64). Because large variation exists in cancer risk among different populations, it is likely that genetic and/or environmental cofactors are important in modifying the consequences of *H. pylori* infection (64). The importance of genetic host factors is suggested by the observation that interleukin (IL)-1 gene cluster polymorphism, suspected to enhance production of IL-1 $\beta$ , is associated with an increased risk of both hypochlorhydria induced by *H. pylori* and gastric cancer (64a). Indeed, IL-1 $\beta$  is highly expressed in gastric mucosa of *H. pylori*-positive subjects (64b) and this cytokine is an important pro-inflammatory factor and a potent inhibitor of gastric acid secretion (64c).

#### V. ISOLATION AND IDENTIFICATION

Isolation is the only method of diagnosis that allows definitive bacterial identification and definition of antibiotic sensitivity. In clinical practice, *H. pylori* is usually cultured from endoscopic gastric mucosal biopsies, which should be transported to the laboratory in isotonic saline to prevent drying and contact with atmospheric oxygen toxic to the bacterium. The bacterial recovery rate can be

improved by transporting the specimens in Stuart's transport medium (65) or in commercially available *H. pylori*-specific transport media (65). *H. pylori* can survive for 2 days or longer at 10°C in saline but becomes nonculturable within 6 hours or less at a temperature above 15°C (65). Therefore, the samples should be processed within 2 hours of receipt or stored at 4°C for subsequent processing as soon as possible.

*H. pylori* is microaerophilic and grows well in an atmosphere of 5–10% O<sub>2</sub> and 5–10% CO<sub>2</sub> on blood-containing media such as Oxoid agar with 5% horse, sheep, or human blood. *H. pylori* grows optimally on plates at 37°C after 3–7 days, but longer incubation times (up to 12 days) may be necessary (66). All strains grow over a relatively narrow temperature range (33–40°C) and, depending on the culture medium, over a wide pH range with good growth at pH 6.9–8.0. Colonies are circular (1–2 mm), convex, and translucent in appearance (Fig. 2) and are surrounded by some hemolysis in blood agar. Identification of *H. pylori* is based on the presence of typical colonies, microscopic features (Gram stain), and positive tests for urease, catalase, and oxidase.

Two categories of diagnostic methods can be distinguished: invasive tests detecting the microorganism in biopsy samples of the gastric mucosa obtained at endoscopy and so-called noninvasive tests performed without the need for endoscopy. The specificity and sensitivity and advantages and disadvantages of such methods have been extensively reviewed elsewhere (67). Briefly, fresh endoscopic biopsy specimens from the gastric antrum and body can be tested for the presence of *H. pylori* urease in commercially available tests (68) or in noncommercial locally made rapid urease tests. Moreover, the invasive tests for *H. pylori* infection include culture, histology, or molecular methods such as detection of *H. pylori* DNA. The noninvasive methods include breath tests based on <sup>13</sup>C or <sup>14</sup>C urea detection. For the <sup>14</sup>C urea breath tests, urea enriched with <sup>14</sup>C is hydrolyzed in the stomach by *H. pylori* urease to produce ammonia and carbon dioxide (<sup>14</sup>CO<sub>2</sub>). The gas is absorbed at the intestinal level into the blood and is eliminated by the lungs in the exhaled air. Breath samples



**FIGURE 2** Morphology of *H. pylori* colonies (laboratory strain) on blood agar-based medium. (Courtesy of Y. Esbensen, Institute of Microbiology, Rikshospitalet, Oslo, Norway.)

are collected before and after the administration of  $^{14}\text{C}$  urea; the proportion of exhaled  $^{14}\text{CO}_2$  is then calculated. Acid suppressive therapy may produce false-negative results; ideally, the test should be performed at least 2 weeks after the termination of such treatment.  $^{13}\text{CO}_2$  breath tests are increasingly used for the diagnosis of *H. pylori* infection (69) and do not expose the patients to radioactive isotopes.

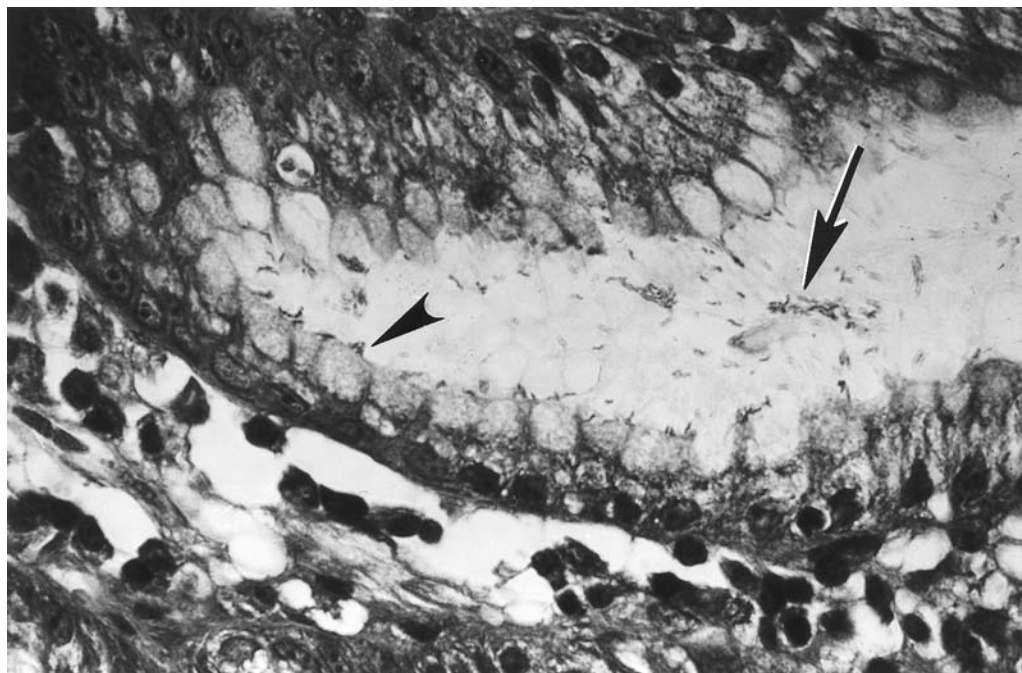
Noninvasive tests further include antibody determinations such as immunoblot analysis or the measurement of specific serum IgG and IgA antibodies with enzyme immunoassay, which can be performed with commercial kits (70). A decrease in IgG antibody titer of 40% or more 3–6 months after therapy can be highly accurate in confirming *H. pylori* eradication (71). Recently, antigen stool assays have been introduced and high sensitivity and specificity have been reported (72). Such assays should be evaluated in studies of *H. pylori* eradication as well as for diagnosis in children, two situations in which stool tests should be of major interest.

## VI. PATHOGENICITY

### A. Microbial Effects

*H. pylori* produces large amounts of the enzyme urease (73), which digests plasma-derived urea into ammonium and  $\text{CO}_2$ . By generating a cloud of ammonium around itself, the bacterium creates a near neutral and protective local microenvironment. Urease activity is essential for the establishment of a chronic *H. pylori* infection in piglets (74).

Histological studies suggest that most *H. pylori* organisms are free-living in the gastric mucus layer and that less than 25% adhere to the epithelium (Fig. 3) (75). Epithelial adhesion sites are



**FIGURE 3** Giemsa-stained section of formalin-fixed gastric mucosal specimen from the antrum of an *H. pylori*-infected patient with duodenal ulcer. Many rod-shaped bacteria are located within the mucus in a gastric pit (arrow) or adhere to the mucus-producing surface epithelial cells (arrowhead). (Magnification:  $\times 1000$ )

formed by depressions in the plasma membrane constituting “indentation” sites or sometimes by a raised plasma membrane constituting a plateau-like extrusion or “adhesion pedestal” (75). Heavily colonized gastric epithelium can be seen adjacent to a segment of intestinal metaplasia where no bacteria are present (76). This demonstrates that *H. pylori* exhibits tropism for gastric epithelial cells in vivo. The development of intestinal metaplasia might be regarded as an adaptive mechanism by which the stomach eliminates *H. pylori*.

*H. pylori* interacts with Lewis<sup>b</sup> blood group substances in the gastric mucus layer and on the epithelium. The Lewis<sup>b</sup>-binding adhesin, BabA, is associated with the presence of the *cagA* pathogenicity island (see below) among clinical isolates of *H. pylori* (77). Bacterial adhesins belong to the large family of outer membrane proteins. Many genes in the *H. pylori* genome encode putative outer membrane proteins (7), so the potential exists for recombination events leading to mosaic expression patterns. Such antigenic variation may represent an effective bacterial mechanism for host defense evasion.

Lipopolysaccharide (LPS), or endotoxin, is essential for the physical integrity and function of the bacterial outer membrane. *H. pylori* LPS generally has low immunological activity compared to its counterpart of other gram-negative bacteria. As an example, *H. pylori* LPS induces significantly lower secretion of cytokines by human mononuclear cells than *E. coli* LPS (78). Lipid A of *H. pylori* LPS contains unusual fatty acids and is underphosphorylated compared to that of other gram-negative bacteria, which may explain its low biological activity and aid persistence of colonization (79).

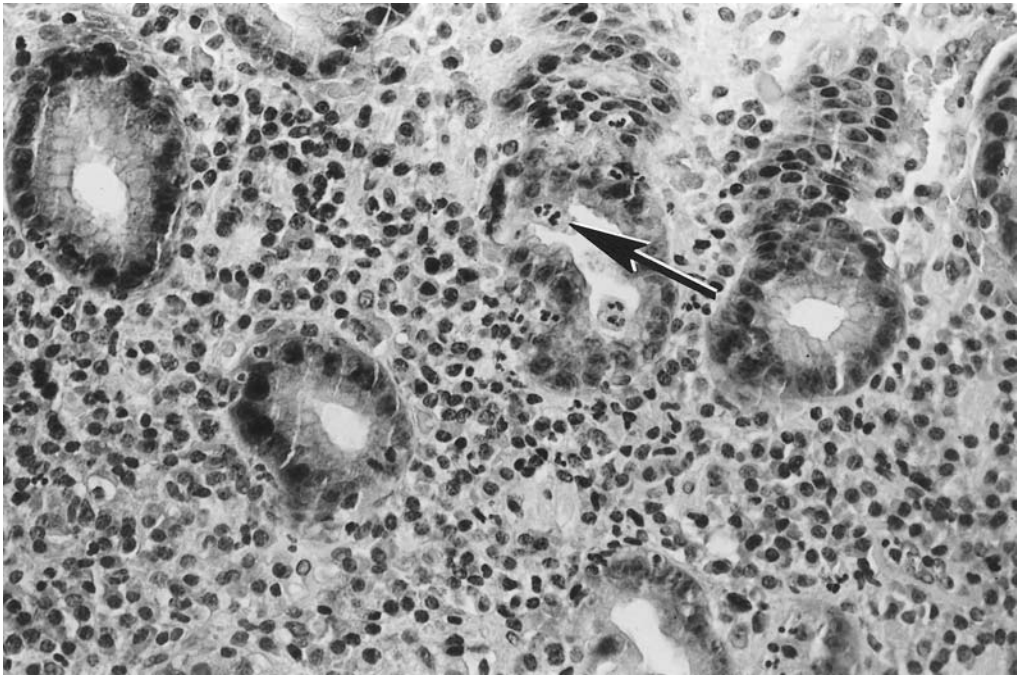
## B. Host Response

The normal gastric lamina propria contains only a few leukocytes, although plasma cells are quite prevalent in antral mucosa (80). In acute *H. pylori* infection, there is depletion of surface mucus, extensive epithelial erosions, and abundant neutrophilic infiltration (11,13). In chronic active gastritis, the lamina propria contains a mixed population of granulocytes (mainly neutrophils) and mononuclear inflammatory cells (Fig. 4). Neutrophils can also infiltrate the foveolar epithelium, often within the isthmus region where the epithelial stem cells are located (81). *H. pylori* may stimulate migration and activity of neutrophils directly or indirectly. Exposure of neutrophils to whole organisms or bacterial products results in chemotaxis (82), generation of reactive oxygen metabolites (83), and release of myeloperoxidase (84). Host factors such as chemotactic cytokines, particularly (IL)-8, bioactive lipids (e.g., leukotrienes and prostaglandins), and complement anaphylatoxins (C3a, C4a, and C5a) may also cause neutrophil accumulation.

Gastric epithelial cells, located at the interface between the microbes and interior of the host, are instrumental in neutrophil recruitment and infiltration of the gastric epithelium in *H. pylori* gastritis (85). Thus, epithelial IL-8 immunoreactivity and secretion in cultured antral biopsy specimens are increased in *H. pylori* infected compared with normal control mucosa (86,87). Gastric epithelial cell lines produce IL-8 after *H. pylori* adherence in vitro, but live bacteria are necessary to elicit this response (88,89). Also, IL-8 can be secreted at least in small amounts by epithelial cells after stimulation with high concentrations of *H. pylori* LPS (89).

Complement is deposited in inflamed gastric mucosa, but *H. pylori* may evade complement-mediated attack in the foveolae where activated complement is not detected (90,90a). In fact, bacteria in this location may be protected both by non-complement activating IgA and by the complement regulator protectin (CD59) that appears to be taken up from adjacent epithelial cells (90a) by which several complement-inhibitory proteins are expressed (90b). However, subepithelial or luminal deposition of immune complexes near the site of mucosal antigen entry may induce an Arthus reaction in which lytic enzymes released by accumulated neutrophils, together with the complement membrane-attack complex, cause localized tissue damage.

Little is known about the local development of specific immunity to *H. pylori*. Moreover, the mechanism of gastric uptake of *H. pylori* antigens in the absence of local organized MALT with



**FIGURE 4** Hematoxylin and eosin–stained section of formalin-fixed gastric mucosal specimen from the body region of an *H. pylori*–infected individual with chronic gastritis. In the isthmus regions many granulocytes (mainly neutrophils, arrow) are seen in the lamina propria, and often migrating into the gastric pits between the epithelial cells. (Magnification:  $\times 400$ )

M cells is unclear. Primed T and B cells that exit from ileal Peyer's patches seed the intestinal lamina propria as well as more remote mucosal effector sites (91). Therefore, antigen uptake of *H. pylori* products and/or whole bacteria by M cells in Peyer's patches may result in homing of primed cognate B cells to the gastric lamina propria, but so far there is no direct evidence for such a migratory route to explain immune responses against *H. pylori* in the stomach.

Disruption of epithelial tight junctions and microerosions of the gastric epithelium induced by *H. pylori* toxins and infiltrating neutrophils enhance the penetration of bacterial products into the lamina propria. Urease and surface proteins of *H. pylori* have been demonstrated immunohistologically deep in inflamed gastric mucosa (82). Such bacterial products can be endocytosed by resident or newly recruited macrophages. Mucosal macrophages located in close proximity to the gastric epithelium (92) supposedly have the ability to act as antigen-presenting cells (APCs) to induce T- and cognate B-cell responses against the bacterium. The development of gastric lymphoid follicles associated with *H. pylori* infection (82) suggests that such immune responses to *H. pylori* are elicited locally. B-cell proliferation in these follicles most likely contributes to the increased number of plasma cells in chronic gastritis (80).

*H. pylori*–specific IgA and IgG antibodies can be detected both in peripheral blood (93–95) and at the gastric mucosal level (94–97) in infected patients. A substantial increase of Ig-producing cells occur in chronic gastritis, particularly of the IgA1 isotype, but *H. pylori* does not appear to possess IgA1-specific nor nonspecific IgA-degrading protease activity (98). Despite the induction of local and systemic humoral and cellular immunity, immune elimination of *H. pylori* from the stomach is inefficient; the infection becomes chronic and persists for years and probably for life in most patients, and they appear to be unprotected against subsequent reinfection (99).

## VII. GENETIC FACTORS INVOLVED WITH VIRULENCE

Histological studies suggest that *H. pylori* infection causes damage to gastric epithelial cells (100). To some extent this damage may result directly from the biological activities of several *H. pylori* products, including ammonia liberated by *H. pylori* urease, vacuolating cytotoxin A (VacA) activity (101), and phospholipases (26).

VacA produces acidic vacuoles in a wide variety of cultured cell lines (102). Prominent vacuolation similar to that observed in cell culture systems is not usually seen in gastric epithelium in situ, suggesting the existence of an in vivo inhibitory effect that perhaps is exerted by the secretory immune system. All *H. pylori* strains possess *vacA*, the gene encoding the actual cytotoxin, yet this toxin is produced by only about 60% of the strains. The *vacA* alleles differ among strains, most markedly in the region encoding the signal sequence (which in the United States may be type s1a, s1b, or s2) and the midregion sequence (type m1 or m2). The *vacA* s1/m1 strains produce higher levels of VacA activity in vitro than the s1/m2 strains, and the s2/m2 strains do not produce detectable activity (103). The signal sequence s1 is closely associated with the presence of *cagA* despite different localization in the genome (104). The *vacA* s1a strains are associated with increased gastric inflammation and duodenal ulceration, whereas the *vacA* s2 strains are associated with less inflammation and lower ulcer prevalence (103). However, Go et al. (105) could not confirm any association between the *vacA* genotype and duodenal ulcer disease.

The *cagA* gene is located at the end of a large (20–40 kb) DNA segment present in all *cagA*<sup>+</sup> strains but absent in *cagA*<sup>-</sup> strains. Altogether, this DNA segment contains over 40 separate genes and has been termed the *cag* pathogenicity island (PAI). A type IV secretion system translocates the *cagA* gene product, CagA, into gastric epithelial cells (106). Within the host cells, CagA undergoes tyrosine phosphorylation by a kinase, and the new phosphoprotein alters the physiology of the affected cells (106). Strains that are *cagA*<sup>+</sup> (approximately 60% in Europe and the United States) elicit more intense proinflammatory cytokine responses in gastric mucosa than do *cagA*<sup>-</sup> strains (107). Serum antibodies to CagA are significantly more prevalent in patients with peptic ulceration (85–100%) than in those with gastritis only (50–60%) (52). Serum antibodies to CagA are also associated with increased risk of gastric carcinoma (52), which may reflect that infection with *cagA*<sup>+</sup> strains represents an increased risk of developing atrophic gastritis and intestinal metaplasia (108). *H. pylori* strains have by definition been divided into type I (virulent), which express CagA and VacA, and type II (nonvirulent), which express neither CagA nor VacA. Although of considerable interest to researchers in this field, determination of the presence of CagA and VacA in *H. pylori* isolates (and/or corresponding serum antibodies) has limited clinical value because most patients infected with Type I strains never develop ulcer disease or cancer of the stomach. Because patients may be infected with more than one strain (109), it is likely that coexistence of type I and II infection may occur.

*CagA*<sup>+</sup> strains induce significantly higher levels of IL-8 in gastric epithelial cell lines than *cagA*<sup>-</sup> strains (88). However, isogenic *cagA*<sup>-</sup> mutants of *cagA*<sup>+</sup> strains retain full activity in these assays, suggesting that other PAI genes are necessary for IL-8 secretion. Indeed, the products of two genes denoted *picA* and *picB*, located upstream of *cagA* on PAI, are necessary for the secretion of IL-8 by epithelial cell lines (110).

## VIII. CONTROL MEASURES

Because it is not well known how *H. pylori* is disseminated, control measures are difficult to implement. Based on epidemiological studies cited above, improvement of socioeconomic standards of living, such as less crowded housing in childhood and uncontaminated water supplies, may reduce the risk of infection. Consumption of untreated water is significantly associated with *H. pylori* infection in subjects under 40 years of age (111). The importance of bacterial transmission through food products remains unknown. Whether long-term breast-feeding reduces the risk of infection in

childhood is also unclear. Nevertheless, sucklings appear to be temporarily protected by *H. pylori*-specific IgA present in breast milk (112). Supplementing breast-feeding in the first months of life with unclean water is not advisable because this practice may increase the risk of *H. pylori* exposure (113).

The U.S. National Institutes of Health consensus conference recommended antimicrobial treatment of all ulcer patients infected with *H. pylori* (114). The European *Helicobacter pylori* Study Group consensus guidelines from 1997 went even further (115). They stated that:

1. Dyspeptic patients less than 45 years old and with no alarm symptoms should be subjected to *H. pylori* diagnosis at the primary care level by noninvasive means ( $^{13}\text{C}$  urea breath test or serology).
2. *H. pylori*-positive patients should be treated with antimicrobial therapy.
3. Indications for eradication of *H. pylori* at the specialist level should be broadened to include *H. pylori*-positive patients with (a) functional dyspepsia in whom no other possible causes of symptoms can be identified (after full investigation including endoscopy, ultrasound, and other necessary investigations), (b) patients with low-grade MALT lymphoma (managed in specialized centers), and (c) patients with gastritis showing severe macro- or microscopic abnormalities.

The original antimicrobial triple therapy designed by Marshall and coworkers to eradicate *H. pylori* from the stomach of infected individuals consisted of bismuth salts, metronidazole, and either ampicillin or tetracycline (116). In highly motivated patients this therapy was reported to be about 90% effective (117), but side effects and low compliance are problematic. At present, recommended eradication treatments are proton pump inhibitor-based triple therapy for 7 days, including two of the following antimicrobial agents: clarithromycin, a nitroimidazole (metronidazole or tinidazole), and amoxicillin. Such therapies are well tolerated and produced greater than 90% eradication rates in some studies (117). Nevertheless, treatment failures are still reported, and the negative factors implicated include poor compliance, inadequate drug delivery, and antimicrobial resistance (118).

The development of a safe and effective vaccine for the prevention of *H. pylori* infection should lower the incidence of both peptic ulcer disease and gastric cancer and may lessen the emergence of drug-resistant *H. pylori* strains. Vaccine development in laboratory animals has been promising. Oral immunization with *H. pylori* urease protects mice against *H. felis* infection (119). Also, an *H. pylori* mouse model has been developed, and oral immunization with purified *H. pylori* antigens protects mice against bacterial infection (120). Interestingly, chronic *H. pylori* infection in mice can be successfully eradicated by intragastric vaccination with *H. pylori* antigens such as recombinant VacA or CagA, administered together with a genetically detoxified mutant of the heat-labile exotoxin of *E. coli* (121). Moreover, such therapeutic vaccination confers efficacious protection against reinfection (121). Regrettably, no vaccine is as yet available for use in humans. However, Kreiss et al. (122) showed that recombinant *H. pylori* urease was safe when given orally without adjuvant in *H. pylori*-infected volunteers. As expected, repeated doses of this antigen failed to induce a mucosal or systemic immune response against urease and did not modify bacterial colonization at the gastric surface (122). Nevertheless, this study represents an important step in the development of a clinical vaccine against *H. pylori*.

The availability of the full-length DNA sequences of two *H. pylori* strains (7,123) offers unprecedented opportunities for vaccine design as the complete inventory of genes encoding virulence factors and potential immunogens are available for selection as potential vaccine candidates.

## IX. SUMMARY

In developing countries, 70–90% of the population carries detectable *H. pylori*. In western countries, the prevalence of this infection is considerably lower, especially in early childhood, although the



infection rate increases with age. Persistent gastric *H. pylori* colonization is thus one of the most common human bacterial infections. The bacteria are mainly free-living in the gastric mucus layer whereas a proportion adheres to the surface epithelium. *H. pylori* causes persistent inflammation in the gastric mucosa, which may lead to loss of the normal mucosal architecture with gradual disappearance of glands (gastric atrophy), a process that however usually takes many years, even decades, to develop. The bacterium plays an important role in the pathogenesis of peptic ulcer disease, and long-standing infection increases the risk of gastric adenocarcinoma outside the cardia, which is among the most common fatal malignancies in the world. Long-standing infection is also linked to the development of gastric MALT lymphoma. Despite the fact that *H. pylori* is a relative newcomer to the scientific community, it has become one of the most intensively studied organisms. Nevertheless, the mechanisms by which the bacterium is transmitted remains poorly understood. Both oral-oral and fecal-oral spread are potential routes of transmission. Also, it still remains uncertain why immunological elimination of *H. pylori* from the stomach is inefficient, despite the induction of local as well as systemic humoral and cellular immunity, and activation of various immune mechanisms by this bacterium.

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# 14

## *Vibrio cholerae*

G. H. Rabbani, David A. Sack, and M. R. Choudhury

*International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh*

### I. BACKGROUND

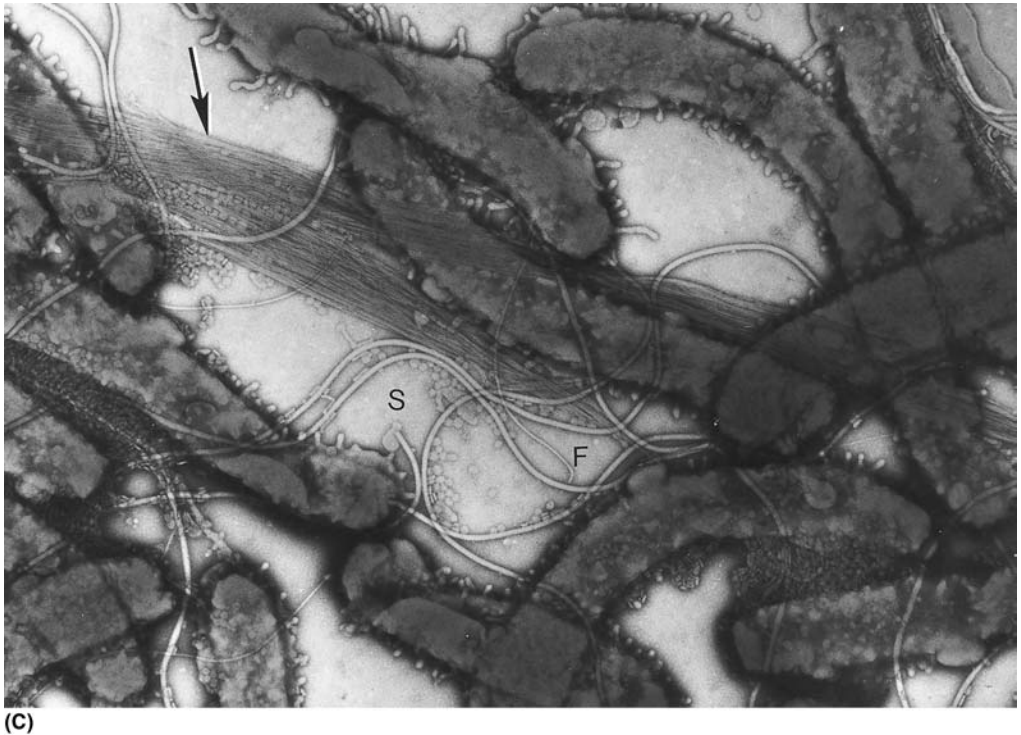
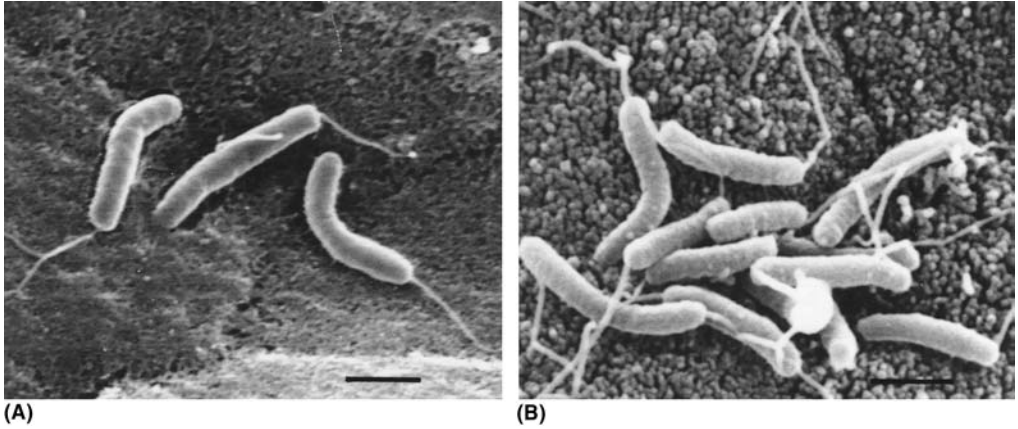
Cholera is a disease of great antiquity. The term “cholera,” first mentioned by Hippocrates, signifies the Greek words, *chole* (bile) and *rein* (flow), thus meaning flow of bile (1–3). Some authorities claim that the word came from *cholades*, meaning the intestine, or the Greek word *cholera*, which means gutter or roof (4). In the Vedic *Sushruta Samhita*, which originated about 500 B.C., the disease cholera was believed to be denoted by the Sanskrit word *visuchika* (5). The writings of modern historians indicate that, for more than 2000 years, cholera must have been a very familiar disease worldwide, because a name for it exists in most cultures, signifying a broad spectrum of the intestinal diseases (1,3).

Since 1817 there have been seven pandemics of cholera. The first six originated from the delta regions of eastern India, particularly Bengal, and swept all over the world, claiming a heavy toll in human lives. The seventh pandemic started in an Indonesian island in early 1960s and has spread to all continents. Although a contagious cause of cholera had been suspected by many authorities, including John Snow as early as 1859, it was Robert Koch who studied cholera in Egypt and India and first isolated the comma-shaped organisms from the victims of cholera in 1883; the bacterium was called *Kommabazillen* (2,6). Since then advances in cholera research have significantly contributed to the understanding of the organism and the disease caused by it.

### II. CHARACTERISTICS

The taxonomy of the family Vibrionaceae, which include *Vibrio cholerae*, is in transition. The current classification has been reviewed by West and Colwell (7). The members of the genus *Vibrio* are halophilic and natural inhabitants of the estuarine and marine environment. The genus *Vibrio* contains several bacterial species, of which *V. cholerae* and *V. parahaemolyticus* are the two most important pathogens of humans. *V. cholerae* is a gram-negative, monoflagellate, short, straight, or curved rod ( $0.5 \times 1.5\text{--}3.0 \mu\text{m}$ ) (Fig. 1). *V. cholerae* is classified on the basis of its somatic antigens (O-antigens) into 60–70 serovars (serotypes) (8). The serovar O:1 includes all the strains responsible for the epidemic and endemic cholera and has two subtypes, Ogawa and Inaba; a Hikojima subtype has been reported rarely. All these subtypes can be further distinguished into two biovars (biotypes), Classical and El Tor. El Tor differs from the Classical biovar in several aspects. El Tor is resistant to Mukherjee’s group IV phage and to polymixin B, it causes agglutination of chicken erythrocytes (9), it produces a hemolysin, and it is Voges-Proskauer positive (10). More than 30 species have been recognized as belonging to the genus *Vibrio*, although many of them differ in DNA characteristics (11,12). All species except one are oxidase positive, and with few exceptions they ferment glucose but no gas. All are chemoorganotropic and require sodium for their growth in vitro.





**FIGURE 1** *Vibrio cholerae*, serotype Ogawa, biotype El Tor colonizing villous epithelium of rabbit small intestine. Note polar flagellum (A) and tips of microvilli (B). Bar, 1  $\mu$ M. Magnification,  $\times 12,000$  (A) and  $\times 13,000$  (B). (Courtesy of Dr. Jacob S. Teppema.) (C) Electron photomicrograph of *V. cholerae* cell preparation negatively stained with 1% phosphotungstic acid, pH 6.8. Note the bundle of filamentous structures, tcpA fimbriae (arrow). Also note the appearance of sheathed (S) and unsheathed (F) flagellae. Magnification,  $\times 25,800$ . (Courtesy of Dr. Ben Tall, Center for Vaccine Development, University of Maryland.)

### III. DISEASES

Cholera, the most severe form of all secretory diarrheas, is caused by infection of the human small intestine by *Vibrio cholerae* of serotypes O1 and O139. The illness is clinically characterized by vomiting, massive rice-watery stool, dehydration, metabolic acidosis, and sometimes death.

#### A. Clinical Manifestations

The clinical manifestations of cholera range from asymptomatic infection to severe diarrhea. The subject has been reviewed by Rabbani and Greenough (13). Many cases are mild and cannot be clinically distinguished from other milder forms of gastroenteritis; such patients rarely seek therapy. In a typical adult case of severe cholera, the diarrhea starts abruptly; the first few motions normally contain fecal materials, but the stool rapidly becomes clear and liquid, containing flecks of mucus but no trace of blood (rice-watery stool). Vomiting of clear fluid may accompany the initial states of diarrhea and contributes to dehydration. However, vomiting rarely persists after adequate rehydration.

Most fluid loss occurs during the first 24–48 hours of the illness. The magnitude of body fluid loss can be assessed clinically by diminished skin turgor, sunken eyeballs, depressed fontanelle (in infants), dry mucous membranes, cold extremities, thready pulse, and orthostatic hypotension (Fig. 2). In severe cases, when adequate fluid replacement is given, the total weight of the diarrheal stool may exceed the body weight of the patient. Diarrhea tends to decline after 24 hours and usually terminates within 1–6 days without leaving significant residual effects if adequate fluid replacement has been provided. Clinical complications are infrequently seen in well-hydrated cholera patients.



**FIGURE 2** Adult male with signs of severe dehydration due to diarrhea caused by *Vibrio cholerae*. Note loss of abdominal skin turgor and wrinkling of fingers (washerwoman's hand) of the patient.

However, without adequate rehydration, fatal complications may arise, including (a) hypovolemic shock (with muscle cramps and anuria) caused by isotonic fluid loss, (b) metabolic acidosis due to loss of bicarbonate ions in the stool and poor perfusion of the tissues, and (c) hypokalemia caused by loss of potassium ions. In rare instances pulmonary edema occurs when severely acidotic patients are treated with normal saline containing no base. Paralytic ileus may occur as a consequence of hypokalemia. In the late stages of pregnancy, cholera carries a risk of fetal death, which may be related to acidosis and hypoxia.

The rapid loss of isotonic fluid in the stool leads to hemoconcentration and increased plasma specific gravity. These are often used as indicators of severity of dehydration. Serum potassium deficits range between 6 and 7 mmol/kg in untreated cases. The serum sodium concentration and total serum osmolality are usually normal, but a moderate elevation of serum chloride is caused by a base deficit, which returns to normal after bicarbonate replacement. The watery stool in cholera contains about 50 mmol of bicarbonate per liter (14), the loss of which contributes to the profound metabolic acidosis in severely dehydrated patients. In untreated cases the serum bicarbonate values are frequently less than 10 mmol/L, and the pH of the arterial blood may be as low as 7.0 (15). However, clinical studies in severely dehydrated adult cholera patients indicate that the acidosis is associated with increased serum anion gap caused by hyperproteinemia, lactic acidemia, and hyperphosphatemia (16). The acidosis is more profound than would be expected on the basis of stool losses of bicarbonate because of superimposed lactic acidemia and renal failure.

## **B. Infection Due to Non-O1 *Vibrio cholerae***

Strains of vibrios, which morphologically resemble *V. cholerae* but do not agglutinate with *V. cholerae* O1 antiserum, have been previously termed as nonagglutinating (NAG) or noncholera vibrios (NCV); they are also indistinguishable biochemically from O1 *V. cholerae*. Some strains of non-O1 *V. cholerae* are known to produce enterotoxin that is structurally and biologically similar to *V. cholerae* O1 enterotoxin; these strains (toxin producers) are also known to cause diarrheal illness in humans (17). In contrast, some non-O1 strains of *V. cholerae* that do not produce CT have been shown to cause cholera-like diarrhea in volunteers (18). However, most non-O1 *V. cholerae* strains lack the ability to produce CT, but they may or may not possess other enterotoxigenic mechanisms.

Although non-O1 *V. cholerae* have been associated with sporadic cases of diarrhea, epidemics caused possibly by non-O1 *V. cholerae* were first reported in 1954 (19). Since then many outbreaks due to non-O1 infection have been reported from different parts of the world, including Czechoslovakia, Australia, Bangladesh, and the United States (reviewed in Ref. 17). In 1986 non-O1 strains of *V. cholerae* have been reported to cause severe diarrhea in Bangladesh with high rates of mortality (25.8%) (20). The reported clinical features included a range of symptoms besides diarrhea and dehydration, such as abdominal pain, pyrexia, and bloody diarrhea, which are rarely present in "typical" cases of cholera. If the infection is caused by a CT producing non-O1 strains such as occurred in Bangladesh during the epidemic in 1992/3, the clinical disease is indistinguishable from classical cholera (21).

## **C. Treatment**

The fundamental principle in treating watery diarrhea, including cholera, is to correct dehydration and to replace further losses of diarrheal fluid and electrolytes. This concept of replacement therapy was recognized by several medical workers as early as 1831, when intravenous fluid infusion was shown to prevent deaths in patients with severe cholera (22,23). In the past 25 years there has been considerable improvement in rehydration therapy, resulting in fewer deaths regardless of severity (14). Rapid infusion of intravenous fluid containing an appropriate amount of salts can be life-saving in severely dehydrated patients. In less severe cases, rehydration can be achieved by administration of fluids containing glucose and electrolytes in the form of oral rehydration solution (ORS).

The electrolyte composition of ORS is based on the average electrolyte composition of the

diarrheal stool, which may vary widely depending on the microbial etiology of the diarrhea, stage and the severity of the illness, and the age of the patient. The ORS recommended by UNICEF and the World Health Organization has the following composition: sodium, 90 mmol/L; chloride, 80 mmol/L; bicarbonate, 30 mmol/L; potassium, 20 mmol/L; glucose, 111 mmol/L. This solution is clinically useful and safe for the treatment of diarrhea from all causes and in all age groups (24). Although ORS has been successfully used in the management of diarrheal diseases, continued attempts have been made to simplify and improve its composition, preparation, and distribution. Controlled clinical trials have shown that in ORS formulation, glucose may be replaced by sucrose and bicarbonate by citrate without altering its efficacy and safety.

Widespread health education and distribution of ORS have significantly reduced diarrheal morbidity and mortality in rural communities. Family studies in several developing countries have shown that early rehydration therapy can be initiated at home using solutions prepared from household sugar or molasses and common salt. The use of high carbohydrate-containing cereals to replace glucose has been recently suggested because polymers can provide more sodium cotransporting substrate to the intestine at lower luminal osmolality (25). In Bangladesh, a rice-based ORS has been developed and used with success in the treatment of secretory diarrhea caused by *V. cholerae* O1 and *Escherichia coli* (26,27). Recent observations indicate that reducing the osmolality of ORS from 290 to 249 mOsmol/kg by reducing the amount of glucose (from 111 to 89 mM) and sodium (from 90 to 67 mM) increases its therapeutic efficacy in children with cholera (28). However, more studies are needed to confirm and further elaborate upon these findings.

Although ORS has the potential to be universally applied, there are still certain limitations to its usage. Initially there was concern about the risk of hypernatremia in infants receiving ORS containing 90 mmol/L of sodium. However, several clinical studies using this concentration of sodium have shown that hypernatremia has not been a significant problem (27) when mother's milk and free water are provided to infants. Severe vomiting may interfere with the ingestion of ORS in some patients, but this has not been an obstacle to successful ORS therapy. In addition to ORS therapy, the importance of nutritional support during diarrhea has been increasingly emphasized. A large body of evidence indicates that, contrary to a belief held in many communities, food should not be withheld during diarrheal illness. Liberal feeding along with rehydration therapy has been shown to promote weight gain and positive nitrogen balance in children with diarrhea (29). In studies in which food is incorporated into the hydration solution or fed separately, there has been a reduction in both severity and duration of diarrheal illness (30).

## D. Antimicrobial Agents

When used as an adjunct to fluid therapy in cholera patients, appropriate antimicrobial agents can reduce the volume of stool and the duration of diarrhea and can shorten the period of excretion of vibrios in the feces (31,32). Antimicrobial treatment may therefore lead to greater savings of rehydration fluid and nursing care, both of which are in short supply in rural communities. *V. cholerae* is sensitive (in vitro) to a number of antimicrobial agents, including tetracycline, chloramphenicol, trimethoprim, streptomycin, erythromycin, and quinolones. However, tetracycline is the most commonly used antibiotic. A dose of 500 mg every 6 hours for 3 days is considered sufficient for adults. *V. cholerae* is rapidly killed in the intestine by the antibiotic, and fecal cultures are usually bacteriologically negative after 24 hours. Nonabsorbable drugs such as furazolidone (5–7 mg/kg) may also be clinically useful if the infecting *V. cholerae* is susceptible, but would require a longer duration of treatment (33). However, they remain the treatment of choice in children and pregnant women. Other drugs, including doxycycline, have been useful, but tetracycline still remains the most popular. In recent years the widespread use of antibiotics has led to the emergence of plasmid-mediated drug resistance in *V. cholerae*. Resistance to tetracycline and other antibiotics has been reported but is clinically important only in restricted areas. Quinolones, including ciprofloxacin, are useful in the treatment of resistant organisms (34). Single-dose antimicrobial therapy is useful in epidemic situations and disaster conditions.

#### IV. EPIDEMIOLOGY

Cholera is a disease of great epidemic potential. Since 1817, six pandemics of cholera, originating from the deltas of the big rivers in Bengal, swept all over the world. The seventh pandemic, beginning in early 1960s in an Indonesian island, spread into the western hemisphere, including the United States. The epidemiology of cholera is poorly understood, particularly its periodic resurgence in endemic areas and the intermittent shift between the Classical and the El Tor biovars.

Humans are the only natural host known to develop symptomatic infection caused by toxigenic *V. cholerae* O1 and O139. No animals other than planktonic copepods and marine crustaceans have been reported to play a significant role in the environmental transmission of the organisms. In endemic areas, cholera is more common in children than in adults. Attack rates decline with increasing age, being highest in children less than 5 years of age but rare in breastfed children and uncommon after the age of 40 years (35). Epidemiological evidence suggests that fecally contaminated water is the principal vehicle of transmission of cholera. The importance of food in cholera transmission has been recently recognized. Seafood including shellfish, oysters, and shrimp have been particularly implicated, especially when eaten raw, undercooked, or prepared with polluted water (36). Although *V. cholerae* can grow in rice, fish, eggs, and milk, environmental studies in Bangladesh have shown that most foods are usually not contaminated with *V. cholerae* (37).

During the seventh pandemic, spread of cholera continued to occur through seaport or river ports, particularly in Africa during the epidemics in the 1970s (38–40). Cholera contamination of stored water supply aboard a ship was responsible for an outbreak among the crew members (41,42). However, the cholera outbreaks aboard two ships in Hamburg in 1892 were due to consumption of water taken from the river Elbe (43). In Japan, spread of cholera in the coastal region was due to pollution of seawater by ships from abroad. Subsequent introduction of preventive measures including diagnosis and treatment significantly reduced the spread of cholera into the province (44).

Patients with active disease are believed to be the principal source of infection. The role of asymptomatic carriers in the transmission of cholera has been suggested but not found to be important. It has been shown that 90% of acute cases no longer excrete vibrios in the feces for more than 3 weeks after the infection (45). El Tor vibrios tend to persist longer in the carrier state than the Classical vibrios, but this difference is not epidemiologically significant. It has been shown that *V. cholerae* O1 may independently survive in the aquatic environment in association with zooplanktons (46) and that *V. cholerae* remain associated with various aquatic flora including water hyacinth, duckweed, and green algae. A blue-green alga, *Anabaena variabilis*, has been shown to act as a reservoir of *V. cholerae* (47). Similarly, recent isolation of toxigenic *V. cholerae* O1 from the coastal waters of the United States and Australia, where cholera cases were unknown, suggest the importance of aquatic environment as a natural microhabitat of these organisms.

The seasonal incidence of cholera varies in different parts of the world and correlates more with poverty, poor sanitation, and overcrowding than with any particular climatic conditions. In Calcutta there are two peaks, in May and October, with the lowest prevalence in February. Outbreaks seem to correlate more with temperature than with rainfall. In the Philippines cholera is common during the rains, while in Bangladesh cholera comes after the wet season and declines during the dry season (48). Since 1961, there has been a rapid change in the prevalence pattern of El Tor and Classical biovars of *V. cholerae* all over the world. The El Tor biovar, which was responsible for the current pandemic, was replaced by the Classical strain initially in India and later in Bangladesh. The definitive reasons for this change remain speculative.

Cholera appeared in Latin America in 1991 for the first time in the twentieth century. Peru was the first country to report cholera during the epidemic (49). The disease was spreading fast in other countries. By August 26, 1992, 640,000 cholera cases and 5,600 deaths were reported by 19 countries; these numbers exceeded those reported for the entire world during the previous 5 years. In the same wave of the epidemic, cholera entered into the United States in association with the travelers who visited the Latin American countries during the epidemic.

In several instances, food has been incriminated as the vehicle of transmission of cholera from Latin America to the United States. In the United States, the first case of cholera after 1911 occurred

in a shrimp fisherman on the Texas coast in 1973 (50). The isolate was identified as *V. cholerae* O1, biotype El Tor. In spite of extensive investigations, the source of infection remained unidentified. In 1978, 11 persons were attacked with *V. cholerae* El Tor in Louisiana due to consumption of cooked crabs in different sites of the Louisiana coastal marsh. In 1984, one person developed cholera in Maryland, who had consumed infected crabs collected along the Texas coast. In Florida and Georgia two isolated cases of cholera occurred in 1978 due to consumption of contaminated raw oysters. In the Louisiana outbreak of cholera in 1986, 18 persons were attacked in 12 different clusters; all were infected by eating crabs and shrimps collected from different sites along the Louisiana coast (51). In the fall of 1991, a single cholera case was identified in an oil rig barge in Texas, which was followed by 14 secondary cases of cholera with one asymptomatic infection (52). The source of infection in the index case was traced to consumption of infected seafoods from local water. The secondary cases were infected by consuming rice prepared with water, which was contaminated by the feces of the index case through cross-connection between sewer drain and drinking water supply. Since 1973, a total of 65 cholera cases have been associated with the Gulf Coast reservoir in the United States. In all cases, the isolated *V. cholerae* O1 were El Tor biotype and Inaba serotype; all strains produced cholera toxin and hemolysin on blood agar and possessed a characteristic bacteriophage, VcA3 (53). Genetically, all strains had the same restriction digest pattern and ribotype pattern. All these data support the hypothesis that *V. cholerae* O1 strains have been persisting as a free-living organism along the Gulf Coast for the past 20 years, independent of exogenous introduction during the pandemics.

The African continent has been free from cholera for the past 70 years; however, cholera reappeared in Africa in 1971, and 30 of the 46 countries started reporting cholera in succession. In a cholera outbreak in Mali in 1984, the results of a case-control study have shown that it was associated with eating leftover millet gruel by the villagers in an arid region (54). In this community, millet is a major food item, which is prepared once a day and stored at room temperature for many hours. This food is often consumed by people in groups without proper cleanliness. In another outbreak in Guinea, consumption of leftover rice with peanut sauce has been incriminated as the vehicle of transmission. In contrast, leftover rice eaten with tomato sauce, having acidic or unfavorable pH for *V. cholerae*, was not associated with cholera cases (54). There were also two reports of cholera outbreaks that occurred due to contamination of rice during preparation of a funeral feast by women who had cleaned the bodies of patients who died of cholera. During 1991–92, 21 African countries reported a total of 153,367 cholera cases with 14,000 deaths. During the tribal conflicts in the central African nation of Rwanda in 1994, more than 500 cholera deaths were reported, and thousands fled to neighboring countries. In the township of Goma, in Zaire, cholera broke out among 500,000 Rwandan refugees and killed 25,000 people within a few weeks (55).

## V. ISOLATION AND IDENTIFICATION

In the laboratory vibrios can usually be grown on MacConkey agar plates, but some strains may need special media for optimum growth. Combinations of thiosulfate, citrate, bile salt, and sucrose (TCBS) agar (56) and tellurite, taurocholate, and gelatin agar (TTGA) (57) are considered very useful for growing *V. cholerae*. TCBS agar is a highly selective media for *Vibrio* species, with the exception of *V. hollisae*, although there may be variations of the inhibitory effects between different laboratories depending on the age of the culture. TTGA is less selective than TCBS agar, and most vibrios are able to degrade gelatin in this medium forming a turbid zone around the colony. Enrichment of media is not required for isolation of *V. cholerae* from fresh diarrheal stool, but it is useful for isolating the organisms from asymptomatic carriers or patients in late convalescence. Alkaline peptone water (pH 9.0) with 1% sodium chloride and alkaline tellurite–bile salt broth of Monsur are good selective enrichment media for isolation of *V. cholerae* O1 (57).

Stool specimens should be collected from patients as early as possible during the course of the illness, preferably before starting treatment with an antimicrobial agent. Specimens collected during the later stages of disease may not yield growth of *V. cholera* because of the declining number of

vibrios in the feces. Specimens could be taken with cotton swabs treated with calcium alginate or polyester fibers. In actively purging patients, samples could be taken using rectal swabs. If a delay in plating is anticipated, the swabs should be placed in Cary-Blair transport medium in screw-capped bottles, and refrigeration should be avoided. After collection, the stool specimens should be directly inoculated onto the agar plates as soon as possible. The swabs are washed in 0.5 mL of peptone water to emulsify, and a loopful of the emulsion is inoculated into the agar plate. The plate is incubated overnight and the vibrio colonies are carefully examined.

For the isolation *V. cholerae* from food and water samples, different enrichment media may be necessary. A combination of alkaline peptone water for the primary enrichment and tellurite-bile salt broth for the secondary enrichment is usually recommended. For isolating *V. cholerae* from seafood, the material is usually homogenized and inoculated into the enrichment broth. However, such homogenates often contain inhibitory substances and may retard the expected growth of the organisms. It is recommended that the seafood samples should be cut into small pieces, added to 10 times the volume of enrichment broth, shaken vigorously for 1–2 minutes or homogenized, the pieces removed, and then the broth incubated at 35°C.

Characteristic appearances of the colonies help in the identification of *V. cholerae* isolates when grown on selective medium such as TCBS agar; however, when the sample contains other indigenous vibrios from marine sources, the identification could be difficult and may require special procedures. Preliminary identification may be achieved by testing for glucose fermentation in Kligler iron agar, nitrate reduction to nitrite, oxidase production, relative growth on nutrient agar with and without salt, and in Moeller's broth containing lysine and ornithine decarboxylases and arginine dihydrolase. *Vibrio* species associated with human infections are oxidase positive, ferment glucose without gas production, reduce nitrate, and grow only on broth containing 1% salt, with the exception of *V. cholerae*, *V. mimicus*, and some strains of *V. metschnikovii*. Susceptibility to the vibriostatic agent O/120 (2,4-diamino-6,7-diisopropylpteridine phosphate) may be useful for differentiating *V. cholerae*, *V. mimicus*, and *Aeromonas* spp. The primary isolates could be further identified by tests including Voges-Proskauer reaction and acid production from L-arabinose, lactose, maltose, sucrose, mannitol, and salicin. Serotypes of *Vibrio* species could be determined by using type-specific anti-serum in the reference laboratories. Biotypes of *V. cholerae* could be determined by the test for hemolysin production, i.e., by incubating the organisms in sheep blood agar culture at 35°C for 24 hours in anaerobic conditions without carbon dioxide (58).

## VI. PATHOGENICITY

The knowledge about the pathogenesis of cholera has been changing during the past 25 years. Early suggestions included denudation of intestinal epithelium (proved to be postmortem change afterward), ultrastructural injury to intestinal mucosa, alteration of microcirculation of lamina propria, elaboration of gastrointestinal hormones (gastrin or secretin), and finally hypersecretion of the epithelial cell, as possible pathogenic mechanisms of cholera. A comprehensive review of this literature has been presented by Rabbani and Greenough (13).

Successful colonization of the small intestinal mucosa by *V. cholerae* depends on the adhesion of the vibrios to the epithelial surface. *V. cholerae* strains that are unable to colonize the epithelium do not produce disease. Adhesion secures the bacteria against the effects of intestinal motility and facilitates the delivery of the toxin that is produced. The glycoprotein component of the mucus may contain a variety of molecules, which may act as specific receptors for bacteria. *V. cholerae* (59) and *Escherichia coli* (60) both have been shown to possess carbohydrate-mediated attachment to intestinal cells. The importance of bacterial pili in the colonization process has only been recently recognized. Taylor et al. (61) characterized a pilus, designated as toxin-coregulated pili antigen (TcpA), elaborated by *V. cholerae* O1 of Classical and El Tor biotypes that is required for colonization in mouse and humans. Herrington et al. (62) subsequently showed that an isogenic mutant of *V. cholerae* lacking TcpA was unable to colonize volunteers. Expression of TcpA and cholera toxin

is regulated by a transmembrane DNA-binding regulatory protein called ToxR. Yamamoto et al. (63) showed that *V. cholerae* O1, irrespective of the biotype and serotype, adhere to the mucus coat of the human small intestine and that adherence to the mucus coat is more extensive than to the villous surface. Finkelstein et al. (64) described a protein “cholera lectin,” made by *V. cholerae*, which is involved in the attachment of the organisms to the mucosal cells, probably by altering mucin, fibronectin, or lactoferrin.

*V. cholerae* O1 produces a potent enterotoxin, which stimulates intestinal fluid secretion leading to massive watery diarrhea in patients. The toxin molecule is a protein comprising two subunits: a single heavy A subunit (28,000 MW), which is noncovalently attached to an aggregate of five light B subunits, each with a molecular weight of 11,600 (65,66). Each A subunit can be split into fragments A1 (22,500 MW) and A2 (5,500 MW) when treated with thiol-reducing agents. Both A and B subunits are initially synthesized as precursors, which are rapidly processed and translocated across the inner membrane into the periplasm. The light subunits are responsible for cell binding (B subunit) and the heavy subunits for direct toxicity (A subunit). B subunits bind strongly to intact cells and to the isolated cell receptors, but are not toxic. Purified A subunits do not bind to cells and are not toxic; however, when A is associated with B subunits, both cell binding and toxicity occur. The cell receptor for binding the toxin molecule has been identified as a specific ganglioside, GM1 (65). The GM1 content of the cell membrane can be increased by an enzyme, sialidase, which is produced by most strains of *V. cholerae*. After binding to the GM1 cell receptor, the A subunit penetrates through the cell membrane into the cytosol by an as yet unknown mechanism and activates an enzyme adenylate cyclase, which requires NAD and ADP ribosylation of a GTP-binding protein (66). This process results in the generation of intracellular second messengers, including cAMP, cGMP, and cytosolic free calcium, leading to increased permeability of the apical membrane to chloride ions and water molecules, together with an inhibition of coupled NaCl absorption. Besides cAMP-mediated secretion, other neurohumoral mechanisms may be involved in cholera toxin-stimulated intestinal secretion; these include prostaglandins, 5HT, VIP, and nitric oxide (67). In addition to cholera toxin, several other toxins have been described, including a hemolysin (58), a Shiga-like toxin (68), and a hemagglutinin (69); these toxins have not yet been fully characterized.

Most definitive studies showed that minimal morphological changes occur in the small bowel in response to cholera vibrios or its enterotoxin. Findings such as increased mucus production by the goblet cells of the epithelium, hyperemia of the vessels of the lamina propria, and increased rate of mucosal epithelial turnover may be caused by concomitant disease or effects by cholera toxin on mucus secretion itself. These observations were confirmed by subsequent studies in animal models (70). It has been shown that *V. cholerae* does not penetrate the gut epithelium, so that during natural disease no other tissues than the gut are exposed to its potent enterotoxin. That the effect of the enterotoxin is limited to the gut mucosa is indicated by the observation that the enterotoxin is never detected by the most sensitive technique either in the thoracic duct lymph or superior mesenteric venous blood during experimentally induced cholera (70). However, when nonintestinal tissues are exposed to cholera toxin by artificial means, a response occurs that is characteristic of the particular tissue being examined. Most studies indicate that the secretion of isotonic fluid by the small intestine in response to cholera enterotoxin is the most important event leading to clinical disease. In experimental animals, small intestinal secretion of isotonic fluid occurs after a brief exposure to enterotoxin and lasts for a period of 12–24 hours (71). The fluid loss occurs entirely from the small intestine, involving all segments, in both humans and experimental animals. There is no evidence to suggest that salivary, gastric, biliary, or pancreatic secretion contributes to any degree in cholera diarrhea. In experimental canine cholera as well as in humans, fluid output per unit length of bowel is greatest in duodenum and least in ileum. The electrolyte content of the intestinal fluid in patients with cholera and in experimental animals greatly varies from the duodenum (where the bicarbonate concentration is less than that of plasma) to ileum, where it is two to three times that of simultaneously obtained plasma. The electrolyte composition of intestinal fluid in cholera is given in [Table 1](#).



**TABLE 1** Electrolyte Composition of Stool in Cholera and Noncholera Diarrhea and Different Rehydration Solutions<sup>a</sup>

	Electrolyte concentrations (mmol/L) <sup>b</sup>				
	Sodium	Potassium	Chloride	Bicarbonate	Glucose
Cholera stool	80.0	21.0	68.1	45.7	2–5
Noncholera stool (ETEC)	38.0	8.7	50.0	14.2	1–3
Oral rehydration solution	90.0	20.0	80.0	30.0	111
Normal saline	154.0	0.0	154.0	0.0	0.0
Half-strength Darrow's solution	61.0	17.5	52.0	26.0	150.00
Dacca solution	134.0	13.0	98.0	48.0	0.0

<sup>a</sup> All electrolytes except potassium are higher in cholera stool than in noncholera stool, but in cholera the range of variation is very narrow in contrast to that in noncholera stool [enterotoxigenic *Escherichia coli* (ETEC)].

<sup>b</sup> Mean  $\pm$  SD.

## A. Prostaglandins in Cholera

That the prostaglandins are involved in the pathogenesis of cholera is shown by the fact that non-steroidal anti-inflammatory compounds, including aspirin and indomethacin (potent prostaglandin inhibitors), prevent the secretory effects of cholera toxin in experimental animals (72). The subject has been reviewed by Rabbani (73). A study using intestinal perfusion in cholera patients showed that during active disease the jejunal prostaglandin E (PGE) concentrations were significantly higher (1,721,435 pg/mL) as compared to early convalescent stage (60–270 pg/mL;  $p < 0.001$ ) (74). The PGE concentrations were negatively correlated ( $r = 0.71$ ,  $p < 0.05$ ) with the time after the onset of the disease and positively correlated ( $r = 0.84$ ,  $p < 0.01$ ) with the stool output. It is possible that local intestinal PGE production in severe cholera results in mucosal PGE concentrations above those required for maximal secretory response. This observation might explain why conventional doses of aspirin and indomethacin had no antisecretory effects in cholera patients (75). An alternate hypothesis is that cholera toxin maximally stimulates the secretory system, and any increases of PGE cannot enhance it further.

## B. Colonic Functions in Cholera

In contrast to our understanding of small intestinal function, the colon is a less studied segment of the gastrointestinal tract in relation to cholera and other toxigenic diarrheas. Although the transport functions of the large intestine are in many ways similar to that of the small bowel, many differences make the large intestine a critical determinant of the development of diarrhea. Unlike the small intestine, the large intestine does not actively absorb glucose and amino acid, nor is there any glucose- or amino acid-stimulated sodium absorption, except in the neonatal period. Effects of mineralocorticoids (aldosterone) on ion transport are more profound in the colon than they are in the small intestine. The colon possesses significantly greater capabilities than small intestine for absorbing sodium against considerable concentration gradients. In healthy adults, the colon can absorb a maximum of 5 L of water in 24 hours, with a normal ileocecal flow of approximately 2 L/24 h (76). Several factors, including the volume, composition, and rate of flow of luminal fluid, determine colonic fluid absorption. Because of the great absorptive capacity of the colon, diarrhea does not occur so long as the ileocecal flow rate remains within the limits of the colonic absorptive capacity. In contrast, a small alteration in colonic absorption caused by any disease would result in significant diarrhea in the face of normal small intestinal function and ileocecal flow rate.

In health, human colon absorbs water, Na<sup>+</sup>, and Cl<sup>-</sup> and secretes K<sup>+</sup> and HCO<sup>-</sup>. Aldosterone

and glucocorticoids augment electrogenic Na<sup>+</sup> absorption. During cholera diarrhea, alteration of colonic function certainly occurs but is poorly understood because of the lack of adequate studies. The only study that has assessed the colonic function during acute cholera is that of Speelman et al. (77). These authors carefully conducted colonoscopic perfusion studies using nonabsorbable markers in patients with acute cholera and during convalescence. The results showed that during acute cholera the colon is in a state of dysfunction consisting of diminished water absorption and increased potassium secretion. The net water transport was approximately zero. These results showed that the colon contributes to the clinical expression of cholera by failing to absorb water normally and by secreting potassium at a higher rate. However, there is conflicting evidence in the literature about the effects of cholera toxin in the colon. Results of limited experiments in rabbit and dog indicated no alteration of colonic water and electrolyte transport caused by cholera toxin (78,79). In contrast, more definitive studies by Donowitz and Binder (80) showed that cholera toxin stimulated cyclic AMP production and net fluid secretion in rat cecum. These apparently discrepant findings are difficult to explain but may be related to the differences in methods and species of experimental animals used. More studies will be needed to determine fully the changes of colonic functions in cholera.

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

The genetic factors controlling different aspects of *V. cholerae* pathogenicity including toxin production, motility, adherence, drug resistance, and metabolism have been extensively studied using a variety of classical and molecular techniques. The fundamental genetic structure of *V. cholerae* including transcription, translation, regulation, and transduction is similar to those of other enteric bacteria.

The production of toxin by *V. cholerae* is regulated by structural genes, which are similar to genes encoding the heat-labile enterotoxin of enterotoxigenic *E. coli* (*eltAB*). Analysis of DNA sequences have shown that there is 76% homology between the two genes, and it is believed that the toxin gene was first originated in *V. cholerae* and later acquired by *E. coli* about 130 million years ago (63). The formation of subunits A and B of cholera toxin is regulated by a gene called *ctxAB*, the DNA sequence of which has been determined. It is seen that the sequences encoding the A subunit (*ctxA*) precede the sequence encoding B subunit (*ctxB*) and overlap by 4 nucleotides (ATGA). The *ctxA* appears to be a translation product of 258 amino acids and that of *ctxB* is of 124 amino acids. The *ctx* promoter gene, which resides immediately before the structural gene, is regulated by a positive element *toxR* (81). Recent studies have revealed important mechanisms of genetic regulation of *ctx* genes in *V. cholerae* O1.

The *toxR* gene of *V. cholerae* O1 strain 569B, when introduced into *E. coli* containing cloned *ctxAB* genes, increases cholera toxin production 10-fold. The *toxR* gene is a positive regulator of *ctx* which regulates transcription, and mutant strains defective in this gene show markedly reduced levels of *ctxAB* mRNA (81). The *toxR* gene product ToxR binds to the repetitive sequence TTTTGAT in the *ctx* promoter activating it, and *V. cholerae* strains that produce low levels of toxin have three or four tandem repeats, while the highly toxigenic strain 569B contains eight repeats. Recent studies indicate that *toxR* gene coordinately regulates more than 17 genes in *V. cholerae*, some of which encode virulence characteristics of the organism (82). This type of genetic regulation is a two-way system; one component perceives the environmental conditions and transmits a signal to a regulatory component, which in turn controls transcription of several genes. This mechanism of assessing differences in gene expression would lead to a variety of responses to different environmental conditions in the bowel lumen, epithelial surface, and natural reservoirs. The changes in conditions like temperature, osmolality, and pH can be sensed by the *toxR* gene with appropriate genetic response.

Another 19 kDa transmembrane regulatory gene, known as the *toxS* gene, is located immediately downstream from the *toxR* gene and is transcribed from the same promoter. This gene has been

shown to enhance the activity of *toxR* gene, probably by interacting with the periplasmic portion of *toxR*. It has been suggested that the *toxS* gene enhances the *ctx* gene expression, thereby stimulating toxin production in *V. cholerae*. Interestingly, the highly toxigenic strain of *V. cholerae* O1 569B lacks the *toxS* gene, suggesting the possibility of a third *toxT* gene locus (83).

It has been shown that the expression of the *toxR* gene is regulated by the heat-shock phenomenon, in which a cell responds to changes in adverse environmental conditions by expressing additional genes that help survive the organism in the unfavorable situation. In this respect, Parsot and Mekalanos described a gene in *V. cholerae* with an amino acid sequence very similar to the heat-shock protein HtpG from *E. coli* (84).

That the virulence property of *V. cholerae* O1 is regulated by the *toxR* gene is shown by the experiment in which healthy volunteers were challenged orally with a *toxR* gene–mutated strain of Ogawa 395. There was significant reduction of intestinal colonization by this strain, probably by a concomitant reduction of *tcpA*-encoded pilus production by the *toxR* mutant strain.

Production of toxin by *V. cholerae* O1 strains seems to be regulated by three other genes recently reported but not yet well characterized. These include *htx* locus, *ltx* locus, and *tox-1000* locus genes; mutation of genes in these loci either increase or decrease the production of toxin by *V. cholerae* O1 strains. In addition, some plasmids present in *V. cholerae* O1 have been reported to influence the toxin production. These include C compatibility plasmids, which regulate antibiotic resistance, and sex factor P; the former increase and the latter decreases toxin production in *V. cholerae* 569B (85).

A number of genes other than those controlling toxin production have been identified in *V. cholerae* O1, many of which have been characterized and cloned. These genes regulate different virulence factors other than toxin production. The gene that regulates hemolysin production, which is used to differentiate between El Tor and Classical strains, has been identified to be a *hlyA* gene of 65 kDa. The cloned *hlyA* gene has been employed to evaluate the role of hemolysin in intestinal fluid secretion. Two other additional genes, *hlyB* and *hlyC*, have recently been reported which also regulate hemolysin production in *V. cholerae* O1 strains (86). The gene that encodes O-antigen of *V. cholerae* has been identified and cloned in Inaba serotype from 569B and of the Ogawa serotype from O17. Expression of specific O-antigens A, B, and C are used to classify *V. cholerae* into Inaba, Ogawa, and Hikojima serotypes. The pilus colonizing factor which is essential for adherence of *V. cholerae* to intestinal cells is regulated by the *tcpA* gene, which is present in all pathogenic strains of El Tor and Classical isolates of *V. cholerae* O1. In contrast, the *tcpA* gene is not found in *V. cholerae* non-O1 strains or in nontoxigenic strains of *V. cholerae* O1. The genetic regulation of outer membrane proteins in *V. cholerae* is accomplished by the *ompV* gene, which encodes a 25 kDa major outer membrane protein of *V. cholerae* O1. Zona occludens toxin (ZOT) is a recently described toxin that is distinct from the cholera toxin and is elaborated by *V. cholerae* O1 strain. The gene encoding ZOT is located immediately upstream of the *ctx* locus on the 4.3 kb virulence cassette and appears to be regulated independently of the *ctx* gene (87).

## VIII. CONTROL MEASURES

Recognizing that cholera is primarily caused by ingesting fecally contaminated water should permit development of intervention methods that interrupt the fecal-oral transmission cycle. The development of improved methods of safe water supply and hygienic disposal of waste through improved sanitation in the West have significantly reduced the incidence of intestinal diseases, including cholera. However, in the developing parts of the world, a comparable improvement has not been accomplished. Nevertheless, in most instances the awareness regarding the public health importance of sanitation and safe water use is increasing, and attempts to develop low-cost sanitation systems are being pursued through government and international initiatives.

## A. Safe Water Supply

Since cholera is primarily a waterborne disease, access to safe water for drinking and domestic use would greatly reduce the incidence of the disease. Supplying safe water to a particular community involves multiple disciplines including health planners, economists, public health engineers, geologists, bacteriologists, and the government. A coordinated effort will be required to design a suitable water supply system for a particular community depending on its physiographic, sociodemographic, and climatic conditions; this could be an expensive undertaking, and economic factors must be considered, particularly in the developing countries where resources may be limited. Various types of water supply systems have been described that can be considered for countries with limited resources (88,89). In urban areas, providing piped chlorinated water for drinking purposes could be an effective means to reduce transmission of cholera. In periurban and rural areas, tube wells or protected dug wells may be cost-effective for supplying safe drinking water. However, in many circumstances, these are not available and people have to use unprotected surface water from open ponds, tanks, and rivers; water from these sources is often difficult to treat for purification. Although boiling is usually recommended, it is not practiced in many poor communities because of lack of fuel and cooking utensils. In Bangladesh, prolonged storage of drinking water in earthen jars allowed growth of *V. cholerae*, leading to an outbreak of cholera. In such circumstances, use of alum potash (500 mg/L) has been shown to kill vibrios by acidification and flocculation of water, but the taste may not be acceptable (90). Water purification tablets containing chlorine (and sometimes thiosulfate to remove the taste) are commercially available and could be useful, particularly in epidemic situations. Iodine could also be used to disinfect household drinking water—2 drops of 2% tincture of iodine are adequate for treating 1 L of water.

## B. Safe Disposal of Human Excreta

Large numbers of *V. cholerae* are excreted in the stools of actively purging cholera patients and is the only source of the fecal contamination of the environment. Therefore, safe disposal of human excreta, particularly from cholera patients, must be ensured to reduce the fecal-oral spread of the disease. In the developed countries, introduction of modern sewage treatment and disposal systems has markedly reduced the incidence of diarrheal diseases, including cholera. However, in the developing world similar improvements in sanitation have not taken place because of poverty, lack of effort, and poor technical support. Many different types of sewage disposal systems have been described (88,89). An appropriate system should be selected depending on the type of need, the urban or rural nature of the community, the terrain and geology, the customs and practices of the community, and of course the availability of resources. Pit latrines of the improved, ventilated type could be useful in rural areas, but they must be set at a distance of 25–30 m away from any source of underground or surface water and at least 12 m away from any living premises. The required dimension of a pit latrine suitable for one family would be 2 m deep with a 1 m<sup>2</sup> opening.

## C. Food Safety

Although cholera is considered a waterborne disease, recent evidence indicates the importance of food, particularly seafood, in the transmission of cholera (91). Foods are likely to be fecally contaminated during preparation, particularly by the infected food handlers in an unhygienic environment. The physicochemical characteristics of foods that support survival and growth of *V. cholerae* O1 are high moisture content, neutral or alkaline pH, low temperature, high organic content, and an absence of other competing bacteria. Seafood, including fish, shellfish, crabs, oysters, and clams, have all been incriminated in cholera outbreaks in many countries, including the United States and Australia. Contaminated rice, millet gruel, and vegetables have also been implicated in several outbreaks. Other foods, including fruits (except sour fruits), poultry, meat, and dairy products, have

the potential of transmitting cholera. To reduce the risk of foodborne transmission of cholera, it is recommended that foods be prepared, served, and eaten in a hygienic environment free from fecal contamination. Proper cooking, storing, and reheating of foods before eating and hand-washing with safe water before eating and after defecation are important safety measures for preventing foodborne transmission of cholera.

The World Health Organization (WHO) recommends the following food safety measures to prevent the spread of cholera (92):

- Avoid raw foods (undamaged fruits and vegetables from which the peel can be removed are safe if hygienically handled).

- Cook food until hot throughout.

- Eat food while it is still hot or reheat food thoroughly before eating.

- Wash and thoroughly dry all cooking and serving utensils after use.

- Handle and prepare foods in a way that reduces the risk of contamination (e.g., cooked food and eating utensils should be kept separate from uncooked foods and potentially contaminated utensils).

- Wash hands thoroughly with soap (or ash) after defecating, or after contact with fecal matter, and before preparing or eating foods or feeding children.

With regard to the risk of cholera transmission through food trade, WHO recommends that “although there is a theoretical risk of cholera transmission associated with international food trade, the weight of the evidence suggests that this risk is small and can normally be dealt with by means other than an embargo on importation” (92).

#### **D. Vaccination**

The prospects of an effective vaccine against cholera has been suggested, but progress in this area has been very slow. In the past, several types of cholera vaccine (whole cell, antitoxin, adjuvant) have been developed and tested. Some of these provided short-term, partial protection against cholera but did not otherwise affect the transmission of the disease. In general, the indications are that in terms of epidemic control, the currently available parenteral vaccines are probably not useful. However, a vaccine with greater protective effect and safety could become an important tool in reducing the incidence of the disease. During natural *V. cholerae* infection, immunity against both the organism and its toxin develops, which synergistically protects the host from subsequent infections. Cholera vaccine field trials carried out in Bangladesh, the Philippines, and India using parenterally administered suspensions of killed whole cells of different serotypes of *V. cholerae* showed that the protection is short-lived (3–6 months) and does not exceed 50–60% (93). This indicates that antibacterial immunity induced by this method is not associated with long-lasting protection. The nature of the protective effect of antibodies to cholera toxin in humans is poorly understood. In mammals the injection of purified cholera toxin stimulates production of antibodies, which can neutralize the effects of the toxin, probably by preventing the binding of the toxin molecule to its cell surface receptors. The toxoid vaccines produced by detoxification with chemical treatment have several shortcomings. In one investigation, a formalin-treated toxoid vaccine was immunogenic but reverted to toxicity. Another group of experiments with glutaraldehyde-treated toxin produced a more stable toxoid but with poor immunogenicity (94). The synergistic and protective effects of antibodies to the bacterium and toxoid have been increasingly demonstrated in animals. One such study that combined both components claimed a 100-fold increase in protection.

The orally administered B-subunit vaccine developed to prevent binding of the cholera toxin molecule to GM1 ganglioside cell receptors has now been field-tested in Bangladesh. In animals, the purified B-subunit has been found to be highly immunogenic, nontoxic, and protective against experimental cholera (95). In humans, a single, oral dose of a purified B-subunit suspension induced a significant increase in intestinal IgA antibodies. In the intestinal mucosa, antibody responses to

bacteria and cholera toxin induced by combined B-subunit and whole cell vaccine were comparable with those seen after clinical cholera (95). The combined vaccine protected 64% of the vaccinees against all episodes of diarrhea and 100% against clinically significant diarrhea in North American volunteers who were subsequently challenged with virulent *V. cholerae* O1. Moreover, this oral vaccine induced no side effects when given to small numbers of North American, Swedish, and Bangladeshi volunteers. However, the field trial of the combined whole cell and B-subunit vaccine carried out in Bangladesh indicated a 6-month protective efficacy against cholera was 85% for the combined vaccine and 58% for the killed whole cell vaccine (96).

Vaccine strains of attenuated *V. cholerae* O1 produced by genetic recombinant DNA technology offer great promise for the future development of oral live vaccines against cholera. These strains are A-subunit gene-deleted mutants of *V. cholerae* O1, which are highly protective and immunogenic in volunteers after a single oral dose. However, although these strains were markedly attenuated compared to their parent strains, they caused mild diarrheal symptoms in 50% of the recipients. Subsequently, two more attenuated, mutant vaccine strains were developed, CVD 103 and CVD 103-HgR (97); these were less reactogenic but retained similar protective and immunogenic properties in volunteers. The CVD 103-HgR is a genetic derivative of Inaba 569B that does not produce Shiga-like toxin; this strain has been extensively tested for its safety and immunogenicity in North American, Thai, and Swiss adults and in Indonesian children and was found to be protective in 80–95% cases. After further evaluation this vaccine will be ready for field testing. An oral bivalent B-subunit O1/O139 whole cell cholera vaccine has been developed by adding formalin-killed O139 vibrios to the recently licensed oral recombinant B-subunit O1 whole cell vaccine; this vaccine was found to be safe and immunogenic in Swedish volunteers (98).

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## ***Vibrio parahaemolyticus***

**Mitsuaki Nishibuchi**

*Kyoto University, Kyoto, Japan*

### **I. BACKGROUND**

*Vibrio parahaemolyticus* is a halophilic marine bacterium first isolated from Shirasu, semi-dried juvenile sardine, implicated in an outbreak of food poisoning in Japan in 1950. It was soon shown that gastroenteritis due to this organism accounted for nearly half of bacterial food poisoning cases in Japan where raw seafood is consumed very often and that this organism is abundant in the seawater environment around Japan in summer. The first food poisoning case due to *V. parahaemolyticus* reported outside Japan was from the East Coast of the United States in 1971. Distribution of this organism in the marine environment of the Pacific Northwest was then studied. Clinical cases and distribution of *V. parahaemolyticus* in other parts of the world have been reported since then. *V. parahaemolyticus* has thus been recognized as an important seafoodborne pathogen worldwide (1). Raw or undercooked seafood contaminated with this organism is the main source of infection. Nowadays, various seafoods are transported across the international borders, and such seafood can be contaminated by *V. parahaemolyticus* at considerable levels although the concentration of virulent strains is supposed to be very low. International travelers also often suffer from *V. parahaemolyticus* infection. Very recently, the use of molecular genetic tools allowed the investigation of the epidemiology of *V. parahaemolyticus* infection on a global scale.

### **II. CHARACTERISTICS**

*V. parahaemolyticus* can be isolated from coastal waters when the water temperature is 15°C or above. The organism can only be detected in sediment when the temperature is below 15°C. This bacterium propagates in association with zooplankton, is released into the seawater in warm seasons, and thus has been isolated from various seafoods in the market (1).

Most strains of *V. parahaemolyticus* isolated from fecal specimens of patients with gastroenteritis induce  $\beta$ -type hemolysis when grown on a special blood agar, Wagatsuma agar. This condition is called the Kanagawa phenomenon (KP). Only 1–2% of strains isolated from the environment manifested KP (2,3). Therefore, KP has been considered a marker for virulent strains. KP is caused by a thermostable direct hemolysin (TDH) produced extracellularly by the organism. However, in 1988 some KP-negative strains isolated from clinical sources were shown to produce a TDH-related hemolysin (TRH) (see Sec. VI) but not TDH (4,5). Currently, the strains capable of producing TDH, TRH, or both are considered clinically important (see Sec. IV).

Like most other vibrios, *V. parahaemolyticus* requires salt (NaCl) for growth, is sensitive to low pH, but grows well at high pH. The minimum, maximum, and optimum temperatures for growth are approximately 10, 44–45, and 37°C, respectively (1). The generation time of *V. parahaemolyticus* can be as short as 9 minutes when grown in culture medium and 12 minutes in seafood (1,6).

*V. parahaemolyticus* is a gram-negative rod and motile by a sheathed polar flagellum. It also

**TABLE 1** Relationship Between O and K Antigens of *V. parahaemolyticus* Strains

O antigen	K antigen
1	1, 5, 20, 25, 26, 32, 38, 41, 56, 58, 60, 64, 69
2	3, 28
3	4, 5, 6, 7, 25, 29, 30, 31, 33, 37, 43, 45, 48, 54, 56, 57, 58, 59, 72, 75
4	4, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55, 63, 67, 73
5	15, 17, 30, 47, 60, 61, 68
6	18, 46
7	19
8	20, 21, 22, 23, 39, 41, 70, 74
9	23, 44
10	24, 71
11	19, 36, 40, 46, 50, 51, 61
12 <sup>a</sup>	19, 52, 61, 66
13 <sup>b</sup>	65

<sup>a</sup> Reacts with anti-O10 antiserum.

<sup>b</sup> Reacts with anti-O3 antiserum.

Source: Adapted from Ref. 117.

forms unsheathed lateral flagella when grown on solid media (7). The antigenicity of the polar flagellum is homogeneous among the strains of *V. parahaemolyticus* and other species of the genus *Vibrio* but is distinct from that of the lateral flagella of *V. parahaemolyticus* (8,9). The antigenicity of the O (heat-stable somatic) and K (heat-labile envelope) antigens can vary among the strains, but there is a correlation between the O antigen and the K antigen. Currently approved O and K antigens and their relationship are listed in Table 1. The O:K serotype is useful for epidemiological investigation. Proposals for new O or K antigens need to be approved by Committee on the Serological Typing of *Vibrio parahaemolyticus* in Japan. The lipopolysaccharide responsible for the O antigenicity of *V. parahaemolyticus* does not contain long polymerized polysaccharide chains. Instead, it contains relatively short polysaccharide chains and is similar to the lipooligosaccharides of nonenteric mucosal pathogens such as *Neisseria gonorrhoeae* and *Bordetella pertussis* (10,11).

*V. parahaemolyticus* strains are generally sensitive to most antimicrobial agents except for ampicillin. Norfloxacin showed lowest minimum inhibitory concentrations (MIC) in many examinations (12–14).

### III. DISEASES

The main symptom of patients with gastroenteritis due to *V. parahaemolyticus* is diarrhea, but this is accompanied by various other symptoms. The symptoms reported in Japanese and U.S. cases are listed in Table 2. In these cases, the incubation period ranged from less than one hour to over 76 hours, with an average of 15–24 hours, and illness generally lasted for 4–7 days (6,15).

There were 4724 food poisoning outbreaks due to *V. parahaemolyticus* in Japan during the 1958–1972 period, and 60 patients died in these outbreaks. Investigators of the first Japanese outbreak in 1950, in which 20 of 272 patients died, reported that most diarrhea stools were watery but some were bloody. Catarrh of the stomach, erosion of the jejunum and ileum, dilatation of blood vessels in the brain, and congestion of various organs including hemorrhage in the lungs were

**TABLE 2** Symptoms of Patients with Gastroenteritis Due to *V. parahaemolyticus* Infection

Symptom	Percent patients		
	Japanese cases <sup>a</sup>	Maryland outbreak <sup>b</sup>	Port Allen, LA outbreak <sup>b</sup>
Diarrhea	95	100	95
Abdominal pain	86	86	92
Headache	40	ND	48
Vomiting	36	26	12
Fever	35	23	48
Weakness	29	ND	90
Chill	23	ND	55
Tenesmus	18	ND	ND
Nausea	0.01	26	72

<sup>a</sup> From Ref. 15.

<sup>b</sup> From Ref. 6.

ND, Not described.

recognized in the eight autopsy cases (16). A possibility that cardiotoxic activity of TDH produced by *V. parahaemolyticus* may be related to death has been suggested (17).

*V. parahaemolyticus* was implicated in trauma, septicemia, and pneumonia, but reports of such extraintestinal infections are rare (18,19). Both KP-positive and KP-negative strains were isolated from the extraintestinal specimens.

#### IV. EPIDEMIOLOGY

After the first report of the *V. parahaemolyticus* food poisoning case in Japan (20), epidemiology of *V. parahaemolyticus* food poisoning has been studied in many parts of the world (21–27). The O and/or K antigen serotype and KP phenotype of the strains isolated from the clinical specimens and incriminated food have been examined and used as the tools in many epidemiological investigations. Antisera for O:K serotyping are commercially available (Denka Seiken Co., 3-4-2 Nihonbashi, Kayabacho, Chuo-ku, Tokyo, Japan). The method of agglutination test using the antisera is described in detail by Kaysner et al. (28). Atypical biochemical characteristics such as indole negativity and urease positivity have attracted attention in some investigations (19,29).

As described above, although the KP phenotype has long been used as the marker for virulent strains, strains capable of producing TDH and/or TRH are now considered important. It is not easy to prepare Wagatsuma agar, and interpretation of the hemolysis on this medium may require some experience. Therefore, the KP phenotype has not often been examined in recent investigations. It has been replaced by immunological methods to detect TDH production. These include a precipitation test (30), a reversed-phase passive agglutination test (31), and various ELISA methods (32,33). A kit based on the reversed-phase agglutination reaction using latex beads coated with rabbit anti-TDH immunoglobulin G is commercially available (Denka Seiken Co.). ELISA-based methods for the detection of TRH have been reported (34,35), but a commercial detection kit that allows specific detection of TRH is currently not available.

With the advent of molecular genetic techniques, the methods that allow detection of the genes (*tdh* and *trh*) encoding TDH and TRH, respectively, were made available. Polynucleotide and oligonucleotide probes specific to the *tdh* gene (36,37) and the *trh* gene (35,38) were reported. Polymerase chain reaction (PCR) methods for detection of the *tdh* and *trh* genes were developed and shown to

**TABLE 3** Detection of *tdh*, *trh1*, and *trh2* Genes in Clinical and Environmental Strains of *V. parahaemolyticus*

No. strains examined		Presence or absence of gene		
Clinical	Environmental	<i>tdh</i>	<i>trh1</i>	<i>trh2</i>
22	0	+	-	-
2	0	+	-	+
112	0	+	-	-
17	0	-	+	-
35	5	-	-	+
26	66	-	-	-

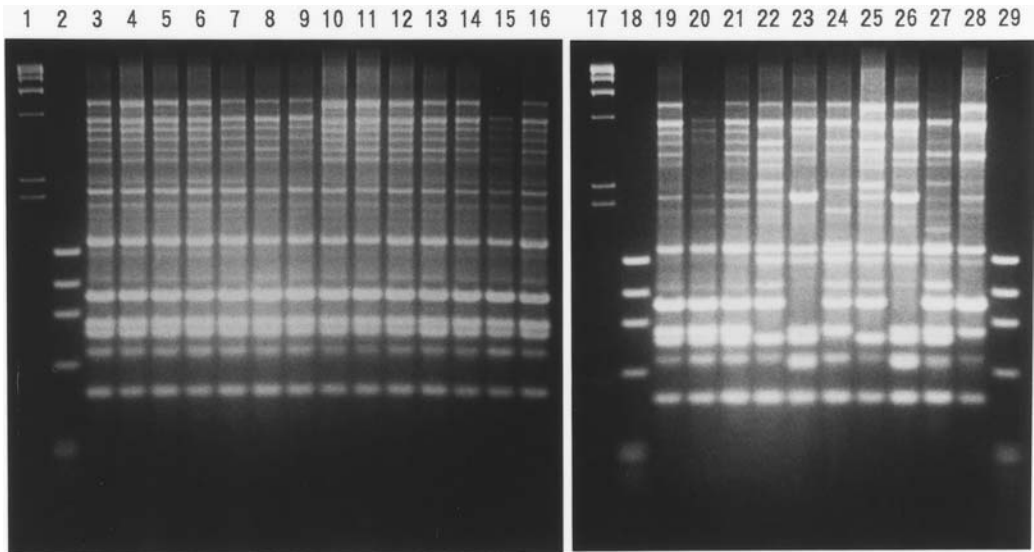
+, Present; -, absent.

Source: Ref. 38.

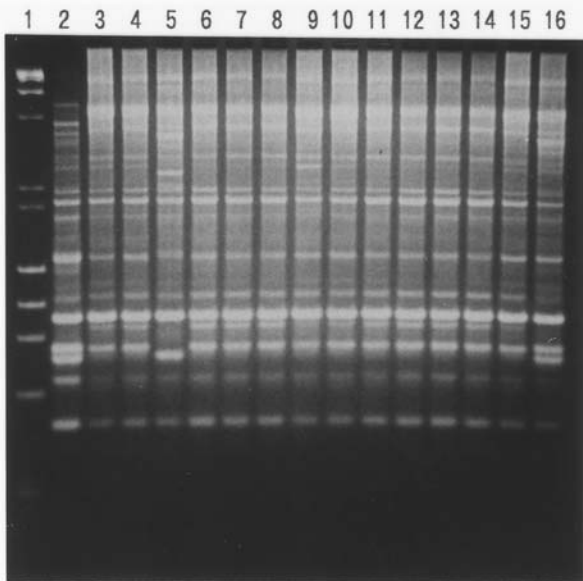
be highly specific (39,40). These PCR primers are commercially available (TAKARA SHUZO Co. Ltd., Otsu, Shiga, Japan). Molecular epidemiological studies demonstrated that the *tdh* and/or *trh* gene is present in most clinical strains but that very few environmental strains carry these genes (35,36,38). The result of one such study is shown in Table 3. However, observations that as much as 12% of the *tdh*-positive strains were KP negative and that most of these strains were isolated from clinical sources were confusing (36). The KP-negative strain carrying the *tdh* gene produced TDH at a very low level but could be converted to a KP-positive strain by a single base change in the *tdh* promoter (41). The chance of such a point mutation can be very high if there is some selective pressure for a high-level TDH producer in vivo. This supports the hypothesis suggested by molecular epidemiological studies that *tdh*-positive strains are clinically important regardless of the KP phenotype. Recently, urease-positive strains have been frequently noted among clinical strains, and there is a very strong correlation between possession of the *trh* gene and urease production (19,40,42–46).

The O:K serotype of both clinical and environmental strains is usually diversified and thus is useful in an epidemiological investigation. Predominance of a single serovar in a majority of infections has rarely been reported. O4:K12 strains producing urease and possessing the *trh* gene were frequently isolated from both clinical and environmental sources on the west coasts of the United States and Mexico in 1980s (40,47,48). This may be an epidemic caused by the strains belonging to a single group. Recently, the incidence of *V. parahaemolyticus* infection is increasing worldwide. A sudden increase in *V. parahaemolyticus* infection in Calcutta, India, after February 1996 was attributed to the emergence of a new clone of O3:K6 strains possessing the *tdh* gene but not the *trh* gene (49). This clone could be distinguished from non-O3:K6 strains and O3:K6 strains isolated in earlier years by an arbitrarily primed PCR method. This new O3:K6 clone was detected among the strains isolated from the travelers originating in Southeast Asian countries after 1995 (Fig. 1). The strains that apparently belong to the same clonal group were frequently isolated in Bangladesh, Laos, Thailand, Taiwan, Japan, and the United States during the 1997–1998 period (50). This may be due to a pandemic spread of the new O3:K6 clone, which has not been seen in the history of *V. parahaemolyticus* epidemiology.

The foods incriminated in *V. parahaemolyticus* infections are mostly seafoods or associated foods including raw seafoods, cooked seafoods, processed seafood materials, and foods containing seafood materials. Various kinds of seafood including fish, shellfish (oysters, clams, etc.), crab, lobster, and shrimp have been implicated (21,23,26). Many cases in Japan are attributable to consumption of raw seafoods, but this is usually not the case in other countries (21,23,26), where cases are more often due to insufficient cooking, recontamination, or improper storage. Foods not con-



(A)



(B)

**FIGURE 1** Genetic fingerprinting by an arbitrarily primed PCR method distinguishes the newly emerged O3:K6 strains from other strains of *Vibrio parahaemolyticus*. (A) Molecular weight markers (lanes 1, 2, 17, 18, and 29); O3:K6 strains newly emerged in India in 1996 (lanes 3–14 and 21); O3:K6 strains of the same clonal group that were isolated from the travelers coming from Indonesia, Thailand, and Singapore to Japan during the 1995–1996 period (lanes 15, 16, 19, and 20); non-O3:K6 strains isolated in India in 1996 (lanes 22–28). (B) Molecular weight markers (lane 1); the O3:K6 strain newly emerged in India in 1996 (lane 2); O3:K6 strains isolated from travelers coming from various Asian countries to Japan before the emergence of the new O3:K6 clone (lanes 3–16).

taining seafood materials may be implicated in food poisoning cases by way of secondary contamination (21).

Food poisoning cases due to *V. parahaemolyticus* are reported only in warm seasons in Japan and the United States, whereas in Thailand, cases are reported throughout the year (1,23,26). The difference in the seasonal incidence of infection is probably associated with seasonal occurrence of the epidemic strains in the marine environment. Although there are a number of studies reporting distribution of *V. parahaemolyticus* in the marine environment in many parts of the world, epidemiological studies investigating occurrence of virulent strains both in clinical specimens and the marine environment in an endemic setting are rare. Wagatsuma (51) reported that KP-positive strains belonging to K11 and K15 serotypes were isolated from both food poisoning cases and the neighboring marine environment in the same season in Japan. Kaysner et al. (52) isolated urease-positive O4 strains possessing the *tdh* gene from shellfish that might be associated with human illness in the Pacific Northwest of the United States. Application of genetic fingerprinting methods to the examination of the strains in this type of the studies would provide stronger support for the association between the clinical and environmental strains.

## V. ISOLATION AND IDENTIFICATION

*V. parahaemolyticus* can be isolated using a selective agar medium. Growth in an enrichment broth prior to the inoculation onto the selective agar medium is recommended for isolation of *V. parahaemolyticus* from environmental samples (seafoods, seawater, sediments, etc.) Clinical samples (stool and swab) may be inoculated onto the selective agar medium with or without the enrichment step. Various kinds of selective agar medium and enrichment broth have been suggested and evaluated (19,28,53–56). Currently, enrichment in alkaline peptone water at 37°C for 6–8 hours and isolation on TCBS agar are most frequently employed (19). Some workers suggest that salt polymyxin broth is more suitable for enrichment of *V. parahaemolyticus* in seafood samples. Our experience has indicated, however, that alkaline peptone water is superior. *V. parahaemolyticus* cells present in refrigerated or frozen seafood may be injured, and thus preenrichment of the samples in nonselective medium is suggested for better recovery of the injured cells (57). Many other tips and detailed protocols for isolation, including the methods for specimen collection and transport, are well described in the literature (19,28,58).

To screen the colonies appearing on a selective agar medium, reactions in the TSI slant medium, or in TSI, KIA, and arginine glucose slants, are employed in some protocols (28,58). A multitest screening medium specifically designed for *V. parahaemolyticus* is also useful for screening the colonies appearing on TCBS agar (49,59). Conventional biochemical tests have been used for identification of the isolated strains. Since *V. parahaemolyticus* is a marine bacterium, addition of NaCl to the test medium to the final concentration of 1% or 2–3% is recommended (19,28). A battery of biochemical tests used for identification of *V. parahaemolyticus* may differ slightly from laboratory to laboratory. Biochemical tests useful for differentiation of *V. parahaemolyticus* from other *Vibrio* spp. of clinical importance are suggested in the literature (19,28,54,55,60,61). Inoculated test media are incubated at 35–37°C in these tests. Since seafood samples may contain various nonpathogenic *Vibrio* spp., many biochemical tests may be required for correct identification of *V. parahaemolyticus* when seafood samples are examined (61). However, such extensive biochemical tests are not performed in routine examinations. Many marine vibrios require incubation temperatures below 35°C, and some species require marine salt-based medium for growth. Thus, such extensive efforts may be impractical.

The O:K serotyping of the identified strains is useful not only for an epidemiological study but for confirmation of the identification. The strains isolated from clinical sources or those implicated in food poisoning cases usually fall under the known O:K serotypes listed in [Table 1](#). There may be unusual strains that might become rough and O antigen typing may be difficult, or their K antigen does not react with any of the established antisera although their O serotypes can be determined.



The atypical strains are encountered more often when the strains isolated from environmental sources are examined.

The number of *V. parahaemolyticus* cells in test samples may be estimated by a three-tube, multiple dilution MPN enrichment method combined with the isolation and identification method (58) or by a membrane filtration method combined with a presumptive biochemical characterization method (28). *V. parahaemolyticus* is frequently detected in seafood in warm seasons, and the number of the cells is usually in the order of 10 per gram but can exceed 1000 per gram (6).

Of molecular genetic techniques, PCR methods are sensitive and can be made specific. Therefore, PCR can be utilized for detection or identification of *V. parahaemolyticus* in food samples. As described above, KP test or the detection of the *tdh* gene is useful for examination of clinical strains but not for identification since most environmental strains are negative in these tests. Recent analysis of *V. parahaemolyticus* genome indicated presence of the nucleotide sequences that appear to be specific to this species. Based on these findings, hybridization probes or PCR methods for specific detection of *V. parahaemolyticus* have been reported. The target sequences in these methods include hemolysins different from TDH and TRH and a sequence of unknown function (62–65). These target sequences were utilized in a universal PCR protocol and a multiplex PCR method that were designed for detection of various foodborne pathogens in food samples (66,67). Specificity of these target sequences is based on the results of examination of the limited number of the strains with known identification. Very recently, PCR methods targeting the variable regions within the *gyrB* and *toxR* genes have been developed for detection or identification of *V. parahaemolyticus* (68,69). These genes are well conserved not only in *V. parahaemolyticus* but in other species as well, and the sequence variation of the genes may reflect phylogenetic relationship among the species.

## VI. PATHOGENICITY

As described above, molecular epidemiological studies demonstrated that not only KP-positive strains but some KP-negative strains that carry the *tdh* and/or *trh* gene are strongly associated with gastroenteritis cases in humans. The ability of KP-positive strains to cause gastroenteritis in humans was demonstrated by volunteer studies and accidental laboratory infection (70–72). More than  $2 \times 10^5$  cells were needed to induce gastroenteritis. Administration of  $10^8$  cells could cause diarrhea in monkeys (73). KP-negative strains were less active in human and animal experiments. KP-positive strains are generally more potent than KP-negative strains in inducing fluid accumulation in rabbit ileal loop models (74,75). Inoculation of more than  $10^7$  cells was needed in this model (74). Definitive evidence that TDH is a major enterotoxin was obtained by comparison of a KP-positive strain and its isogenic TDH-deficient strain (described below) in the rabbit ileal loop test and the Ussing chamber test using the rabbit ileal tissue; the mutant strain lost its ability to induce positive response in both tests (76).

Many characteristics of TDH were clarified using pure preparation of TDH (17). TDH was named after the observation that its hemolytic activity was not inactivated by heating at 100°C for 10 minutes and that its activity was not enhanced by the addition of lecithin (thus its action is direct). It is composed of two identical subunits of  $\sim 21,000$  daltons (estimated by SDS-polyacrylamide gel disc electrophoresis). TDH exhibits hemolytic activity against erythrocytes of various animals except horse. TDH binds to the cells through ganglioside receptors,  $G_{T1}$  and forms a pore in the membrane (77,78). Of the amino acid residues of the TDH subunit, Gly at position 90 and Leu at position 65 from the N terminus appear to be particularly important for hemolytic activity (79,80). Studies suggest that TDH stimulates ion flux in myocardial tissue and in rat and human erythrocytes (78,81,82). TDH was recently shown to use calcium ion as an intracellular second messenger and induce intestinal chloride ion secretion in a rabbit model (83). TDH was also shown to have lethal effects on small experimental animals, cytotoxic activity in respect to various cultured cells, and the ability to increase vascular permeability in rabbit skin (17,84). TDHs encoded by the *tdh* genes

in KP-negative strains were demonstrated to have biological activities similar to those of KP-positive strains (85).

TRH is, like TDH, composed of 189 amino acid residues and shares 62–63% amino acid sequence homology, structural similarity in higher dimensions, and antigenic determinant(s) with TDH (5,38,86,87). Unlike TDH, the hemolytic activity of TRH is destroyed by heating at 100°C for 10 minutes (5). TRH can be divided into two types based on the nucleotide sequence variation in the genes encoding TRH (84% identity between the *trh1* and *trh2* genes), and the profile of hemolytic activity to various species of erythrocytes among TDH and two TRH types differ (5,38). Purified TRH and the *trh*-bearing strains induced fluid accumulation in the ligated ileal loop in a similar manner as TDH and *tdh*-bearing strains (34,87). An attempt was made to demonstrate the role of TRH in pathogenicity using a *trh*-deficient mutant (88). However, the mutant still carried a part of the gene, and definitive conclusions could not be drawn.

General disease symptoms (Table 2) and death due to *V. parahaemolyticus* infection may be explained at least in part by various biological activities of TDH and TRH. Other factors of *V. parahaemolyticus* may also contribute to the disease. These possible virulence factors are not always specific to clinical strains but may play some auxiliary roles. Adherence of *V. parahaemolyticus* to various cells and tissues, including human intestinal tissues, has been reported; the property was better correlated with KP-positive strains in some cases, but the correlation was not always observed (89–94). Pili may be involved in adherence of the organism. Different types of *V. parahaemolyticus* pili have been reported. One of the types was shown to be involved in adherence of an O2:K3 strain to rabbit intestinal tissue, but its role in adherence of the organism to human intestinal tissue is unclear (95). Cell-associated hemagglutinin that is distinct from pili was shown to be implicated in adherence of *V. parahaemolyticus* to rabbit enterocytes and possibly to human colonic cells (96,97). Outer membrane proteins were also suggested to be involved in adherence of *V. parahaemolyticus* to rabbit intestinal epithelial cells (98). *V. parahaemolyticus* strains, regardless of their KP phenotype, were found to invade into the tissue of intestine and other organs in rabbit ileal loop tests and into the cultured cells (99–101). *V. parahaemolyticus* appears to be able to acquire iron from humans through a siderophore termed vibrioferrin (102,103). The system may support the growth of this organism in the human host although its relevance to disease is unclear. Hemolysins distinct from TDH and TRH have been characterized at molecular genetic levels; the genes encoding a thermolabile hemolysin named TLH and a thermostable hemolysin termed delta-VPH were present in all strains, including KP-negative strains, of *V. parahaemolyticus* (62,104,105). The TLH was shown to be an atypical phospholipase (106). Presence of other factors including a lethal factor, Shiga-like toxin, and CHO cell elongation factor has been reported, but these factors have not been well characterized (107–109).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

TDH is the only virulence factor whose pathogenic role has been established by epidemiological data and a large body of experimental data. A review covering genetic studies on the *tdh* gene and related subjects has been published (110). Interested readers are advised to read this review. The essence and additional information are described, and only critical references are indicated below.

The *tdh* gene was cloned not only from representative strains of *V. parahaemolyticus* but from some other *Vibrio* species and their structures compared (Table 4). KP-positive strains carry two *tdh* genes represented by *tdh1* and *tdh2*. KP-negative strains usually have a single *tdh* gene copy. The genes cloned from KP-negative strains are represented by *tdh3* to *tdh5*. The *tdh3* gene is chromosomally located, and the *tdh4* gene is unusual in that it is borne on a plasmid and both of these genes were cloned from the same strain. The *tdh5* gene was cloned from a KP-negative strain that also carried a *trh* gene. Rare strains of *Vibrio cholerae* non-O1 and *Vibrio mimicus* carried, respectively, a plasmidborne gene (NAG-*tdh*) and chromosomally located gene (Vh-*tdh*). All strains of *V. hollisae* examined so far had the *tdh* gene, represented by Vh-*tdh*. Two representative *tdh*-related

**TABLE 4** Representative *tdh* and *trh* genes

Designation	Origin	% Identity with <i>tdh2</i> gene
<i>tdh1</i>	<i>V. parahaemolyticus</i> WP1 (KP+), chromosome	97.0
<i>tdh2</i>	<i>V. parahaemolyticus</i> WP1 (KP+), chromosome	100
<i>tdh3</i>	<i>V. parahaemolyticus</i> AQ3776 (KP-), chromosome	98.6
<i>tdh4</i>	<i>V. parahaemolyticus</i> AQ3776 (KP-), plasmid	98.6
<i>tdh5</i>	<i>V. parahaemolyticus</i> AQ3860 (KP-), chromosome	98.9
NAG- <i>tdh</i>	<i>V. cholerae</i> non-O1 91, plasmid	98.6
Vm- <i>tdh</i>	<i>V. mimicus</i> 6, chromosome	97.0
Vh- <i>tdh</i>	<i>V. hollisae</i> 9041, chromosome	93.3
<i>trh1</i>	<i>V. parahaemolyticus</i> AQ4037 (KP-), chromosome	68.6
<i>trh2</i>	<i>V. parahaemolyticus</i> AT4 (KP-), chromosome	68.8

Source: Ref. 110.

hemolysin genes (*trh1* and *trh2*) are also listed in Table 4. All *tdh* genes and *trh* genes encoded proteins of 189 amino acid residues (including signal peptides), and the predicted secondary structures of the proteins were very similar. When these genes were made to express under the control of exogenous promoters in *Escherichia coli* backgrounds, hemolysins with biological activity were produced. These genes appear to have evolved from a common ancestor by accumulation of base changes so that the fundamental structure and function of the proteins were maintained. Expression of the *tdh2* gene from its own promoter is strong and is the only gene that contributes to KP (111). Expression of another *tdh* gene (*tdh1*) of a KP-positive strain and *tdh3*–*tdh5* genes of KP-negative strains are relatively low (<5% of the *tdh2* gene) and these are attributable to a two-base (*tdh1*) or one-base difference (*tdh3*–*tdh5*) in the important region of promoter sequence from the corresponding bases of the *tdh2* gene (41). The TDH-deficient isogenic strain described above in the pathogenicity section was derived from a KP-positive strain by successive inactivation of the *tdh1* and *tdh2* genes using allelic exchange methods (76). Like *V. cholerae*, *V. parahaemolyticus* has a transcriptional activator, ToxR, in the membrane; the interspecies homology of the *toxR* genes is ~55% (112). The *tdh2* expression is enhanced up to fivefold by the activity of ToxR in a culture medium-dependent manner. Interestingly, a KP-positive strain caused considerably less fluid accumulation in rabbit ileal loops when the *toxR* gene was specifically inactivated. Thus, ToxR appears to play an important role in TDH-mediated stimulation of fluid secretion by the KP-positive strain. Expression of the *trh1* gene was as low as that of the *tdh1* gene (41). The *trh2* gene was expressed at a lower level than was the *trh1* gene (38). The reason for the low-level expressions of the *trh* genes is currently unknown.

Distribution of the *tdh* genes only in a minority of *V. parahaemolyticus* strains in the environment and in some strains of other *Vibrio* species can be explained by horizontal transfer of the *tdh* gene. Plasmids may play a role as a vehicle of transmission. Detection of rare plasmidborne *tdh* genes besides the chromosomal *tdh* gene can be explained by transfer of the *tdh* gene between the two replicons. The mobility of the *tdh* gene is supported by the finding that the *tdh* gene is flanked by insertion sequence–like elements (named ISVs), and this unit exists as a transposon-like structure (113). The ISV sequences are similar to that of a known insertion sequence IS903. However, *tdh* rearranger appears to have taken place in the past with the transposon-like structure undergoing genetic rearrangement as well as accumulation of base changes. Some of the *tdh* genes are now flanked by truncated forms of ISV or only by the terminal inverted repeats of the ISV.

Phylogenetic relationship of the *tdh/trh* genes of various *Vibrio* species and 16S rRNA sequence-based phylogeny of these species did not correlate well (114). This also supports the horizontal transfer hypothesis of the *tdh* gene. The *tdh4* gene of *V. parahaemolyticus* and the NAG-*tdh*

gene of *V. cholerae* non-O1 were present on 35 kb plasmids. Nucleotide sequences of the two *tdh* genes were 100% identical, and the two plasmids were very similar (115). The results suggest a possibility that this type of plasmid may be at least one of the vehicles of *tdh* transmission. However, direct evidence for transfer of these plasmids has not been obtained. Other plasmids or bacteriophages could also have played a role in *tdh* transfer. Very recently, filamentous phages of *V. parahaemolyticus* have been characterized and were shown to have a potential to mediate gene transfer to plasmid and chromosome (116). These phages were related to CTX phage of *V. cholerae* and M13 coliphage of *E. coli*.

The above findings strongly suggest that the *tdh* gene, the major virulence gene of *V. parahaemolyticus*, was acquired from some other organism by horizontal transfer and that only minor population of *V. parahaemolyticus* strains in the marine environment carry the gene. Few studies have been done on the *trh* genes so far, but available data suggest that *trh* genes are similar to *tdh* genes in many respects. The same scenario can probably be applied to *trh* genes.

## VIII. CONTROL MEASURES

Since it is generally accepted that consumption of more than  $10^5$  viable cells of virulent strains (most likely *tdh*- or *trh*-bearing strains) is required to establish *V. parahaemolyticus* infection, control measures to prevent this will be needed.

Although the proportion of *tdh*- or *trh*-positive strains in the environment may be very low, they do exist and can contaminate seafood. In general, *V. parahaemolyticus* can become prevalent in seawater in warm seasons, and raw seafood can carry *V. parahaemolyticus* at high levels. Particularly, shellfish, shrimp, and crabs may accumulate the bacterial cells at high levels in their internal organs. Setting a reasonable upper limit of contamination (e.g., 100 cells per gram of the food, as recommended by the International Commission on Microbiological Specification for Foods) may be one possible way of prevention (19). Such a standard, if possible, would encourage the efforts of depuration and cleaning of surface contamination. However, it may be very difficult to enforce such a public health measure unless there is an urgent need.

General measures to prevent foodborne bacterial infections are important in controlling food poisoning due to *V. parahaemolyticus* infection. An effective measure is a cold treatment of the raw seafood during transportation and marketing. There are many reports that the number of *V. parahaemolyticus* cells slightly decrease or are maintained at refrigeration or freezing temperatures (6,19). The most effective and essential method is proper cooking by heating. This is a well-known practice, but insufficient heating could result in survival of low numbers of *V. parahaemolyticus* cells in the cooked food (6). In addition, attention has to be paid to prevent cross-contamination of other food during cooking. It is not uncommon to encounter *V. parahaemolyticus* food poisoning cases attributable to foods not containing seafood or seafood products (21).

*V. parahaemolyticus* in seafoods or related foods may be killed effectively not only by heating but by other measures including irradiation or treatment with various chemicals or biochemicals (6). The surviving cells present even at low levels in cooked or processed food, however, can propagate rapidly unless the food is stored properly. As described above, *V. parahaemolyticus* grows much faster than other foodborne pathogens under favorable conditions.

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# 16

## ***Vibrio vulnificus***

**Gaskov Clergé\***

*U.S. Food and Drug Administration and  
Howard University, Washington, D.C., U.S.A.*

**Broderick E. Eribo**

*Howard University, Washington, D.C., U.S.A.*

**Ben D. Tall**

*U.S. Food and Drug Administration, Washington, D.C., U.S.A.*

### **I. BACKGROUND**

*Vibrio vulnificus* (*vulnificus* = Latin adj.; inflicting wounds) is a halophilic, gram-negative bacterium that is considered to be one of the most virulent bacterial pathogens known. It is found as a member of the microbial populations associated with estuarine waters and sediments, and various marine species such as plankton, shellfish, and finfish (1–13). Its discovery, however, occurred not as a result of its ability to cause fatal foodborne illnesses, but because of its ability to cause wound infections (14). The first description of human illness produced by this organism is thought to have been recorded by Hippocrates in the fifth century B.C. (15). However, its present-day history began with the description by Roland in 1970 (14) of a previously healthy man who developed vomiting, diarrhea, and a generalized hemorrhagic rash after recreational clamming and bathing off the northeast coast of the United States. Results from a bacteriological analysis of clinical samples yielded a gram-negative bacillus that was subsequently identified by the U.S. Centers for Disease Control and Prevention (CDC) as a strain of atypical *Vibrio parahaemolyticus* or *Vibrio alginolyticus*. In 1975, Fernandez and Pankey (16) reported similar findings in an investigation of three individuals with analogous clinical descriptions of soft tissue infections produced by a group of salt-requiring, lactose-fermenting *Vibrio* species, similar but not identical to *V. parahaemolyticus*. These authors realized that the causative agent was not *V. parahaemolyticus*, nor was it *V. alginolyticus*. However, they noted difficulties with the taxonomic characterization of these strains and other members of the genus *Vibrio*. Noteworthy, their report reemphasized Roland's original findings, and they counseled all clinicians to consider the possibility of this pathogen in any patient with a wound acquired in relation to saltwater or seafood. Baumann and Schubert (17) studied these isolates and designated them enteric group C-2. This was followed by a report by Weaver and Ehrenkranz (18), which surveyed a collection of 34 isolates, including the isolate from Roland's investigation, for taxonomic relatedness based on similarity of biological traits and concluded that these microorganisms should be classified within the genus *Vibrio*; it was their opinion that they should be regarded as a subspecies of *V. parahaemolyticus* or possibly given status as a new species based on these strain's lactose-

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\* Deceased.

fermenting characteristic. This was succeeded by a taxonomic investigation by Hollis et al. (19) that confirmed that lactose fermentation and production of the enzyme  $\beta$ -galactosidase could be used as a criterion to differentiate these isolates from *V. parahaemolyticus* and *V. alginolyticus*.

At this point infectious disease specialists were not sure if they were dealing with one organism that produced two different clinical infections or two closely related pathogens, each capable of causing primary septicemia and wound infections. To answer this question, Blake et al. (20) carried out an epidemiological investigation in 1979 that characterized 39 illnesses in the United States associated with isolation of this newly described, yet unnamed, marine vibrio. It was discovered that some (24 of 39) of the illnesses began with an abrupt onset of systemic symptoms, such as fever, chills, and hypotension, suggestive of septicemia without an apparent primary focus of infection. The remaining 15 illnesses began with the contamination of a wound or ulcer with saltwater or "shellfish drippings." Other important findings were that (a) the primary septicemia syndrome was usually preceded by consumption of raw oysters, often within 24 hours prior to onset of symptoms, (b) 75% of the primary septicemia patients also had preexisting hepatic disease, and (c) most cases (85%) occurred during relatively warmer months of the year (May to October, Northern Hemisphere). From these results it became clear that a single organism was responsible for both of these distinct clinical syndromes and that these organisms could infect humans using two different portals of entry.

To better understand the taxonomic relationship of this unnamed organism to other known *Vibrio* species, Reichelt et al. (21) and Clark and Steigerwalt (22) independently performed in vitro DNA-DNA hybridization analyses on a collection of archival strains. Taken together, the results showed that these organisms were genetically related (by >82% DNA homology) and that they were distinct from all currently recognized *Vibrio* and *Photobacterium* species. On the basis of these data, these lactose-fermenting marine vibrios were given the name *Beneckeia vulnificus* (21). However, Baumann et al. (23), in concurrence with Farmer (24), suggested that these organisms be reassigned to the genus *Vibrio* under their present-day designation, *V. vulnificus*.

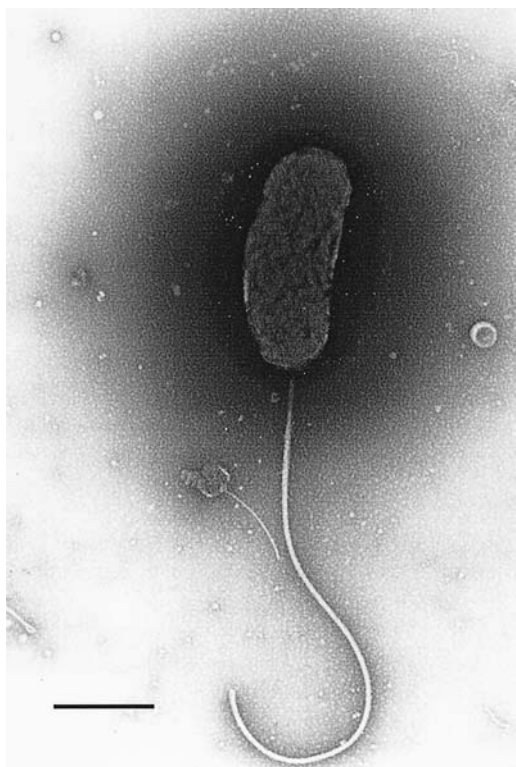
Due to biases inherent to all epidemiological investigations, such as the lack of complete case data and the inability to monitor disease incidence and relative frequencies, Tacket et al. (25) performed a case-control study of *V. vulnificus* isolates obtained from 30 patients submitted to the CDC between 1981 and 1982. Their findings supported those conclusions summarized earlier by Blake et al. (20) that patients with primary sepsis were more likely than controls to have eaten raw eastern oysters (*Crassostrea virginica*) 2 weeks prior to onset of illness and were more likely to have a history of hepatic disease. Additional observations from this study suggested that patients with wound infections were more likely than controls to have had recent exposures of the skin to saltwater or "shellfish drippings" as well as to have a history of hepatic disease. *V. vulnificus* infection was subsequently added to the list of reportable diseases in 1981 (26).

Though the source of infection associated with primary septicemia had been postulated to be the consumption of raw oysters, Johnston et al. (27) were the first to provide proof that this hypothesis was valid in 1983. They isolated *V. vulnificus* both from a blood culture obtained from a septic patient who was diagnosed with relapsing acute myeloblastic leukemia and from his raw oyster meal. However, the patient did not experience diarrhea. In that same year, Pollak et al. (28) also described a case where *V. vulnificus* was isolated from blood and stool cultures of a 65-year-old man who had underlying alcoholic liver disease. It was also possible in this case to demonstrate the presence of high antibody titers to the blood isolate by indirect immunofluorescence, but not by agglutinating or vibriocidal tests. In another report by Johnston et al. (49), *V. vulnificus* was implicated as a cause of acute gastroenteritis. Finally, a report from Romania by Ciufecu et al. (29) presented evidence that *V. vulnificus* strains isolated from environmental waters (Black Sea) were no different from isolates obtained from three adult cases of mild diarrhea. These studies together suggest that acute gastroenteritis may be a third clinical presentation of disease caused by this pathogen. Recent evidence supporting this has been promulgated by the epidemiological findings of Klontz et al. (30) and those of Shapiro et al. (31), which demonstrate that primary septicemia, wound infections, and gastroenteritis are distinct disease entities caused by this marine vibrio and further-

more argue that the epidemiology and pathogenicity of *V. vulnificus* will only be better understood when the ecological nuances of the microbe-host dynamics in the aquatic ecosystem are elucidated.

## II. CHARACTERISTICS

*V. vulnificus* is a gram-negative, halophilic, oxidase-positive, facultative anaerobic, encapsulated bacillus measuring approximately 0.5–0.8  $\mu\text{m}$  in width and 1.4–2.6  $\mu\text{m}$  in length with an ultrastructure typical of most other gram-negative bacteria (Fig. 1). They do not form endospores or microcysts, but become spherical when grown under culture conditions where low levels of nutrients are found, culminating in a viable but nonculturable dormancy state (32). To date, characterizations of *V. vulnificus* strains, at both the phenotypic and genotypic levels, have not recognized unique traits that can be used to estimate pathogenicity for humans. However, characterization of strains has led to the subdivision of the species into three biotypes or biogroups based on phenotypic, serological, and host range differences (12,13,33–37). Biotype 1 strains were originally thought to be pathogenic only for humans and biotype 2 strains only for eels. However, recent evidence brought forth by Hoi et al. (35) demonstrates that biotype 1 can infect eels, and evidence reported by Amaro and Biosca (38) shows that biotype 2 strains can infect humans. Biotype 3 strains have only recently been isolated (36,37,39), and little is known about their ecology, pathogenicity, or host range. We would also like to point out that mass mortalities caused by *V. vulnificus* have been observed among cultured shrimp species such as *P. monodon* and *P. japonicus* in Taiwan (40), and *V. vulnificus* has



**FIGURE 1** Transmission electron photomicrograph of glutaraldehyde fixed *Vibrio vulnificus* cells stained with 2% sodium phosphotungstic acid, pH 6.8. Bar marker = 0.1  $\mu\text{m}$ .

also been isolated from surface lesions associated with moribund black porgy fish (*Acanthopagrus schlegeli*) (41).

Originally, *V. vulnificus* strains could be phenotypically separated from the other marine vibrios by its ability to ferment lactose. With the greater use of genetic and biochemical techniques in studies designed to examine the relatedness of members of this species and those of the genus *Vibrio*, we now know that *V. vulnificus* is not the only vibrio capable of fermenting lactose; strains of *Vibrio cholerae*, *Vibrio mimicus*, *Vibrio metschnikovii*, *Vibrio fluvialis*, and *Vibrio nigripulchritudo* can also ferment lactose. Thus, the utilization of lactose by *V. vulnificus*, as suggested by Baumann et al. (17), should be regarded with caution as a taxonomic trait for this species. The phenotypic characteristics of this organism are shown in Tables 1 and 2 and indicate that *V. vulnificus* can be phenotypically distinguished from other marine vibrio species by a number of distinct traits. For example, *V. cholerae* requires only trace amounts of Na<sup>+</sup> for growth and the fermentation of lactose

**TABLE 1** Biochemical Test Results and Other Properties of *Vibrio vulnificus*<sup>a</sup>

Test	Percentage positive <sup>b</sup>
Indole production (HIB, 1% NaCl)	97
Voges-Proskauer, Barritt (1% NaCl)	80
Arginine dihydrolase, Moeller's (1% NaCl)	0
Lysine decarboxylase, Moeller's (1% NaCl)	99
Ornithine decarboxylase, Moeller's (1% NaCl)	55
D-Glucose, acid production	100
D-Glucose, gas production	0
Acid production from:	
L-Arabinose	0
D-Arabitol	0
Cellobiose	99
Lactose	85
Maltose	100
D-Mannitol	45
Salicin	95
Sucrose	15
Nitrate-6-nitrite	100
Oxidase	100
Lipase (corn oil)	92
ONPG test	75
Growth in nutrient growth with:	
0% NaCl	0
1% NaCl	99
8% NaCl	0
10% NaCl	0

<sup>a</sup> Each test is part of a recommended set of tests suggested by CDC for routine identification of *Vibrio* species. 1% NaCl in parentheses indicates that 1% NaCl had been added to the standard media to enhance growth; HIB, heart infusion broth; ONPG, *o*-nitrophenyl- *D*-galactopyranoside.

<sup>b</sup> Percentage positive after 48 h incubation at 36°C.

Source: Adapted from Ref. 43.

**TABLE 2** Characteristics That Distinguish *Vibrio vulnificus* from Other *Vibrio* Species and Related Genera

Test	<i>V. vulnificus</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>	Other vibrios	<i>Aeromonas</i>	<i>Plesiomonas</i>
Arginine dihydrolase	–	–	–	–/+	+/-	
Lysine decarboxylase	+	+	+	–/+	–	
Ornithine decarboxylase	– <sup>a</sup>	–	+	–/+	+/-	
ONPG	+ <sup>a</sup>	–	–	–/+	+	
Indole production	+ <sup>a</sup>	+	+	+/-	+/-	
Voges-Proskauer	–	+	–	–/+	+/-	
Fermentation of:						
Lactose	+ <sup>a</sup>	–	–	–	–/+	/–
Salicin	+ <sup>a</sup>	–	–	–/+	–/+	
Sucrose	–	+	–	+/-	+/-	
Growth in 1% peptone w/:						
0% NaCl	–	–	–	–/+	+	
1% NaCl	+	+	+	+	+	
8% NaCl	–	+	+	–/+	–	
10% NaCl	–	–	–	–	–	

Symbols: +, most strains (~75%) positive; –, most strains (~75%) negative; +/-, strains mostly positive; –/+, strains mostly negative.

<sup>a</sup> *V. vulnificus* biotype 3 strains are lactose and salicin fermentative negative and negative for ONPG test; biotype 2 strains are ornithine decarboxylase negative.

Source: Adapted from Refs. 17 and 43.

is delayed; *V. nigrapulchritudo* produces a black pigment; *V. alginolyticus* is negative for both the ONPG (*ortho*-nitrophenyl-D-galactopyranoside) test and lactose fermentation; and *V. parahaemolyticus* is positive for acid production from L-arabinose. *V. fluvialis* is positive for acid production from L-arabinose, arginine dihydrolase positive, and lysine decarboxylase-negative, and negative for acid production from salicin; and *V. metschnikovii* is VP-positive and oxidase-negative and unable to convert nitrate to nitrite (42,43). Motile strains of *V. vulnificus* express a polar, sheathed flagellum, and expression of lateral flagella has not been found. Although *V. vulnificus* strains were originally reported to be sucrose-negative by Hollis et al. (19), the CDC has observed over recent years an increase in the number of sucrose-positive strains (43). Even though it is considered to be an obligate halophile, the Na<sup>+</sup> requirement can be satisfied through the use of NaCl in the growth medium; the optimal NaCl concentrations appears to be between 1 and 3%, although the 0.5% NaCl present in many routine laboratory media, such as blood agar, trypticase soy agar, and brain heart infusion agars, provide enough Na<sup>+</sup> for very good growth (3,44). Recently, Azanza et al. (45) recommended using 0.1% peptone in 3% NaCl as a diluent for enumeration studies because a significant loss in viability of *V. vulnificus* was demonstrated in both broth cultures and seeded oyster homogenates when PBS was used as the diluent. Our experience is that 0.9–1.0% saline or artificial seawater at 20 ppt will suffice as an adequate diluent for many studies including preparation of inocula for infectivity studies and identification using the API 20 E identification kits (46). Kelly reported that the optimal temperature for growth of *V. vulnificus* is 37°C (3). However, it will grow at other temperatures. Aeration by agitation will also favor increased growth (44).

Though there are other marine vibrios that are bioluminescent, such as *Vibrio fischerii* and *Vibrio harveyi*, there has only been one report of a luminescent strain of *V. vulnificus* that was isolated from a patient with a fatal wound infection. This is the first description of a human infection caused by a luminescent bacterium (47). Biochemically, strains of biotype 2 and 3 closely resemble strains of biotype 1. Important taxonomic and biochemical traits used to differentiate the three biotypes are shown in Table 3. Biotype 1 strains can be differentiated from biotype 2 strains based on positive reactions for ornithine decarboxylase, indole production, and mannitol fermentation, and a

**TABLE 3** Biochemical Characteristics of the Three *Vibrio vulnificus* Biotypes

Test	Biotype		
	1	2	3
Oxidase	+	+	+
Arginine dihydrolase	–	–	–
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	–	+
Sucrose fermentation	–	–	–
Indole production	+	–	+
D-Mannitol fermentation	+	–	–
D-Sorbitol fermentation	–	+	–
Citrate utilization (Simmons')	+	+	–
Salicin fermentation	+	+	–
Cellobiose fermentation	+	+	–
Lactose fermentation	+	+	–
ONPG test	+	+	d

+, Most strains (~75%) positive; –, most strains (~75%) negative; d, delayed.

Source: Adapted from Ref. 37.



negative reaction for D-sorbitol fermentation. Biotype 3 strains are indole-positive, salicin-negative, cellobiose-negative, citrate-negative, and lactose-negative and have a delayed reaction for ONPG.

Besides biochemical analyses, bacteria can be further characterized by using bacteriophage typing methods (48). Considerable information is known about the Classical, El Tor, and O139 bacteriophage (or phage particles) infectious for *V. cholerae*, the type species of the genus (17, 43,49). Nevertheless, little is known about phage particles infectious for *V. vulnificus*. To date, only three studies have been published (50–52). Pelon et al. (50) found phage particles infectious for *V. vulnificus* in estuarine waters, sediments, plankton, crustacean, molluscan shellfish, and the intestines of finfish of the U.S. Gulf Coast. Encapsulated strains were more susceptible to infections by the phage than unencapsulated strains, suggesting that the capsule may be involved in attachment of the phage. The identification of phage receptor(s) and their role in pathogenicity is currently not well understood. DePaola et al. (52) further characterized several phage particles isolated from a variety of ecniches by transmission electron microscopy (TEM) and showed that they morphologically belonged to three different families of double-stranded DNA phage representing the Podoviridae, Styloviridae, and Myoviridae phage families. One morphotype, identified as a member of the Podoviridae family, was found to be ubiquitous in Gulf Coast eastern oysters. This morphotype has an elongated capsid (mean, 258 nm; standard deviation, 4 nm;  $n = 35$ ) and was also found in sediment and in the water column. Oysters were found to have much greater phage densities than other habitat samples. Within oyster tissues and fluids, the lowest densities of phage particles were seen in hemolymph and mantle fluids (51). Phages were found throughout the year in Eastern oysters collected from estuaries adjacent to the Gulf of Mexico (Apalachicola Bay, FL; Mobile Bay, AL; and Black Bay, LA) and the abundance of phage particles ranged from  $10^1$  to  $10^5$  phage particles per g of oyster tissue.

Another widely used system of characterizing pathogenic bacteria is to determine plasmid diversity and molecular variation by restriction endonuclease fragmentation pattern (REFP) analysis (53,54). Marine vibrios such as *Vibrio anguillarum* are known to carry virulence plasmids (55, 56). Davidson and Oliver (54) found that of 42 clinical and environmental *V. vulnificus* isolates, 12% carried plasmids. In contrast, these authors found that 20 of 32 (62.5%) unidentified lactose-fermenting *Vibrio* spp. possessed plasmids with masses of 2.1–150 MDa. It was concluded that *V. vulnificus*, unlike most other *Vibrio* spp., shows a general lack of these extrachromosomal DNA elements.

*V. vulnificus* is the leading cause of reported deaths from foodborne illness in the United States (26). Studies comparing the virulence of isolates from different sources have yielded contradictory results (6,13,57–60). *V. vulnificus*'s ability to cause invasive disease in humans and in a variety of economically important seafood hosts demonstrates not only its unique (unknown?) qualities as a pathogen to overcome species barriers, but also its importance as a major bacterial pathogen, which greatly impacts aquaculture, marine fish farming, and public health issues.

### III. DISEASES

*V. vulnificus* is an opportunistic pathogen that is responsible for causing fulminating foodborne disease and debilitating wound infections in humans (20,61). *V. vulnificus* alone is responsible for 95% of all seafood-related deaths in the United States. An average of 50 cases per year in the United States (62) is reported to the CDC, which suggests that *V. vulnificus* infections in the United States are rare but may be underreported. *V. vulnificus* disease can clinically present as primary septicemia, wound infections, or gastroenteritis. It can enter the body by two different portals of entry, such as ingestion of raw or improperly cooked seafoods or through the contamination of an open wound or sore with seawater or seafood drippings. A survey of 426 cases of primary septicemia is shown in Table 4. Among primary septicemia patients, 91% had consumed raw oysters within one week of becoming ill. The incubation period is relatively short: approximately 26 hours. The most meaningful nonintestinal symptoms of illness include fever (92.3%) and chills (84.5%). It is interesting to note

**TABLE 4** Summary of Primary Septicemia, Wound Infections, and Gastroenteritis Produced by *Vibrio vulnificus*<sup>a</sup>

	Septicemias ( <i>n</i> = 426)	Wound infections ( <i>n</i> = 349)	Gastroenteritis ( <i>n</i> = 33)
Age (y)	53.7	57.6	41.0
Sex, % males	86.2	79.0	60.0
% of patients with:			
Fever	92.3	75.3	72.0
Chills	84.5	48.5	71.5
Hypotension	46.3	20.3	33.3
Nausea	59.5	37.5	71.0
Vomiting	43.6	37.5	48.5
Diarrhea	34.3	6.5	100.0
Abdominal pain	43.6	0	94.6
Secondary skin lesions <sup>b</sup>	67.6	80.0	3.0
Septicemia	100.0	46.6 <sup>d</sup>	3.0
% of patients with chronic diseases: <sup>c</sup>			
Cancer	9.5	6.5	20.8
Liver disorders	81.8	58.8	16.6
Diabetes	15.0	14.4	9.0
Alcoholism	33.1	22.6	14.0
% of patients with:			
Raw oyster consumption	91.0	11.0	84.6
Seawater/shellfish exposure	15.2	88.0	23.0
Amputation/debridement	25.4	67.7	0
Fatal outcome	57.2	30.4	0
Medium incubation time (h)	26.0	14.0	20.0
Death post admission (d)	4.2	4.6	0
Length of hospitalization (d)	14.8	13.6	8

<sup>a</sup> Values represent % of patients unless otherwise noted.

<sup>b</sup> Secondary skin lesions include hemorrhagic rash, cellulitis, bullae, and ecchymosis.

<sup>c</sup> Conditions are not mutually exclusive.

<sup>d</sup> Overall percentage was calculated from a subset of 15 cases.

Source: Refs. 30, 31, 37, 44, 64, 68, 85, 298, 299.

that many primary septicemia patients also demonstrated intestinal symptoms such as nausea (59.5%), vomiting (43.6%), abdominal pain (43.6%), and diarrhea (34.3%). However, *V. vulnificus* isolation from stool in primary septicemia patients has not correlated with presence of diarrhea. Interestingly, Johnston et al. (63) did note that in patients with primary septicemia, diarrhea occurred before onset of septic symptoms; however, only one of three patients had a stool specimen cultured and no pathogen was isolated. The most striking clinical manifestation displayed by 67% of patients with primary septicemia (summarized in Table 4) is the presentation and development of secondary cutaneous lesions described as a maculopapular rash, primarily affecting the legs (80%) (64). The affected extremity usually has marked erythematous swelling due to extensive intravascular thromboses and skin lesions described as ecchymosis, petechial hemorrhage, purpura, cutaneous bullae, necrotizing fasciitis, gangrenous changes, pyomyositis, and cellulitis. Other affected areas reported in case histories have been arms, entire body surfaces, abdomen, face, buttock, back, chest, and scrotum. The lesions do contain viable *V. vulnificus* cells (64). The skin eruptions are most often

present at the time of admission or usually develop within the first 24 hours of hospitalization and become more severe by the hour. Park et al. (64) reported that 91% of 70 primary septicemia patients had secondary skin lesions on admission. Minor lesions described as papules, wheals, and pustules have also been noted (64). Limb involvement has been described as a compartment syndrome where blood flow within an anatomical space is restricted due to edema and often the affected area becomes necrotic, requiring extensive surgical debridement or amputation (65–67).

As previously mentioned, immunocompromised individuals are at greater risk for developing serious illness than are immune-competent individuals (31). Predisposing health conditions include acquired immunodeficiency syndrome (AIDS) (68), aplastic anemia (69), refractory anemia (70), Diamond-Blackfan syndrome (71), diabetes mellitus, hepatitis B infection, leukemia (72), multiple myeloma, Hodgkin's lymphoma, metastatic cancer, gouty arthritis, gastric disease (requiring stomach acidity control through use of antacids or cimetidine), chronic renal failure (73), systemic lupus erythematosus (SLE) (73), steroid chemotherapy, liver dysfunction (and other metabolic iron disorders, such as alcoholic cirrhosis and chronic hepatitis) (20), hemochromatosis, thalassemia, anemia, and thrombocytopenia (20). In the majority of the primary septicemia cases summarized in Table 4, 98.0% of the patients had a preexisting condition; 86.2% of the patients were male, and the medium age was 53.7 years.

Liver disease was found in almost 81.8% of all sepsis cases and according to Shapiro et al. (31) can be present in 87% of all fatal septicemia cases compared to 74% of nonfatal infections. Of the primary septicemia patients summarized in Table 4, 91% ate oysters within 7 days of their illness. Shapiro et al. (31) reported that of the primary septicemia patients who ate oysters investigated in their study, 84% ate their oyster meal at a restaurant or oyster bar. The average number of oysters consumed was 10 (range 1–24).

Of the three modes of infection, a higher mortality rate is associated with cases of primary septicemia. Of the 426 primary septicemia cases summarized in Table 4, 57% resulted in a fatal outcome. Among those patients who develop hypotension (within 12 hours of admission), the mortality rate has been reported to be as high as 90%, with death culminating within 48 hours postadmission (68,74). The average time to death posthospitalization in the cases summarized in Table 4 varied considerably—approximately 4 days, with death occurring in spite of antibiotic and hypotensive therapies. Hospital stays for nonfatal patients are generally long and on average last for greater than 2 weeks. Shapiro et al. (31) reported that among primary septicemia patients, the average duration of illness was 3 days (range 1–89) in fatal infections and 16 days (range 2–110) in nonfatal infections. Several questions remain unanswered. Why is it that in some primary septicemia patients there is often lower limb involvement? Could this be a consequence of unrecognized perivascular disease? Little information is known about why the secondary skin lesions occur, other than that they contain viable organisms. Could this affinity for localization of the organism in the skin be related to the cell-mediated immunoproliferative processes and antigen presentation roles of skin dendritic cells, such as is seen with the Langerhans cells of the skin (75)?

In addition to being foodborne, *V. vulnificus* causes wound infections by entering already existing or concurrent wounds, ulcers, and insect bites. A survey of 349 cases of wound infections caused by this organism is shown in Table 4. Such wound infections are almost always related to exposure of a wound to seawater or shellfish drippings (88.0%) (Table 4). Reported wound scenarios include puncture wounds caused by a bite from a marine animal (e.g., crabs, stingray), stab or laceration wound from using a shellfish/fish cleaning utensil, lacerations obtained from commercial or recreational fishing gear use, rocks or debris in or near seawater, or contamination of an already existing wound or insect bite with seawater or shellfish drippings (44). It should be noted that such infections are common occupational injuries in commercial fishermen and have been referred to as “fish poisoning,” “salmon poisoning,” or “seal finger” (44). In a study reported by Shapiro et al. (31), 50% of persons with wound infections caused by *V. vulnificus* sustained the wound at the time of exposure and 21% reported a preexisting wound. Once *V. vulnificus* has breached the wound opening, systemic symptoms can occur even more rapidly than those seen in primary septicemia. This is reflected in the shortened incubation period (14 h vs. 26 h), as shown in Table 4. The

principal symptoms typically include intense pain (related to compartmental aspects of the infection), erythema, and edema at the site of injury, progressing to rapidly developing lesions, vesicles, hemorrhagic bullae (76), urticarial plaques, and to necrotizing fasciitis (65,77–81), where the necrotized area involves subcutaneous tissue and fascia, usually sparing muscular tissue. However, cases of rapidly progressing myositis and rhabdomyolysis have been reported (82–84). Nonetheless, because of widespread obliterative vasculitis and vascular necrosis, compounded by the rapidly spreading nature of the tissue necrosis seen in many wound infection patients, surgical debridement is routinely performed. Amputation is often needed to prevent the spread of infection (67.7%) (Table 4) and subsequent deep tissue necrosis that occurs at infection sites (74,85). Although mortality rates following wound infections have been reported to be as high as 43% (86), a mortality rate of 30% was calculated for the 349 cases cited in Table 4. Analyzing a subset of 72 patients (obtained from Refs. 16,30,63,85,87) where it was known whether septicemia was also involved, two distinct outcomes became evident: patients with wound infections that had an accompanied bacteremia had a fatality rate four times higher (31.2%, 10/32 cases analyzed) than that observed in those patients with wound infections without bacteremia (7.5% fatality rate, 3/40 cases). The presence of bacteremia in a wound infection patient may also explain the appearance of secondary bulbous lesions in some wound infection case descriptions (30,31,87).

As seen in cases of primary septicemia, wound infection symptoms also include fever (75%) and chills (48%). Other systemic symptoms such as hypotension, nausea, and vomiting are present, but at a much lower frequency. However, the existence of preexisting disease is still important in that over 57% of all patients with wound infections summarized in Table 4 presented with some underlying disease such as liver disease, diabetes mellitus, or alcoholism.

Gastroenteritis is the least studied disease syndrome caused by *V. vulnificus*. It is characterized by vomiting (48.5%), diarrhea (100%) or abdominal pain (94.6%), a stool culture yielding *V. vulnificus*, negative blood cultures, and no evidence of a wound. The average age of the 33 cases summarized in Table 4 is 41, of which 60% are males. The diarrhea is usually described as watery and profuse, but self-limiting. As has been observed in cases of both primary septicemia and wound infections, the existence of underlying chronic disease, such as liver disease (20,68,88–90) and alcohol abuse (91,92), is also an important contributor to disease severity. It is possible that gastroenteritis patients may also have primary septicemia (3%) (Table 4) that is not documented by blood cultures (31). The mean incubation period is approximately 20 hours, and consumption of raw seafood, especially oysters (84.6%), has been linked to disease. There have been no reported deaths associated with this disease entity.

There have been many other reports of various clinical manifestations caused by this organism, including epiglottitis (93), pneumonia (85,94), endometriosis (95), meningitis (96), coinfections with other marine vibrios (97,98), sepsis from solid organ transplantation (99), osteomyelitis (100), and peritonitis (101), and a few cases have included histopathological findings (44,76).

Most often, biotype 1 strains are the etiological agent responsible for the infections, and they usually occur as individual cases. However, biotype 3 has been reported to cause outbreaks of wound infections accompanied with bacteremia (37), and both biotype 1 and 2 strains can cause zoonotic outbreaks in cultured eels (33) and shrimp (40). Currently the global nature of the three biotypes is not known. Males seem to be more likely to get a *V. vulnificus* infection than are females; 75% of all cases summarized in Table 4 were males. This suggests that sex of the patient is an important profile entity. However, the demographic data summarized in Table 4 may not reflect regional cultural eating and food-preparation practices. For example, Bisharat et al. (37) reported that only 58% of the patients studied in the biotype 3 outbreaks in Israel were males. Does this lowered attack rate for males suggest the presence of cultural biases within this region, or are there unknown factors associated with biotype 3 virulence that led to the lower male infection rate? Additional studies are needed to further elucidate the pathogenesis, ecology, and factors responsible for the emergence of biotype 3 strains. A good geographic area to begin such investigations will be in countries that border the Mediterranean Sea. Lastly, since *V. vulnificus* is associated with many seafood hosts, of which the health status is not known, human disease caused by this organism, as well as other

marine vibrios, will become an increasingly more important food safety issue (102). This threat is compounded further because the demand for seafood is increasing and is currently being met only through the harvest of seafoods from aquaculture and marine fish farming sources. All of these issues highlight the need for surveillance programs to monitor the health status of our seafoods.

#### IV. EPIDEMIOLOGY

*Vibrio vulnificus* is an estuarine bacterium capable of causing rapidly fatal infections in humans. It is indigenous throughout estuaries and marine waters of the world (6,7,103–110). The organism has been isolated from plankton, shellfish, and water samples taken off the southeastern United States and Gulf Coast (57,111,112) and during the summer months, as far north in latitude as Portland, Maine (113), and Seattle, Washington (1). It has been cultured from waters and shellfish along the coasts of Puerto Rico (7), Taiwan (74,85,106,114), Hong Kong (115), India (116), Israel (137), Brazil (103,104), Romania (29), Korea (64), and Japan (79,117). Both biotype 1 and 2 strains have been isolated from environmental and clinical samples in Denmark (33,18–120), Italy (107), Australia (110), and Spain (121,122). Biotype 3 strains have only recently been found in Israel (37).

In the United States during 1997–1998, a total of 141 cases of *V. vulnificus* infections were reported to CDC’s voluntary surveillance system of culture-confirmed *Vibrio* cases (<http://www.cdc.gov/ncidod/dbmd/diseaseinfo/cstevib99.pdf>) (Table 5). Most of the reported cases involved wound infections (64/141, 45%) and primary septicemia (62/141, 44%), followed by 6 (4%) cases of gastroenteritis. The preponderance of the reported gastroenteritis and wound infections occurred between May and August, and the majority of the primary septicemia cases occurred between May and November. Among the *V. vulnificus* infections, 41 deaths were reported, which represented 89% (41/46) of all reported *Vibrio*-related deaths. Among the 300 reported hospitalizations due to *Vibrio* infections, 124 (41%) were because of *V. vulnificus* infections. These data support other evidence that suggests that *V. vulnificus* infections in the United States are the leading cause of deaths and hospitalizations among all *Vibrio* infections.

As stated, most cases of worldwide disease caused by *V. vulnificus* occur during the warm months of the year. It is interesting to note that both gastroenteritis and wound infections in the United States reported by Evans (<http://www.cdc.gov>) began to decrease several months prior to those of primary septicemia cases. Thus, within the overall seasonal *V. vulnificus* disease spectrum, there may be slightly different occurrence patterns present for each disease entity as well as for specific geographic locales. It is currently not known if each disease occurrence subpattern is a trend that has recently emerged or if the subpatterns reflect risk exposure biases that are tied intimately to

**TABLE 5** *Vibrio vulnificus* Infections Reported to CDC’s Voluntary Gulf Coast *Vibrio* Surveillance System

	Year		Total # of infections/total
	1997 (%)	1998 (%)	
# of Infections	58	83	141
Syndromes:			
Gastroenteritis # (%)	2 (3)	4 (5)	6/141 (4)
Septicemia # (%)	22 (38)	40 (48)	62/141 (44)
Wound Infection # (%)	30 (52)	34 (41)	64/141 (45)
Other <sup>a</sup> # (%)	4 (7)	5 (6)	9/141 (6)
Complications:			
Hospitalized # (%)	50/54 (93)	74/78 (95)	124/132 (94)
Deaths # (%)	21/51 (41)	20/71 (28)	41/122 (34)

<sup>a</sup> Includes eye, otitis, peritonitis, pneumonia, and urinary tract infections.

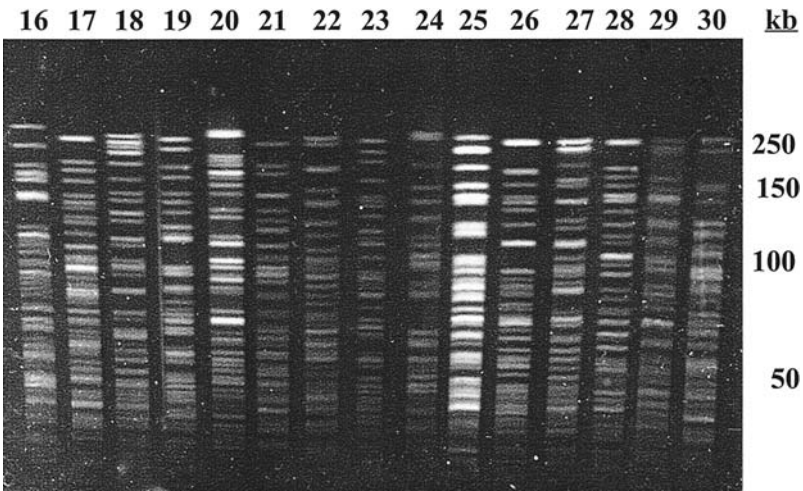
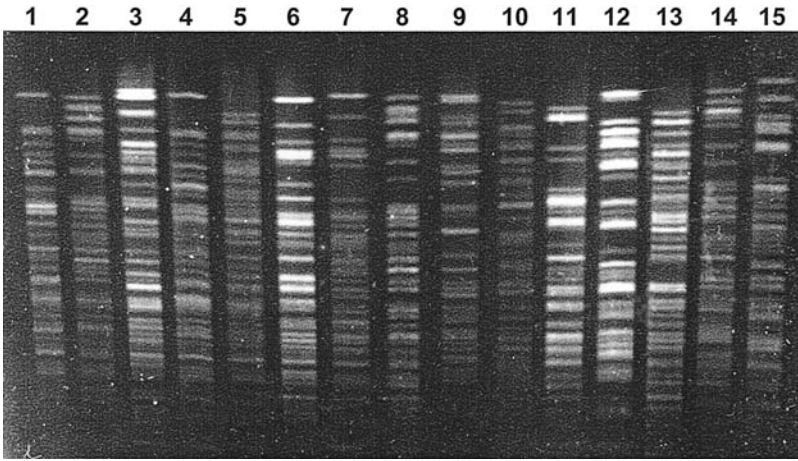
the ecology of this organism, such as its association and population dynamics with phytoplankton/ algal species and other hosts, oyster uptake/feeding patterns, and climatic changes. Other biases may be related to how the oysters are harvested or human risk behavior patterns.

Since shellfish, especially oysters, are seen as the primary vehicle for transmission of human *V. vulnificus* disease, it is interesting to note that most, if not all, *C. virginica* found in mid-Atlantic and Gulf Coast waters are heavily infected with *Perkinsus marinus*, an oyster pathogen responsible for severe oyster population losses throughout this region (123). All oysters from these waters also contain *V. vulnificus* (6,10). Only recently has the ecology of these two organisms been unraveled (46). Studies reported by La Peyre (124,125) have indicated that *P. marinus* produces a serine protease, a major virulence factor capable of digesting oyster connective tissues by degrading extracellular matrix proteins and acting as a potent immunosuppressant to reduce oyster hemocyte motility and lysosomal activity in oyster hemolymph (126). It has been postulated that the protease may be responsible for the overwhelming immunosuppression that leads to or culminates in the inability of oyster hemocytes to kill and degrade intracellular *P. marinus* cells (125,126). Recently, in vitro studies by Tall et al. (46) demonstrated that *P. marinus* protease-treated hemocytes were initially slower to internalize *V. vulnificus* than untreated hemocytes and that the serine protease produced by *P. marinus* also suppresses the vibriocidal activity of oyster hemocytes to effectively eliminate *V. vulnificus*, potentially leading to conditions favoring higher numbers of vibrios in oyster tissues. The results affirm the hypothesis that in the feral setting, higher numbers of *V. vulnificus* in oysters harvested from waters with temperatures above 25°C may also be due to increased numbers of *P. marinus* (escalation of infection) and its immunosuppressive activities controlled by the serine protease (46). This and other studies (127,128) suggest that the serine protease expressed by *Perkinsus* spp. can modulate, specifically suppress, the vibriocidal hemocytic response against multiple *Vibrio* spp. More importantly, however, these studies stress the importance of verifying the health status of the oyster (or clam) as a host and transmission vehicle before attempting to assess the levels of vibrios in these economically important marine species. The consequence of this observation has public health significance, especially in understanding how *V. vulnificus* persists in the oyster and potentially rises to unsafe levels in edible oyster shell stock.

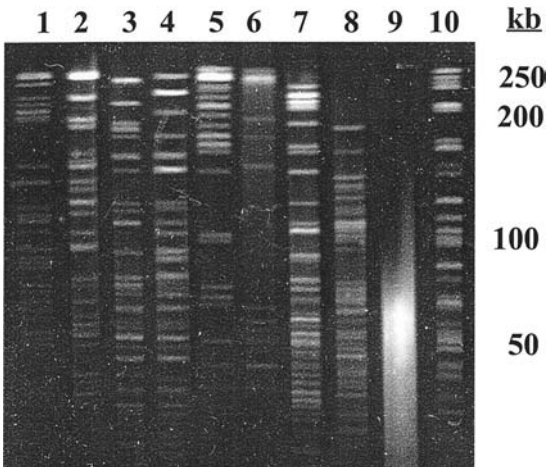
The infectious dose capable of causing human disease is currently not known, but data from epidemiological studies clearly suggest that it may be fewer than 1000 organisms (130) and that the primary reservoir for *V. vulnificus* septicemia is the variety of marine seafoods, either raw or improperly cooked, especially oysters, that humans consume (25,131). However, shrimp, crabs, eels, and other fish (sashimi or sushi) have also been linked to disease (132). Because the epidemiology of this organism is so closely related to its ecology, Singer et al. (133) looked at a collection of *V. vulnificus* strains to determine if genomic differences existed among strains that were obtained from a variety of environmental and clinical sources by pulsed-field gel electrophoresis (PFGE) analysis. Their results showed that extensive diversity was seen not only among *Vibrio* species, but also within the *V. vulnificus* isolates analyzed, suggesting that strains in the environment that infect humans are not dominated by derivatives from a single clone (Fig. 2). Since oysters are the predomi-

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**FIGURE 2** (A) Pulsed-field gel electrophoretic restriction patterns of *V. vulnificus* isolates from 10 different sources using *Sma*I. Lanes 1 6, blood; lane 7, CSF; lanes 8 and 9, wound; lane 10, corneal ulcer; lanes 11 13, clinical isolates; lane 14, shrimp; lane 15, eel; lane 16, clam; lanes 17 20, oysters; lanes 21 30, environmental isolates. Each isolate gave a unique DNA fingerprint (except lanes 15 and 16). The eel isolate (15) from Japan and the clam isolate (16) from Oregon gave indistinguishable patterns. (B) Pulsed-field gel electrophoretic restriction patterns of representative *Vibrio* species using *Not*I. Lanes 1 and 2, *V. vulnificus*; lanes 3 and 4, *Vibrio cholerae*; lanes 5 and 6, *Vibrio parahaemolyticus*; lanes 7 and 8, *Vibrio fluvialis*; and lanes 9 and 10, *Vibrio hollisae*. Molecular size standards in kb are noted in each figure. Note extensive differences in patterns signifies diversity among the genus *Vibrio*.



(A)



(B)

nant vehicle of transmission in primary septicemia, Buchrieser et al. (134) sought to determine if an individual oyster was populated by numerous genetically polymorphic strains of *V. vulnificus* or by a single clone of *V. vulnificus*, which may then be responsible for disease. Their results showed that a single individual oyster can harbor several different *V. vulnificus* pulsed-field types and that the average oyster meal consisting of 12 oysters can contain hundreds of strains (134,135). These problems have significantly hindered establishment of tolerance levels for *V. vulnificus* in seafood products. However, a study by Jackson et al. (130) has provided some insights into what might comprise significant case-associated *V. vulnificus* levels. These researchers followed over a 3-year period the total *V. vulnificus* levels in oysters harvested from a commercial site in Apalachicola Bay, Florida, and traced cases of infection back to this location. Their data showed that human infections occurred when *V. vulnificus* levels in oyster meat reached or exceeded  $10^3$  organisms per gram and case-associated levels ranged from  $10^3$  to  $10^5$  organisms per gram. Furthermore, these investigators analyzed over 150 blood isolates from three human infections and showed that a single PFGE profile was unique to each patient, suggesting that *V. vulnificus* infections result from proliferation of a single pathogenic strain (130). Using ribotyping methods, Tamplin et al. (135) found that clinical strains isolated from different individuals were less than 50% related. This observation suggests that the strains were not of the same clone and that these findings supported an earlier hypothesis proposed by Rubin (136) and promulgated by Jackson et al. (130), which asserts that within mixed populations each bacterium acts alone or is independently capable of causing infection. As proposed by Jackson et al. (130), it would be important to examine whether *V. vulnificus* isolates obtained from the same individual from both blood and skin/cellulitis affected sites are related to one clone or if each represents the expansion of multiple clones present in the same individual.

Since the estuarine environment is the natural habitat for *V. vulnificus*, it is not surprising that most infections are found in temperate and tropical coastal areas of the world where seafoods and water can be vectors for transmission to humans. In the United States most cases occur either in the states bordering the Gulf of Mexico or the southeastern Atlantic coast or states that import oysters from these areas (31). However, reports of inland infections not solely attributed to the interstate transportation of seafood products have been reported (137). In Japan, a large percentage of cases occurs along the more temperate southwestern shores of the island chain.

The worldwide occurrence of *V. vulnificus* in estuarine environments is most often influenced by changes in salinity and temperature. Increased water temperature appears to favor survival and growth (138,139). Though the influence of salinity has been controversial (3,7,138–140), a salinity range of 10–15 ppt has been reported to be the optimum. There is no correlation between *V. vulnificus* and fecal coliform levels, the indicator organisms that the National Shellfish Sanitation program uses to measure the sanitary quality of shellfish and their growing waters (131). *V. vulnificus* is readily detectable by plating samples onto solid nutrient media. However, the organism usually is not isolated from water samples that are collected during the winter (6,11,35,57,118), a phenomenon now attributed to what is called a “viable but nonculturable state” (VNC), which was originally described by Brayton et al. (141) for *V. cholerae* and by Linder and Oliver (142) for *V. vulnificus*. It is thought that this inability to culture these species from low-temperature environments is due not to cell death, but to the transition of cells from a viable growth stage to the state of nonculturability, which is defined as an inability of cells to produce colonies on appropriate solid media after prolonged incubation (32,143–147). The bacteria ingress into the VNC state, easily doing so at a medium temperature of 5°C. This result suggests that temperature downshift plays a major function in the induction of the VNC state. The cells can be resuscitated by subjecting them to higher temperatures (143). An increase in the total cell counts was not observed in such cultures, which indicates that the resuscitated cells came from the original cell population and was not due to growth of a few culturable cells. Characterization at the molecular level of the processes that lead to the nonculturable state, as well as those responsible for the genesis of resuscitation, is of considerable interest. These studies stress the importance of the VNC state, and future investigations may help define survival and adaptation strategies used by this microorganism.



## V. ISOLATION AND IDENTIFICATION

The isolation and identification of *V. vulnificus* from a food, environmental source, or a clinical specimen is probably quite rare in many inland laboratories, though a few cases have been reported (137). This is not true, however, for those laboratories serving a coastal geographic area where the majority of cases are found and the vast plurality of clinical isolates are obtained from wound and blood specimens. Usually these specimens are free of other competing bacterial contaminants and are processed with no particular attention to the isolation of *Vibrio* species; most blood culture protocols that use blood agar plates (5% sheep erythrocytes mixed either in a trypticase soy agar or blood agar base) as the primary isolation medium are quite sufficient for recovery of *V. vulnificus*. However, unusual clinical sources do include urine and respiratory aspirates, which may have a plethora of competing microorganisms present (43). After an 18- to 24-hour incubation period on blood agar medium at 35°C, *V. vulnificus* appears as regular colonies, ~3 mm in diameter, usually with a double zone of hemolysis, typified as a narrow zone of  $\beta$ -hemolysis (complete clearing) surrounding the colony with a wider zone of  $\alpha$ -hemolysis (greenish discoloration) (44).

Many of the isolation techniques used in the clinical microbiological laboratories work well with *V. vulnificus* (43). Most *V. vulnificus* strains grow well on MacConkey, Endo, xylose-lysine deoxycholate, and Hektoen enteric agar plates, therefore, use of these standard stool isolation media for stool specimen processing will probably ensure isolation. However, there is considerable variation in growth, colonial morphology, and tinctorial appearances on these media, and *V. vulnificus* grows poorly or not at all on other enteric isolation media such as eosin–methylene blue, *Salmonella-Shigella*, and deoxycholate agars (148). One major factor in how well the organism grows on these media is that *V. vulnificus* needs NaCl for optimum growth and activity (17).

The importance of obtaining rectal swabs or stool specimens prior to antibiotic therapy cannot be overemphasized in the clinical diagnosis of gastroenteritis, and whenever possible these specimens should be inoculated on isolation plates with minimal delay (43). Stool specimens should be transported as soon as possible to the laboratory or inoculated immediately into alkaline peptone (AP) water (APW) enrichment broth or Cary and Blair semisolid transport medium (43) if transit time is less than 8 hours. It is recommended that buffered glycerol-saline not be used as a transport medium (43). However, tellurite-taurocholate-peptone broth has been extensively used with success as an enrichment transport medium at the International Center for Diarrheal Disease Research in Dhaka, Bangladesh (43).

Traditionally, a loopful of stool or swab is streaked onto thiosulfate–citrate–bile salts–sucrose (TCBS) or modified cellobiose–polymyxin B–colistin (mCPC) agar (148,149) and inoculated into an APW enrichment tube. The TCBS agar plates are placed at 35°C and mCPC agar plates at 40°C for 18–24 hours. The APW enrichment cultures are streaked onto TCBS or mCPC after 6–18 hours of incubation (usually 12–16 h) and incubated as previously stated.

Today, most clinical microbiological labs located in tropical areas now include both TCBS and APW enrichment in their routine stool isolation protocols either year-round or during the warm periods of the year. On TCBS agar, most *V. vulnificus* are green or sucrose-negative; on mCPC agar *V. vulnificus* colonies are yellow surrounded by a yellow zone due to cellobiose fermentation. However, because of an increasing number of sucrose-positive strains being encountered (43), mCPC and other selective and differential isolation media such as *V. vulnificus* enumeration (VVE) (150), sodium sulfate–polymyxin B–sucrose (SPS) (151), and VV media (152) are now being included in clinical isolation protocols. All suspected colonies can be identified using biochemical tests already described or by using a commercial biochemical identification system, such as the API 20 E system, which has been tested comparatively and works well for the identification of marine vibrios (153–155). It is recommended that a cell suspension of the unknown isolate be made with added salt (1% NaCl or 20‰ marine salts) instead of the 0.85% saline salts solution recommended by the manufacturer (153). CDC recommends that a panel of 23 biochemical tests be used (see [Table 1](#)). Alternative computer-aided identification programs currently being developed at CDC are based on mathematical analysis of the results of 45–50 simple biochemical tests (156).

APW enrichment broth is widely used for the enrichment and isolation of *V. cholerae*, but Roberts et al. (157) found that it was less effective for *V. vulnificus* than direct plating to TCBS for environmental samples. Alternatively, SGP and PNC broths can be used to enrich for *V. vulnificus* (151,158). Kitaura et al. (151) found that SGP was useful in isolating *V. vulnificus* from environmental samples. Hsu et al. (158) found that rapid detection of *V. vulnificus* could be enhanced by optimizing the components of the APW enrichment broth. PNC [5% peptone, 1% NaCl, and 0.08% cellobiose (pH 8.0)] enhanced the growth of *V. vulnificus* compared to APW. Additionally, PNCC (PNC with 1.0–4.1 U of colistin methanesulfonate per mL) was shown to increase the growth of low levels of *V. vulnificus* while suppressing nontarget bacteria.

*V. vulnificus* in food and environmental samples can be enumerated by using a MPN series (159) and confirmed by biochemical testing or by using an immunological test, such as an ELISA procedure utilizing a monoclonal antibody to a species-specific intracellular antigen (10). Alternatively, a direct plating procedure for enumerating *V. vulnificus* in oysters has been described by Miceli et al. (150). However, comparative studies using all of the above described *Vibrio* isolation media and methods have not been done.

Other rapid identification tests include a coagglutination assay using *Staphylococcus aureus* Cowan 1 cells armed with specific *V. vulnificus* antflagellar antibody (160); an indirect immunofluorescence (IFA) procedure (161), fatty acid analysis by capillary gas-liquid chromatography (162), a DNA probe Southern blot hybridization procedure specific for the hemolysin-cytolysin gene (112,163–165) or with a probe complementary to specific 16S ribosomal RNA sequences of *V. vulnificus* (121,166–169), and by polymerase chain reaction assay using a 519 bp portion of the cytotoxin-hemolysin gene as a template (170,171). The IFA was useful in detecting cells in blood, lesion, and frozen tissue specimens from experimentally infected mice (161) and from a blood culture obtained from a human case of septicemia (28). A sandwich enzyme-linked immunosorbent assay (ELISA) for detection of *V. vulnificus* hemolysin production has been developed by Parker and Lewis (172). This assay compared favorably with the FDA standard immunoassay developed by Tamplin et al. (10). The sandwich ELISA had a 95% sensitivity and a 99% specificity rating and a positive predicted value of 98%. The DNA probe procedure has the added benefit that it can detect both viable and viable-but-nonculturable cells (173).

## VI. PATHOGENICITY

Several factors have been described as potential virulence determinants for *V. vulnificus*, including the expression of exocellular enzymes (104,174), a metalloprotease (175), a cytotoxin/cytolysin/hemolysin (165,176–178), and other secreted proteins, which may cause or contribute to host tissue damage (179). Surface structures such as a polysaccharide capsule (180–182), which is thought to play a role in resistance to the bactericidal effects of serum and phagocytosis (183–187), an endotoxic lipopolysaccharide (188–191), a siderophore-iron uptake system (192–196) flagella (160), and the expression of fimbriae (197–199), which are thought to be involved in adherence and invasion should also be considered as virulence factors (16,198,200–204).

Animal studies have been helpful in reproducing some aspects of the disease syndromes, specifically wound and septicemia infections produced by *V. vulnificus* (205). However, it has not been correlative for assigning the infectious dose 50 for human infections, nor have animal studies been helpful in the study of intestinal disease caused by this microorganism.

### A. Exocellular Proteins

Clinical characteristics of *V. vulnificus* infections imply that the organism is capable of invading healthy tissue. This invasion is either a consequence of the action of substances produced and released by the organism that digest tissue elements such as the extracellular matrix, an essential

component for normal arrangement and function of all tissues, or invasion into host tissues is due to an active bacterial internalization process (16,174,203,204,206–208).

The extracellular matrix network is constructed by cells from secreted glycoproteins and proteoglycans. *V. vulnificus* produces a variety of compounds that potentially can affect these host components, which include a constitutively expressed collagenenase, DNase, lipase, phospholipase, chitinase,  $\beta$ -*N*-acetylhexosaminidase, mucinase, chondroitin sulfatase, hyaluronidase, and fibrinolysin (58,179,209,210). Oliver et al. (58) examined representative strains from both clinical and environmental sources and found consistent production of all of these exoproteins and that origin or source of the strains did not correlate with mouse lethality.

## B. Elastinolytic Zinc Metalloproteases

Metalloproteases, containing zinc as an essential metal ion for enzyme catalysis, are elaborated by many bacteria and are classified into 30 major families, which include the thermolysin-like, elastase-like, *Serratia* protease-like metalloproteases, and the neurotoxins of *Clostridium tetani* and *Clostridium botulinum* type B (211). *V. vulnificus* expresses a thermolysin-like metalloprotease (175,212–214), which was originally described by Kreger and Lockwood (174). It was later isolated and characterized by Kothary and Kreger (214), who assigned it a molecular weight of 50.5 kDa and found that it had both caseinolytic and elastinolytic activity. Miyoshi et al. (213) also showed that the metalloprotease underwent autoproteolytic conversion to a 35 kDa active form, losing both a 186-amino-acid N-terminal propeptide and a 10 kDa C-terminal peptide in the process. Furthermore, it has been shown by a number of investigators that the metalloprotease possessed similar biological and immunological properties with the common zinc-metalloproteases expressed by *V. cholerae* (211), *V. mimicus* (175,176,214,215), *V. tubiashii* (216), *V. anguillarum*, *Vibrio proteolyticus*, and *Pseudomonas aeruginosa* (211).

The extracellular metalloprotease is thought to be important in aiding invasion of the bacterium into tissues by degrading elastin and collagen and may be responsible, at least in part, for the extensive tissue necrosis observed clinically during infections (217). Other studies by Miyoshi et al. (218) demonstrated that the metalloprotease proteolytically activates the Hageman factor (factor XII), generating bradykinin, a 9-amino-acid inflammatory polypeptide (219,220). The kinins bradykinin and lysylbradykinin are important mediators of inflammatory responses and are potent vasoactive peptides with wide-ranging properties, including the ability to (a) increase vascular permeability, (b) cause vasodilation, pain, and smooth muscle contraction, and (c) stimulate arachidonic acid metabolism. Activation occurs at sites of negatively charged molecules, such as collagen fibers associated with the interstitial tissue space (221). High molecular weight kininogen (HMWK) and prekallikrein (circulating as a 1:1 stoichiometric complex, together with the Hageman factor) binds to collagen, and the metalloprotease then activates the Hageman factor to convert prekallikrein to kallikrein, which then digests HMWK to release lysylbradykinin, which is then converted to bradykinin. More research is needed to determine if the metalloprotease acts alone or in combination with other *V. vulnificus* bacterial products in conditioning the Hageman factor–collagen activation site. However, it is likely that the protease activation of the kinin system is involved in the intravascular dissemination. Miyoshi et al. (222) have carried these experiments a step further by injecting the metalloprotease intradermally into dorsal skin of a rabbit and then following the level of in vivo hemorrhagic activity. The results showed that the metalloprotease was found to be significantly correlative with that of the in vitro proteolytic activity for the reconstituted basement membrane gel, which consists primarily of laminin and type IV collagen. Only type IV collagen was easily digested by the metalloprotease. Additionally, type IV collagen antibodies, but not antibodies against laminin, showed sufficient protection against the hemorrhagic reaction. Capillary vessels are known to be stabilized by binding of the basal surface of vascular endothelial cells to the basement membrane. Therefore, specific degradation of type IV collagen may cause destruction of the basement membrane and subsequent breakdown of capillary vessels, resulting in leakage of blood components including erythrocytes into the affected area. Alternatively, this may explain why the organism

targets itself to the skin and why a hemorrhagic rash, the most notable symptom observed during infection, occurs.  $\alpha_2$ -Macroglobulin, a plasma protein, has been shown to be a potent inhibitor of the metalloprotease (223). Because of this liability, Narukawa et al. (224) developed an  $\alpha_2$ -macroglobulin-resistant derivative of the metalloprotease, which was also stable in vivo. The metalloprotease was modified with activated monomethoxy polyethylene glycol. The modified protease retained full activity to a peptide substrate and 10–20% activity to protein substrates and was resistant to entrapment by  $\alpha_2$ -macroglobulin.

Another role that the metalloprotease may play is in iron metabolism. It is thought that *V. vulnificus* obtains iron from a variety of heme proteins through proteolytic action of the metalloprotease (225,226). Nishina et al. (227) showed that both clinical and environmental isolates of *V. vulnificus* could grow in a synthetic medium supplemented with heme protein as the iron source. In support of this, Fouz et al. (228,229) demonstrated that proteolysis by an outer membrane preparation, which was enriched for a 36 kDa protein (presumably the metalloprotease) expressed by *V. vulnificus* biotype 2 cells, caused the release of iron from native hemoglobin and hemin. Similarly, Miyoshi et al. (226) showed that the purified metalloprotease could liberate protoheme (iron-protoporphyrin IX) through proteolytic digestion of these heme proteins; the iron itself or in association with protoporphyrin IX is then transported into the cell. Further observations by Miyoshi et al. (230) also provide additional support that *V. vulnificus* can obtain iron from hemoglobin bound to haptoglobin types 1 and 2 and synthetic iron sources, such as Fe-alpha, beta, gamma, and delta-tetraphenylporphine tetrasulfonic acid, but not that bound to haptoglobin type 2-1. Results from a study by Nishina et al. (227) confirmed that metalloprotease was needed for heme liberation. The protease digested all of the heme proteins tested and elicited the liberation of heme from the proteins. Lastly, an in vivo mouse peritonitis model employed by Helms et al. (231) demonstrated that heme-containing molecules enhanced the lethality of infections by *V. vulnificus*. The lethality of inocula of the bacteria injected intraperitoneally (ip) was increased by concurrent injections (ip) of hemoglobin, methemoglobin, or hematin, but not by myoglobin. These investigators obtained similar results in mice with phenylhydrazine-induced hemoglobinemia.

Several investigators have shown that the protease can cause a variety of erythrocytes from vertebrate species (rabbit, sheep) to agglutinate (232–234). The C-terminal portion of the protease is thought to be essential for hemagglutination, binding to the erythrocyte membrane, and for proteolysis of membrane proteins (213). Together, these results suggest that *V. vulnificus* is capable of extracting iron from hemoglobin in vivo for use as a nutriment and that protease contributes to the efficient utilization of heme and iron metabolism by *V. vulnificus*.

### C. Cytotoxin/Hemolysin

Kreger and Lockwood found in 1981 that there was an extracellular factor(s) in culture filtrates obtained from *V. vulnificus* seed cultures that was cytolytic for Chinese hamster ovary cells (174). Later, Gray and Kreger (178) purified the cytolytic toxin and demonstrated that it was a heat labile, hydrophobic protein that was inhibited by large amounts of cholesterol, was partially inactivated by proteases and trypan blue, had a molecular weight of 56 kDa, possessed a single isotype with an isoelectric point of 7.1, could lyse mouse erythrocytes, and was lethal for mice at a dose of 3  $\mu\text{g}/\text{kg}$ . It was later shown by Yamanaka et al. (235) that the cytotoxin/hemolysin was active against a variety of erythrocytes including sheep, horse, bovine, rabbit, and chicken, and that the activity was closely associated with both the binding ability of the cytotoxin/hemolysin and the stability of erythrocytic membranes. Yamanaka et al. (236) had previously determined that the mechanism of hemolysis caused by the *V. vulnificus* hemolysin was temperature dependent and that release of  $\text{K}^+$  and hemoglobin followed a colloid-osmotic mechanism similar to that described for *Staphylococcus aureus*  $\alpha$ -toxin and the *Escherichia coli*  $\alpha$ -hemolysin. These data were supported by results summarized by Kim et al. (237), which showed that the hemolysis was also temperature dependent, but the binding of the hemolysin was temperature independent and the cytolysin-induced hemolysis was accompanied with conversion of the membrane-bound cytolysin into a 210 kDa oligomer,

corresponding to a tetramer of native cytotoxin molecules. These researchers also found that nonesterified cholesterol inactivated the cytotoxin by converting active monomeric toxin into inactive aggregates of the 210 kDa oligomer. Together, these data support the hypothesis that the monomeric toxin binds to the cholesterol receptor as it associates with the erythrocytic membrane, then oligomerization occurs during lateral diffusion of the monomer in the fluid lipid bilayer. These data also suggest that the oligomer is not active unless it is already bound to the membrane.

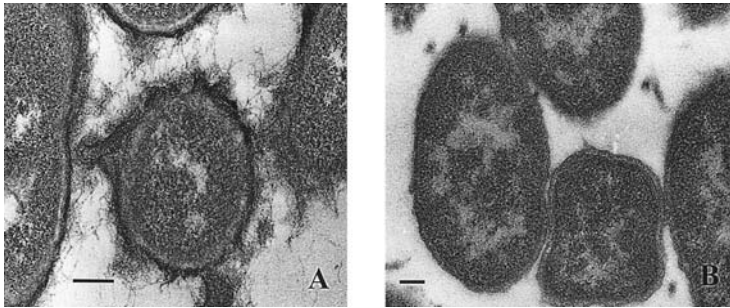
Gray and Kreger (238) showed that antitoxin antibodies were produced *in vivo* during the development of the disease in mice and in a human that survived *V. vulnificus* disease. In another study (239), these authors showed that a single intradermal injection of the purified toxin into the skin of mice produced extensive edema, caused the disorganization of collagen bundles, and generated an accumulation of cell debris and plasma proteins, which yielded extensive damage to fat, capillary endothelial, and muscle cells. A mild inflammatory cell infiltration confined to the subdermal area of the injection site was also induced. Yamanaka et al. (240) in a later communication also showed that the hemolysin can lyse cell membranes of mast cells in a dose-dependent fashion. Okada et al. (241) determined that *V. vulnificus* may produce a second hemolysin that had a molecular weight of 36 kDa, which did not cross-react antigenically with the anti-56 kDa hemolysin antibodies.

Recently, Kook et al. (242) reported that the hemolysin produced by *V. vulnificus* dilated rat thoracic aorta via elevated cGMP levels without affecting nitric oxide synthase. They showed that hemolysin action could be attenuated with 6-(phenylamino)-5,8-quinolinedione (LY 83.583), which inhibits the catalytic domain of guanyl cyclase and cholesterol, which in turn blocks hemolysin incorporation into the membrane; the removal of ATP, a cofactor of particulate guanyl cyclase, also attenuated hemolysin activation and that the addition of ATP gammaS, a nonphosphorylating analog, restored it. These data suggest that the hemolysin activates particulate guanylyl cyclase via hemolysin incorporation into the vascular smooth muscle cell membrane in cooperation with certain unidentified cytosolic component(s). In recent years, contradictory reports by Massad et al. (243) and later by Wright et al. (244) have demonstrated that there may not be any correlation between hemolysin production and virulence. Using ethyl methanesulfonate (243) transposon mutagenesis (244) and marker exchange to inactivate the 56 kDa cytotoxin gene, both groups of investigators compared the virulence of hemolysin-negative mutants to fully virulent *V. vulnificus* wild-type strains. The nonhemolytic mutants were found to be as virulent as their parent strains. While these data suggest that the 56 kDa cytotoxin may not be involved in the pathogenesis of *V. vulnificus* infections, its role appears to be less important than other factors, such as encapsulation. However, the role of the two hemolysins, separately or together, is currently not known.

## D. Polysaccharide Capsule

Capsules are produced by organisms in a variety of habitats, many of which are found in the marine environment (245). Expression is considered a basic cellular function, as judged by its early evolution and development (245). Pathogenic bacteria are often classified on the basis of the complex polysaccharides found on the surface, usually capsular polysaccharides or lipopolysaccharides. It is common in the clinical microbiology laboratory to use reactivity with antisera that specifically recognize these various cell surface carbohydrates for identification purposes.

Early studies reported by Kreger et al. (183,184) and others (186,246) supported the concept that the virulence of *V. vulnificus* was associated with its ability to resist phagocytosis, as well as its resistance to the bactericidal action of human serum (185–187,247) and its inability to activate complement (186). These results taken together suggest that *V. vulnificus* expressed an antiphagocytic surface antigen, such as a capsule. Amako et al. (180) and Kreger et al. (184) both demonstrated in 1984 that *V. vulnificus* expressed such a surface structure. Amako and colleagues examined two clinical strains for the presence of a polysaccharide capsule by using a ruthenium red staining procedure. The proportion of ruthenium red-stained cells correlated well with mouse virulence and with the susceptibility of the organisms to the bactericidal activity of normal human serum. Amako's observations on the ultrastructure of the capsule were confirmed by several other studies (201,248–



**FIGURE 3** Transmission electron photomicrograph showing encapsulated (A) and unencapsulated (B) phase variant *Vibrio vulnificus* cells stained with Alcian blue. Bar makers represent 0.1  $\mu\text{m}$ .

250). Figure 3 shows the ultrastructure of the capsule stained with Alcian blue and lysine in both encapsulated and unencapsulated phase variants. These studies further demonstrated that encapsulation correlated with colonial opacity and that variation in the opacity of colonies formed by the organism was accompanied by variation of capsular formation. Furthermore, encapsulated strains were also more resistant to the bactericidal action of human serum, possessed greater antiphagocytic activity, and were highly lethal for mice.

Multiple capsule types have been described for *V. vulnificus* (182,251), but virulence does not appear to correlate with any one particular capsule type (251,252). Reversible phase variation for opaque and translucent colony morphologies has been described, and both biotype 1 and 2 strains exhibit these properties (181,190,248,249). Capsule expression in biotype 3 has not been characterized. Phase variation or the spontaneous conversion of opaque or encapsulated strains to translucent colonies (and vice versa) was found to occur at similar rates ( $\sim 10^{-4}$ ) (248–250), and Kaysner et al. (253) showed that phase conversion can occur in vivo.

Capsular polysaccharide was purified from a virulent strain of *V. vulnificus* by Reddy et al. (254). Nuclear magnetic resonance spectroscopic analysis of the purified polysaccharide showed that the polymer is composed of a repeating structure with four sugar residues per subunit: a residue of 2-acetamido-2,6-dideoxy-hexo pyranose in the alpha-gluco configuration (QuiNAc), a residue of 2-acetamido-2,6-dideoxy hexopyranose in the alpha-galacto configuration (FucNAc), a residue of 2-acetamido-2,6-dideoxy hexopyranose in the alpha-manno configuration (RhaNAc), and a residue of 2-acetamido-2,6-dideoxy hexouronate in the alpha-galacto configuration (GalNAcA). This was the first reported occurrence of RhaNAc in a bacterial capsular polysaccharide.

Currently there are over 15 capsular serotypes (251). Capsular polysaccharide (CPS) from three opaque *V. vulnificus* strains was purified and characterized by Simonson and Siebeling (182). The purified acidic capsule contained considerable amounts of hexosamine, and given alone by injection, the purified capsule was poorly immunogenic for rabbits and mice; repeated injections produced little detectable anticapsular antibody. To improve immunogenicity, they conjugated the capsule to keyhole limpet hemocyanin. They then armed *S. aureus* cells with each of the three anticapsular antibodies and showed that each antibody could only co-agglutinate the homologous opaque strain. These findings suggested the existence of at least three capsular types and are reminiscent of earlier work performed with *E. coli*, which is known to produce over 70 capsular polysaccharides (255). Bush et al. (252) attempted to improve upon these results with a chemotyping method for bacterial capsular polysaccharides based on carbohydrate analysis of an acid hydrolysate of the capsule. Using a high-performance anion-exchange chromatography procedure coupled to an electrochemical detection method, they were able to semi-quantitatively produce a capsule “fingerprint,” which was used to discriminate among isolates of *V. vulnificus* that expressed different capsular polysaccharide structures. The procedure was applied to a collection of 120 isolates of *V. vulnificus* from both clinical cases of septicemia and from such environmental sources such as seawater, sediments, and shellfish.

**TABLE 6** Population Dynamics of Biofilm Formation by Encapsulated and Unencapsulated Phase Variants of *Vibrio vulnificus* on Glass Coverslips

Phase variant	Time (h)	CFU/ml, CS	CFU/ml, Sup.	Incubation/ colonizing
Unencapsulated	0	$7 \times 10^3$	$9 \times 10^5$	0.7
Unencapsulated	3	$9.3 \times 10^4$	$6.9 \times 10^6$	1.3
Unencapsulated	18	$1.1 \times 10^7$	$1.2 \times 10^{10}$	0.1
Encapsulated	0	$2 \times 10^2$	$8.5 \times 10^5$	0.02
Encapsulated	3	$2.2 \times 10^3$	$2.2 \times 10^6$	0.1
Encapsulated	18	$1.3 \times 10^7$	$1.4 \times 10^{10}$	0.1

CS, coverslip.

Source: Ref. 201.

They found a number of unusual sugars, including many amino sugars and a wide variety of capsular carbotypes.

Apart from its involvement in protection against host bacterial defense mechanisms, the capsule may also play a role in adherence and biofilm formation (245). Tall et al. (201,256), in two separate studies, present evidence that unencapsulated *V. vulnificus* strains may be more adept at colonizing cultured tissue cells (256) and sterile surfaces, such as glass coverslips (201), than were counterpart encapsulated phase variants (Table 6).

## E. Outer Membrane and Lipopolysaccharide

Another structural layer (8–10 nm) associated with the cell surface of gram-negative bacteria is the outer membrane. This fine structure is visually indistinguishable from that of a unit membrane. It does, however, differ both in chemical composition and in function from the cytoplasmic membrane. The outer membrane is considered to be the structural surface layer that has its inner surface adjacent to the peptidoglycan layer and its outer surface sticking out from the cell surface, interacting with the external milieu. This outer wall layer is highly irregular. The outer membrane is covalently attached to the peptidoglycan layer by lipoproteins. It is thought that the protein portion of the lipoproteins is involved in this attachment. Phospholipids are located mainly on the inner side next to the peptidoglycan layer, and the outer side contains a lipopolysaccharide (LPS) that has its lipid portion (lipid A) embedded in the membrane and its polysaccharide portion (O antigen or O polysaccharide) sticking out from the bacterial cell surface (257,258). This outer LPS layer plays a major role as a porous structure that limits the access of harmful molecules to the inner part of the cell, yet allows diffusion of low molecular weight solutes. 2-Keto-3-deoxyoctonic acid, a normal component of the LPS of typical Enterobacteriaceae species, was not found as a constituent of the LPS of *V. vulnificus* (188). Hexadecenoate (C16:1) was the predominant fatty acid, and hydroxy fatty acids composed over 40% of the total fatty acid content of the lipid A portion of the LPS (188). There was no apparent difference between the LPS of virulent and avirulent strains of *V. vulnificus* biotype 1, nor were there differences in LPS isolated from opaque and translucent colony variants (190). There are several immunologically distinct LPS types recognized for biotype 1 strains, but there seems to be only one LPS type for biotype 2 strains.

McPherson et al. (189) found that intravenous injections of purified LPS isolated from *V. vulnificus* were not lethal for mice. However, intravenous injections of *V. vulnificus* LPS in rats produced significant cardiovascular damage (ischemic heart damage), which led to death. Intraperitoneal injections of *V. vulnificus* LPS into rats also produced a pyogenic response. These studies suggest that the LPS may be a factor contributing to the virulence of this organism and that mice are less sensitive to *V. vulnificus* LPS than are rats (189). It is known that divergent species of animals vary greatly in their susceptibilities to the fatal effects of LPS (260). One of the dominant catalysts

believed to augment septicemia is the overproduction of tumor necrosis factor (TNF), leading to overstimulation of nitric oxide synthase, in response to LPS (62). When the nitric oxide (NO) synthase inhibitor *N*-monoethylarginine was administered to rats along with LPS from *V. vulnificus*, the lethality due to the endotoxin was reversed (189). These data suggest that the stimulation of TNF followed by overproduction of NO induces endotoxic shock in response to *V. vulnificus* LPS.

In addition to LPS, the outer membrane also contains a number of proteins. A few of the most important ones are porins and protein receptors, such as siderophores. Koga and Kawata (259) examined the major outer membrane proteins of various isolates grown under different conditions and found that a major outer membrane protein expressed in cells grown in nutrient broth containing 3% NaCl possessed a molecular weight of 48,000. However, this protein was not produced in strains grown in chemically defined media. Other outer membrane proteins identified by these researchers possessed molecular weights of 33,000 and a 40,000. Cells grown in iron-deficient media produced one to three new outer membrane proteins with molecular weights ranging from 74,000 to 85,000. The 48,000 molecular weight protein and the smaller molecular weight proteins (33,000–40,000) were considered to be porin-like proteins because of their resistance to trypsin.

## F. Siderophore–Iron Uptake System

Bacteria, like most organisms, require iron for cellular functions. In mammals, most of the iron is either stored intracellularly or bound to serum proteins such as transferrin or lactoferrin and is generally unavailable for use by microorganisms. Elevated levels of serum iron seem to be necessary for *V. vulnificus* to multiply and survive in the human host (261). To overcome this nutritional limitation, *V. vulnificus* produces two high-affinity iron-acquisition complexes called siderophores. Siderophores are low molecular weight, highly specific iron chelators, which are produced by the organism under low iron growth conditions. Simpson and Oliver (192) showed that *V. vulnificus* produces both a hydroxamate and a phenolate or catecholate siderophore constitutively. Interestingly, *V. vulnificus* appears to be the only bacterium known to express both types of siderophore together. In *V. vulnificus*, transcriptional regulation by iron depends on the *fur* gene (278). The N-terminal amino acid sequence of a 77 kDa protein purified from the *V. vulnificus fur* mutant had 67% homology with the first 15 amino acids of the mature protein of the *V. cholerae* heme receptor, HutA. In another study reported by Webster and Litwin (262), a second iron-regulated protein, a 72 kDa outer membrane protein purified from a *V. vulnificus fur* mutant, was shown to have 53% homology with the first 15 amino acids of the mature protein of the *V. cholerae* vibriobactin receptor, Viu. Biotype 2 strains also produced both phenolate- and hydroxamate-type siderophores of an unknown nature and also express two new outer membrane proteins of around 84 and 72 kDa in response to iron starvation (195). Okujo et al. (193) described a third type of catecholate siderophore and gave it the name vulnibactin. As mentioned before, the zinc-metalloprotease probably plays a role in digestion of the serum iron-binding proteins like transferrin, hemoglobin, and hemoglobin-haptoglobin so that the siderophore can then use the iron as a nutrient, thereby promoting growth (231).

## G. Flagella

*V. vulnificus* produces a sheathed polar flagellum—usually a single flagellum. To date it is not known if *V. vulnificus* can express lateral flagella similar to that expressed by *V. parahaemolyticus*, *V. alginolyticus*, or *V. fluvialis* (160,263) or if it has the genes for such structures. Tassin et al. (264) were the first to show that the flagellar antigen expressed by *V. vulnificus* was species-specific and that its antigenicity is limited to the flagellar core protein. For a number of reasons it is difficult to visualize the flagellum within time limitations of immunofluorescence and slide agglutination assays (264). However, Tassin et al. (264) described a tube flocculation assay capable of identifying 97% of the *V. vulnificus* strains among 467 environmental *Vibrio* isolates. The tube test, though reliable, is labor intensive and uses anti-flagellum sera produced in rabbits immunized with formalin-

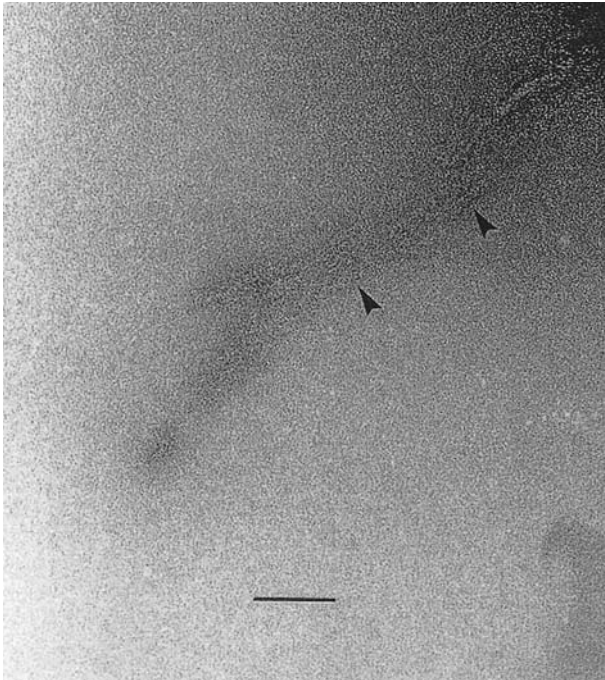


killed whole cell immunogens, which then have to be extensively absorbed to remove anti-capsule and anti-LPS activity. Because of these limitations, Simonson and Siebling (263) developed a rapid coagglutination serological slide assay, which takes advantage of the Fc receptors expressed by *S. aureus* cells. The assay was able to detect microgram quantities of *V. vulnificus* flagellar antigen in isolates one step beyond primary isolation. They further developed the co-agglutination assay using monoclonal antibodies prepared against purified flagellar core protein (160).

## H. Expression of Fimbriae: Adherence/Invasion

Systemic illness in humans caused by *V. vulnificus* usually presents as primary septicemia that occurs after ingestion of raw seafood, such as oysters. This suggests that the organism colonizes the intestinal epithelium, then establishes a systemic infection after transversing the intestine.

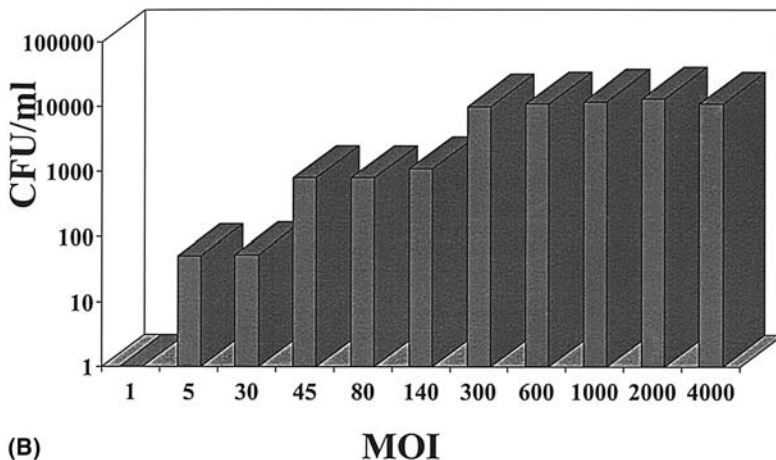
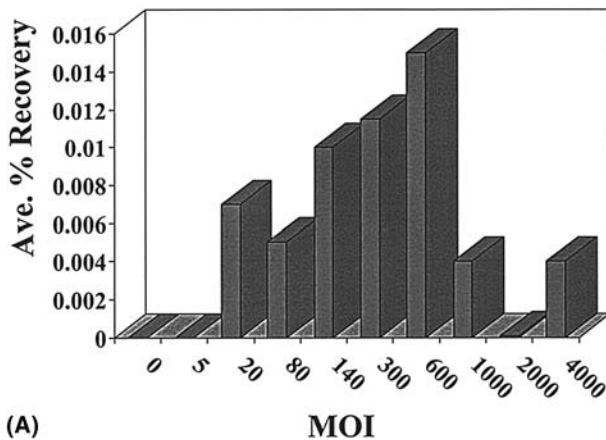
The presence of fimbriae was first reported by Gander and LaRocco (197), but the nature of the mechanism of attachment and the contribution of the fimbriae in adherence is ill-defined. Paranjpye et al. (198) presented evidence suggesting that *V. vulnificus* expresses type 4 pili. Studies by Tall et al. (199) revealed 3.5 nm fibrillar structures composed of linear strands, multiple strand bundles, or wiry aggregates radiating from the bacterial surface (Fig. 4). Using a KSCN/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation procedure, they obtained a crude fibrillar extract (CFE) that consisted of single filaments, filaments in bundles, and also possessed hemagglutination (HA) activity. CFE obtained from biotypes 1 and 2 of *V. vulnificus* hemagglutinated sheep, chicken, bovine, human O, and eel RBCs. Additionally, CFE obtained from biotype 1 cells also hemagglutinated human A and B RBCs (204). It is currently not known if the fimbriae reported by Tall (199) and by Gander and LaRocco (197) are the type 4 pili that Paranjpye (198) described or if *V. vulnificus* expresses multiple fimbrial types.



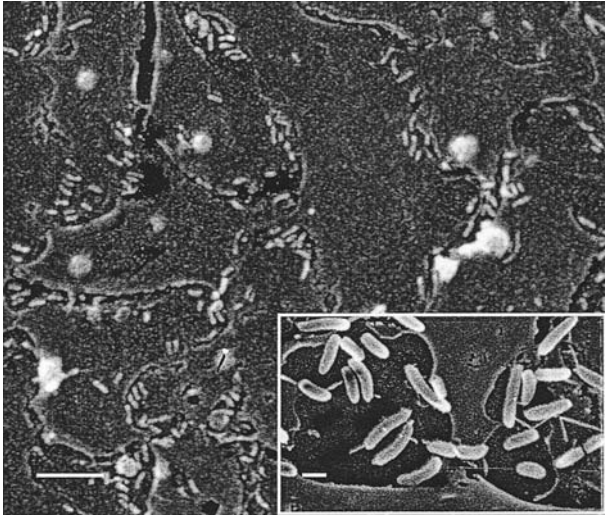
**FIGURE 4** Electron photomicrograph of glutaraldehyde fixed *Vibrio vulnificus* cells stained with 0.5% uranyl acetate and 0.5% trehalose. Note flexible appendages composed of individual 3.5 nm linear fibrillar strands and multiple fibrillar bundles. Bar marker represents 0.1  $\mu\text{m}$ .

Adherence of a pathogen to its target tissue is thought to be a prerequisite for triggering internalization of bacteria by host cells (265). Studies by numerous researchers have shown that such invasion mechanisms require signal transduction, the trafficking mechanisms that a cell uses to respond to specific environmental signals. These signals involve a cascade of phosphorylation-dephosphorylation reactions, which control cell morphology, migration, differentiation, and proliferation.

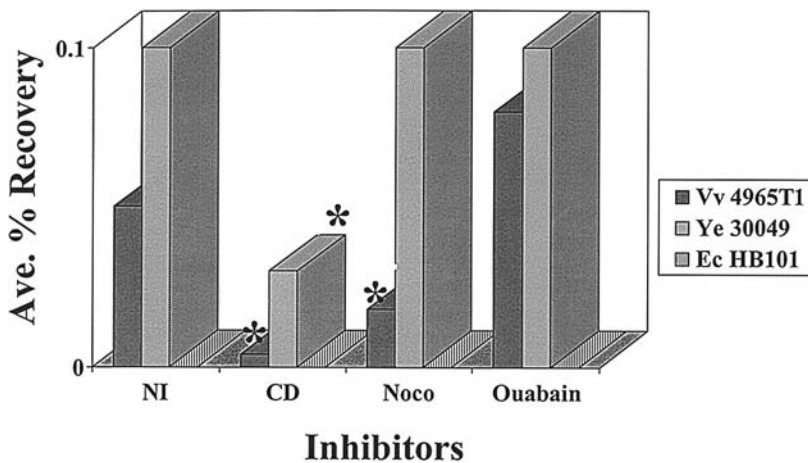
Invasion of *V. vulnificus* into tissue-cultured cells was first demonstrated by Elkind et al. (266). These researchers showed by both electron microscopy and quantitative studies that *V. vulnificus* could invade Hep-2 cells in tissue culture. Their results also suggest that there might be differences in invasion efficiencies between clinical and environmental isolates; of 17 clinical strains, 6 possessed the ability to enter cells as opposed to zero of 3 environmental strains. Tall et al. (202,208) followed these studies with adherence and invasion studies using Atlantic menhaden liver epithelial cells. To monitor the effect of available bacteria on host cell uptake, these researchers assessed the percent recovery of *V. vulnificus* over a wide range of starting multiplicities of infection (MOI) (number of *V. vulnificus* cells/host cell) using the gentamicin kill-invasion assay (Fig. 5) (267). The resulting percent recovery increased steadily from a starting MOI of ~2 to ~300, where it reached



**FIGURE 5** (A) Effect of varying the starting MOI on invasion efficiency of *Vibrio vulnificus* into Atlantic menhaden liver (AML) cells. (B) Effect of varying the starting MOI on the number of cells recovered per well of *Vibrio vulnificus* infected AML cells.



**FIGURE 6** Scanning electron photomicrograph showing adherence of *Vibrio vulnificus* limited to 5–7 foci of infection affiliated with the apical and peripheral surfaces of the AML cell, 1 h post-infection. Each focus consists of 2–5 bacteria. Bar marker in the lower magnification micrograph represents 10  $\mu\text{m}$  and bar marker in the inset represents 2  $\mu\text{m}$ .



**FIGURE 7** Invasion of *Vibrio vulnificus*, *Yersinia enterocolitica*, and *Escherichia coli* into AML cells in the presence and absence of Cytochalasin D, Nocodazole, and Ouabain into AML cells. \*Significant comparison with % recovery result from cells not treated with inhibitors,  $P < 0.05$ . Abbreviations: NI, No inhibitor; CD, Cytochalasin D; and Noc, Nocodazole, 1 h postinfection. Per cent recovery data were calculated and analyzed using the Student t-test and one factor analysis of variance followed by Dunns' or Student-Newman-Kuels multiple comparisons of means.

**TABLE 7** Internalization Mechanisms Utilized by *Vibrio* spp. in Atlantic Menhaden Liver Cells

	<i>V. vulnificus</i>	<i>V. mimicus</i>	<i>V. fluvialis</i>	<i>V. parahaemolyticus</i>
Cytochalasin D (MF)	+	+	–	+
Colchicine (MT)	+	+	+	+
Ouabain (RME)	–	+	–	–

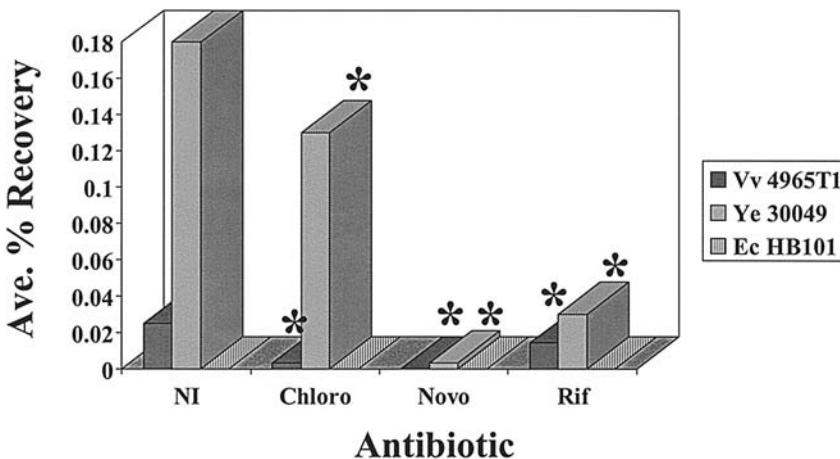
+, Inhibition (decrease in recovery to that obtained from cells not treated with inhibitor);

–, no inhibition (similar or an increase in recovery to that obtained from cells not treated with inhibitor).

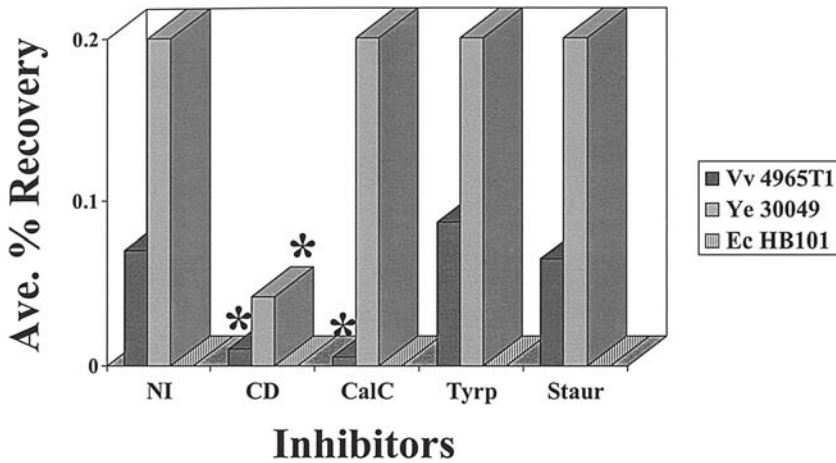
Source: Adapted from Ref. 268.

a maximum of 0.02%, and decreased sharply and consistently thereafter. In contrast, the total number of bacteria recovered relative to MOI also increased with MOI, peaking at a MOI of ~300, after which the total number of bacteria recovered remained unchanged. Electron microscopy of infected AML cells showed 5–7 clusters of 2–5 bacteria per cell associated with the apical and peripheral surfaces (Fig. 6). Taken together, these data suggest that there was a strict physical limitation on cell entry of *V. vulnificus*.

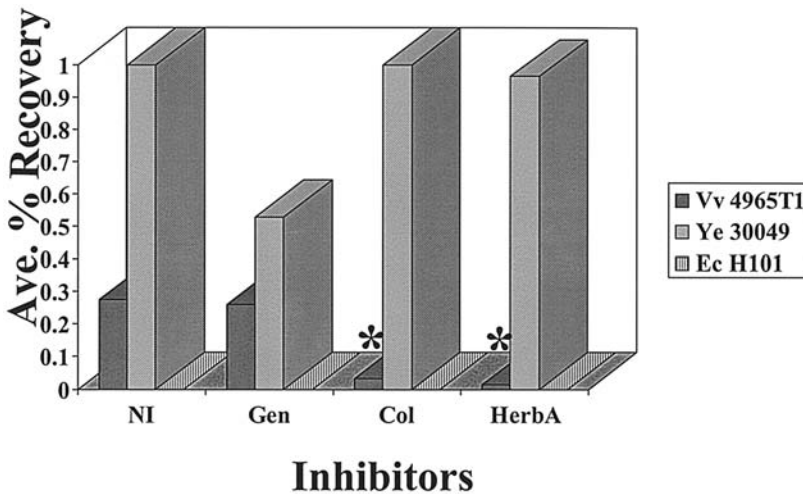
The internalization mechanism(s) triggered by *V. vulnificus* was studied by invasion assays conducted with different inhibitors that act on eukaryotic and prokaryotic structures and processes (204,206,267–269). Results demonstrated that nocodazole and colchicine, both inhibitors of microtubulin polymerization, and cytochalasin D, an inhibitor of microfilament polymerization, significantly reduced bacterial uptake when compared with untreated controls (Fig. 7). However, ouabain, an inhibitor of receptor-mediated endocytosis, did not (269). The results of these results suggest that uptake of *V. vulnificus* is dependent on microfilament and microtubulin polymerization/depolymerization. Tall et al. (268) reported that other marine vibrios have similar or related internalization requirements (Table 7). Comparatively, *V. mimicus* internalization depended on all three uptake



**FIGURE 8** Invasion of *Vibrio vulnificus*, *Yersinia enterocolitica*, and *Escherichia coli* into AML cells in the presence and absence of Chloroamphenicol, Novobiocin, and Rifampicin, 1 h postinfection. \*Significant comparison with % recovery result from cells not treated with antibiotics,  $P < 0.01$ . Abbreviations: NI, No inhibitor; Chloro, Chloroamphenicol; Novo, Novobiocin; and Rif, Rifampicin. Per cent recovery data were calculated and analyzed using the Student t-test and one factor analysis of variance followed by Dunns' or Student-Newman-Kuels multiple comparisons of means.



**FIGURE 9** Effect of Cytochalasin D, Calphostin C, Tyrphostin 47, and Staurosporine on invasion of *Vibrio vulnificus*, *Yersinia enterocolitica*, and *Escherichia coli* into AML cells, 1 h post-infection. \*Significant comparison with % recovery result from cells not treated with inhibitors,  $P < 0.05$ . Abbreviations: NI, No inhibitor; CD, Cytochalasin D; Cal, Calphostin C; Tyrp., Tyrphostin 47; and Staur, Staurosporine. Per cent recovery data were calculated and analyzed using the Student t-test and one factor analysis of variance followed by Dunns' or Student-Newman-Kuels multiple comparisons of means.



**FIGURE 10** Effect of Genistein, Colchicine, and Herbinycin A on invasion of *Vibrio vulnificus*, *Yersinia enterocolitica*, and *Escherichia coli* into AML cells, 1 hour post-infection. \*Significant comparison with % recovery result from cells not treated with inhibitors,  $P < 0.05$ . Abbreviations: NI, No inhibitor; Gen., Genistein; Col, Colchicine; and HerbA, Herbinycin A. Per cent recovery data were calculated and analyzed using the Student t-test and one factor analysis of variance followed by Dunns' or Student-Newman-Kuels multiple comparisons of means.

pathways. *V. parahaemolyticus* was internalized via the microfilament and microtubule pathways, and *V. fluvialis* depended only on microtubules. The role of bacterial protein and DNA syntheses in uptake was investigated by incorporating chloramphenicol, rifampicin, and novobiocin into their invasion assay. Results showed a decrease in uptake when these synthetic pathways were inhibited by these antibiotics, suggesting that both de novo bacterial protein and DNA syntheses are needed for uptake (Fig. 8) (269).

The effects of mammalian cell signaling were studied by incorporating various signal transduction inhibitors into the invasion assay. Results showed that uptake was regulated by host cell protein tyrosine kinase action and somewhat by PKC activity (Figs. 9 and 10) (207).

In summary, these data provide evidence indicating that uptake of *V. vulnificus* occurs at a limited number of sites on the AML cell surface and was dependent on expression of bacterial surface ligands and on the involvement of host cytoskeletal elements and protein kinase activities.

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

Studies dedicated to the determination of the genetic factors involved in virulence of *V. vulnificus* arose shortly after the beginning of the molecular biology age; most of the studies, if not all, have been published since the mid-1980s. The first study using these techniques was that of Wright et al. (165), which described the cloning of a 3.2 kb DNA fragment containing the cytolysin/hemolysin gene. They also applied DNA hybridization techniques, which determined that all 54 *V. vulnificus* strains tested possessed the homologous cytolysin gene sequence and that this technology could be useful in identification of the organism. This was followed by the studies of Wortman et al. (210) describing the cloning of the chitinase gene, a putative virulence factor. Davidson and Oliver (54) then tried to determine if the presence of plasmids had any significance in virulence. However, this investigation failed to demonstrate a correlation with the presence of plasmids (molecular mass 2.1–150 MDa) and a variety of phenotypic traits involved in virulence such as cytotoxicity to CHO cells and mouse lethality. This study did find a correlation with the presence of a 6.5 MDa plasmid and resistance to pteridine 0/129. Since then many studies have utilized molecular biology techniques/DNA techniques for the detection of the cytolysin gene in an assortment of assays including PCR (170,171,173) and DNA probe hybridization (163–165,168,270). Other molecular biological assays reported by researchers that have been useful in the identification of *V. vulnificus* from a variety of samples are nested PCR (271,272) and RAPD PCR (167,273,274) assays applying the genes for the 16S or 23S rRNA as target primers or probes (166). Other genes encoding for virulence factors that have been cloned are the epimerase gene, which is essential for capsule synthesis (275), a novel hemolysin (276), metalloprotease (277), *fur* gene responsible for the transcriptional regulation of iron (278), siderophore (196), and heme utilization (279). Bang et al. (280) have since shown that hemolysin production in *V. vulnificus* increased after the addition of cyclic AMP and was undetectable in a putative cAMP receptor protein mutant. These findings suggest that the regulation of the expression of the hemolysin may be under a carbon catabolite, global regulatory system.

In summary, with the exception of the polysaccharide capsule, it has been difficult to identify specific virulence factors and genes that contribute to the pathogenicity of *V. vulnificus*. However, there is overwhelming evidence to suggest common themes of microbial pathogenicity (198,265).

## VIII. CONTROL MEASURES

With the exception of *V. cholerae* and *V. parahaemolyticus*, relatively little is known about the susceptibility of any of the vibrios to various food-preservation methods (261). It is thought that the prevention of seafoodborne disease caused by *V. vulnificus* cannot be accomplished by reliance on a single control measure. Prevention requires knowledge about the foods serving as vehicles of transmission, food-handling errors associated with the processing of these foods that increase the risk of infection, ecology of the organism in the environment and seafood hosts, factors that place

humans at risk of infection, and identification of the mechanism(s) responsible for the persistence and emergence of *V. vulnificus* from one seafood host.

The first potential control measure is the control of fecal contamination associated with the harvest and processing waters. Though increased *V. vulnificus* levels in the environment and in seafoods do not seem to be related to increased levels of fecal contamination, it is still an important concern in fundamentally sound seafood sanitation programs (281).

Oysters normally contain up to  $10^5$  CFU of *V. vulnificus*/g oyster meat. Motes et al. (282) found that *V. vulnificus* counts in oysters naturally contaminated with *V. vulnificus*, which were relayed to offshore Gulf of Mexico waters (salinity, 30–34 ppt, suspended in racks at a depth of 7.6 m) were reduced to  $10^3$ - to  $10^4$ -fold within 7–17 days. *V. vulnificus* levels were reduced when relaying times were increased to 49 days. Offshore suspension relaying may be a method that industry can employ to reduce *V. vulnificus* levels in raw oysters. Another intervention method, depuration, wherein filter-feeding bivalves are allowed to purify themselves through the pumping of bacteria-free water through their tissues, is of considerable value in removing many human pathogens (283–285) but is of little use in reducing populations of naturally occurring *Vibrio* microflora present in mollusks (11).

While many studies have mostly demonstrated that the marine vibrios, as a group, are relatively sensitive to cold, seafoods have also been reported to be protective for vibrios at refrigeration temperatures (261). At these temperatures (4–8°C), Hood et al. (286,287) found that *V. vulnificus* could increase in numbers when oyster meats were stored. However, Cook and Rupple found that the storage of shellstock oysters at 10°C prevented multiplication of *V. vulnificus*, but did not kill the organism (288). This is presumably due to the induction of cold-responsive proteins (289). Oliver (290) further showed that death of *V. vulnificus* in oyster homogenates held at 4°C was attributable to both cold shock and deleterious factors in oyster homogenates.

Rupple and Cook (291) found that normal commercial processing did not significantly reduce the levels of *V. vulnificus* in oyster meats, but storage of processed meats, in containers, packed on ice usually produced a 1–2 log unit reduction in numbers of *V. vulnificus* after 3 and 7 days, respectively. The tenacity of *V. vulnificus* to persist in oysters following freezing and storage at –20°C, with or without vacuum packing, was found to be dependent on the length of frozen storage time for cells packaged without vacuum, with a decrease from approximately  $10^5$ – $10^1$  CFU/g of oyster meat (261) being achieved. To prevent multiplication during warm months, shellstock must be chilled to below 13°C within 2 hours (292).

Cooking and food-handling errors associated with the processing of these foods that increase the risk of infection are the next control measures to consider. Obviously, thorough cooking will kill all vibrios and eliminate them as a health hazard. In fact, all of the vibrios are sensitive to heat, although a wide range of thermal inactivation rates and conditions have been reported (261). Cook and Rupple (293) reported that decimal reduction times of 78 seconds at 47°C were useful in decreasing the number of *V. vulnificus* in raw oysters and that heating oysters for 10 minutes in water at 50°C was sufficient to reduce *V. vulnificus* populations to nondetectable levels. Thermal D values (times required to reduce the viable population of a given strain by 90%) and Z values (absolute values of the temperature required to reduce 1 log unit scale of D values) for encapsulated *V. vulnificus* cells were reported by Kim et al. (294) to be higher than that for unencapsulated cells.

The use of ionizing radiation for reducing *V. vulnificus* levels in shellstock oysters has been studied by Dixon (295). A dose of 1 kGy was found to decrease *V. vulnificus* levels by 5 log units; higher doses (1.5 kGy) were found to totally eliminate *V. vulnificus* from oysters, but also resulted in higher oyster mortalities. With the global economy and importation of seafood supplying most of the seafoods eaten in the United States, alternative practices such as hydrostatic pressure treatments (296) are being developed.

Once cooked, the handling of the seafood is also critical for preventing disease. Even if the food is cooked, there is ample opportunity for cross-contamination to occur by hands and other cooking surfaces. Time and temperature are important considerations. If cooked seafood is not eaten immediately or kept at a temperature below 4°C or above 60°C, multiplication of the vibrios may

occur. Heated and unheated polyphosphates have been shown to be lethal or highly inhibitory to a wide variety of foodborne pathogens (261). However, Oliver and Kaper (261) reported that 1% tripolyphosphate has no lethal effect on *V. vulnificus*, suggesting that such substances would not be useful as a wash or sterilization agent.

Another compelling control measure is the season when the seafood species are harvested. There is a potential for partial control by restricting consumption of raw seafoods to certain months. Data from the Gulf Coast *Vibrio* Surveillance Report (<http://www.cdc.gov/ncidod/dbmd/diseaseinfo/cstevib99.pdf>) suggests that restricting harvesting of Eastern oysters from the Gulf of Mexico to the colder months (December through April) may help decrease the overall number of illnesses. However, much more research on the ecology, epidemiology, and the identification of the mechanism(s) responsible for the persistence and emergence of *V. vulnificus* from one seafood host to humans is needed before disease-free seafoods can be produced.

A final control measure would be for persons at high risk, such as those with low gastric acid or who are immunocompromised, to avoid raw seafood. Since it has been demonstrated that protective humoral antibodies to formalin-killed encapsulated strains of *V. vulnificus* can be raised in mice and rabbits, the use of immunoglobulins derived from donors, both in passive immunoprophylaxis and as an accessory to therapy in patients with severe *V. vulnificus* infections, could be beneficial (62). However, clinical trials using such immune reagents have not been carried out.

In principle, regulating the harvesting, transportation, and storage of shellfish can minimize the public health risk of exposure to *V. vulnificus*. The incorporation of warning labels and shipping tags has also helped. However, sanitary regulations cannot completely prevent infections since *V. vulnificus* are natural inhabitants of coastal waters. The best approach is still developing the means to educate the consumer. Prevention of disease will most easily be accomplished if consumers can be educated to change their behavior or avoid behaviors that place them at risk. Research has shown that repeated reminders and having the appropriate literature available (i.e., placed in space and time to the point at which a health behavior decision must be made) increases the likelihood of compliance (297).

In conclusion, infections from *V. vulnificus* can be life-threatening and present a serious and growing public health hazard. Where does the primary responsibility lie for prevention of disease? Most likely, it is a partnership between the shellfish/seafood industry, governing parties, and the consumer, all of whom must share the burden and act socially and responsibly.

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## Other *Vibrio* Species

**Mahendra H. Kothary**

*U.S. Food and Drug Administration, Washington, D.C., U.S.A.*

### I. BACKGROUND

Vibrios are part of the normal flora of an aquatic environment, and therefore most human infections are acquired by exposure to water or consumption or handling of foods harvested from such environments. During the last 20 years, an increasing number of infections have been attributed to species other than *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Other vibrios that are responsible for infections include *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio hollisae*, *Vibrio damsela*, *Vibrio alginolyticus*, *Vibrio metschnikovii*, *Vibrio cincinnatiensis*, and *Vibrio carchariae*. Most of these nine pathogenic vibrios have been the subject of excellent reviews (1–9). The aim of this chapter is to update the information, especially that pertaining to the epidemiology, diseases, putative virulence factors, and genetics of the virulence factors. Most of the information presented in this review is retrieved from publications in English literature.

#### A. *Vibrio mimicus*

In 1981, Davis et al. (10) studied a pathogenic *Vibrio* that was classified as atypical *V. cholerae* because of its inability to ferment sucrose and named it *Vibrio mimicus*. Isolates studied were obtained from the United States, Bangladesh, Mexico, New Zealand, Philippines, Guam, and the Orient. Thirty of the 51 isolates were recovered from stools, ear infections, and a wound infection. The environmental isolates were obtained from water, oysters, prawn, and water nuts. Since this original description, the pathogen has been recovered in many other countries and has been isolated from other seafoods. Gastrointestinal infections are usually associated with consumption of seafood, while ear infections are a result of exposure to seawater.

#### B. *Vibrio fluvialis* and *Vibrio furnissii*

A group of vibrios previously referred to as Group F vibrios and Group EF6 was described and given the name *Vibrio fluvialis* by Lee et al. in 1981 (11). The species was further divided into two biogroups. Biogroup I was isolated from the environment and from humans and did not produce gas during fermentation. Biogroup II, also referred to as *V. fluvialis* aerogenic, was isolated only from the environment and produced gas during fermentation. In 1983, Brenner et al. (12) used DNA-DNA hybridization to show that strains of Biogroup II were related to those of Biogroup I but were sufficiently different to constitute a new species that was subsequently named *Vibrio furnissii*. Later it was shown that this pathogen could also be isolated from patients with diarrhea. Both pathogens have been isolated from river water, estuarine water, marine molluscs and crustacea, and from hu-

mans with diarrhea. *V. fluvialis* has also been isolated from fish. It is distributed worldwide, and gastrointestinal illness is usually associated with consumption of seafood.

### **C. *Vibrio hollisae***

Strains of vibrios that originally belonged to two groups named EF13 and Enteric Group 42 were combined to form this new species called *Vibrio hollisae*. The role of the pathogen as a possible cause of human diarrheal disease was first described in 1982 by Hickman et al. (13). It has been isolated from estuarine water, marine life, and from humans with diarrhea and other infections. Isolates have been recovered from all over the world, and gastrointestinal illness is associated with consumption of seafood contaminated with the pathogen.

### **D. *Vibrio alginolyticus***

In 1968, Sakazaki (14) proposed that biotype 2 *Vibrio parahaemolyticus* should be called *Vibrio alginolyticus* because it has various biochemical properties that are different from *V. parahaemolyticus* (Table 1). It can easily be differentiated from *V. parahaemolyticus* by its ability to ferment sucrose and consequently grow as a yellow colony on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Like *V. parahaemolyticus*, it is a common inhabitant of marine environment throughout the world. It has also been isolated from soft tissue, wound, ear, and eye infections and has been associated with a few cases of gastroenteritis. However, in many instances its role in pathogenicity cannot be determined since various other microorganisms are also isolated from the same human clinical sample.

### **E. *Vibrio damsela***

In 1981 a *Vibrio* isolated from skin ulcers occurring naturally in damselfish was named *Vibrio damsela* (15). It was originally referred to as group EF-5 *Vibrio* species. Subsequent to this original description, various unidentified *Vibrio* strains isolated from human clinical specimens were determined to be *V. damsela*. The pathogen is found in the marine environment where it causes skin lesions on some fish.

### **F. *Vibrio metschnikovii***

In 1884 *Vibrio metschnikovii* was isolated from a fowl that had died of choleraic disease, and *Vibrio proteus* was isolated from feces of patients suffering from cholera (16). In 1978 these two strains and similar organisms isolated from river water, sewage, lobster, fowl, cockles, and prawns were classified as *Vibrio metschnikovii* (16). In 1988 Farmer et al. (17) extensively characterized different isolates by DNA-DNA hybridization and phenotype. The pathogen is widely distributed in the aquatic environment and in rare instances has been isolated from some animals and from human clinical specimens. Most human isolates have been recovered from blood, urine, wound, and feces.

### **G. *Vibrio cincinnatiensis* and *Vibrio carchariae***

In 1986, studies conducted by Brayton et al. (18) indicated that a *Vibrio* isolated from blood and cerebrospinal fluid of a patient suffering from bacteremia and meningitis in Cincinnati, Ohio, represented a new species. This vibrio was named *Vibrio cincinnatiensis*. Very little is known about this pathogen.

In 1984, Grimes et al. (19) isolated two halophilic vibrios from a brown shark that had died in captivity at an aquarium. One of these vibrios was identified as *V. damsela*, while the other was found to be a new species and was named *Vibrio carchariae*. The organism has also been recovered from a human clinical sample, but not much is known about this pathogen.

**TABLE 1** Significant Tests for Differentiating *Vibrio* Species Other than *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*

Test	<i>V. mimicus</i>	<i>V. fluvialis</i>	<i>V. furnissii</i>	<i>V. hollisae</i>	<i>V. damsela</i>	<i>V. alginolyticus</i>	<i>V. cincinnatiensis</i>	<i>V. metschnikovii</i>	<i>V. carchariae</i>
TCBS	G	Y	Y	NG	G	Y	Y	Y	Y
0% NaCl	+	-	-	-	-	-	-	-	
3% NaCl	+	+	+	+	+	+	+	+	
6% NaCl	-	+	+	+	V	+	+	+	
8% NaCl	-	V	+	-	-	+	-	V	+
10% NaCl	-	-	-	-	-	+	-	-	
Arginine dehydrolase	-	+	+	-	+	-	-	V	-
Lysine decarboxylase	+	-	-	-	V	+	V	V	+
Ornithine decarboxylase	+	-	-	-	-	+	-	-	
D-Glucose, gas production	-	-	+	-	-	-	-	-	
Indole	+	-	-	+	-	+	-	-	
Methyl red	+	+	+	-	+	+	+	+	
Voges-Proskauer	-	-	-	-	+	+	-	+	V
Oxidase	+	+	+	+	+	+	+	-	
ONPG	+	V(40)	V(35)	-	-	-	+	V(50)	-
Sensitivity to O/129, 10 µg	R	R	R	R	S	R	R	S	S

Abbreviations: TCBS, thiosulfate-citrate-bile salts; Y, yellow colony; G, green colony; NG, no growth; V, variable reaction; S, sensitive; R, resistant; +, 80% or more strains positive; -, 80% or more strains negative; V, variable reaction. Values in parentheses indicate % of the strains that exhibit that property.

## II. CHARACTERISTICS

### A. Classification

The genus *Vibrio* belongs to the family Vibrionaceae, which also includes the genera *Aeromonas*, *Plesiomonas*, and *Photobacterium*. All members of the family are gram-negative, straight or curved rods. They do not form spores and are motile by means of single or more polar flagella.

### B. Biochemical

All the species in the genus *Vibrio* are chemoorganotrophs and catabolize D-glucose and other carbohydrates with production of acid but not gas (except *V. furnissii*). Sodium ion is required for the growth of many species; all the species except *V. mimicus* require the addition of NaCl to growth media. Various media and tests that are used for identifying members of the Enterobacteriaceae family are also useful in identifying the vibrios. However, these media have to be supplemented with extra NaCl. The optimal temperature for growth varies considerably; most of them grow well at 37°C but will also grow at 20°C.

## III. DISEASES

### A. *Vibrio mimicus*

In the first detailed study of *V. mimicus*, Davis et al. (10) reported that 24 of the 30 clinical isolates were recovered from feces. In a subsequent study involving 21 patients, the pathogen was associated predominantly with gastrointestinal illness (20). Among 19 patients, symptoms associated with gastrointestinal illness include diarrhea (94%), nausea, vomiting, and abdominal cramps (67%), fever (44%), and headache (39%). Diarrhea lasted a median of 6 days (range 1.5–10), with 3 of the patients having bloody diarrhea. Bloody diarrhea was also observed by Mitra et al. (21) in 2 of the 7 patients who had diarrhea. In another study (22) involving 33 patients, the symptoms of the gastrointestinal illness include diarrhea (100%), vomiting (100%), and cramps (93%). A majority of the gastroenteritis cases can be treated with oral rehydration therapy and antibiotics normally used for cholera cases. Severe cases, however, may require hospitalization.

*V. mimicus* has also been implicated as a causative agent of some extraintestinal infections. Davis et al. (10) reported that 4 of the 30 clinical isolates were recovered from patients with ear infections. Shandera et al. (20) described two isolates that were recovered from patients who had ear infections. Isolation of the pathogen from a wound infection has also been reported (10). The pathogen has also been implicated as the causative agent of bacteremia in 2 cases (23,24). The patients with the ear infections had reported recent exposure to seawater, while the 2 patients with bacteremia had diarrhea and underlying conditions such as altered immune response in one and a liver disease in the other. Isolation of the organism from blood, coupled with the observation of bloody diarrhea in some cases, suggests the possibility that *V. mimicus* may be invasive.

### B. *Vibrio fluvialis*

*V. fluvialis* has been implicated in outbreaks and sporadic cases of diarrhea. In a study conducted during a 9-month period (1976–1977), samples from 518 of the 10,674 patients with diarrhea showed the presence of the pathogen (25). Most of the patients from whom the pathogen was isolated were infants, children, and young adults. The stools sampled during this outbreak had an average of a million organisms per mL. Other symptoms associated with watery diarrhea in 34 of the patients include vomiting (97%), abdominal pain (75%), dehydration (67%), and fever (35%). About 75% of these patients had pus cells and erythrocytes in their stools. Levine et al. (26) reported that 86% of the patients with gastroenteritis had bloody stools. Presence of red blood in stools of a 1-month-old infant has also been observed (27). Gastroenteritis in an infant younger than 1 month has been



reported (28). In a small outbreak in India, the pathogen was isolated from stools of 9 of the 14 people who had gastroenteritis (29). In the United States *V. fluvialis* is usually associated with sporadic cases (24,26,27,30–35). In two cases the infections were fatal (24,30).

In a few instances *V. fluvialis* has also been recovered from wound infections resulting from a boating injury (33) and leech therapy (36), from cecostomy drainage (33), and from a patient with acute suppurative cholangitis (37).

### **C. *Vibrio furnissii***

The role of *V. furnissii* as a causative agent is questionable because it has usually been isolated together with other bacteria that also cause gastroenteritis. The pathogen has been implicated in sporadic and outbreak cases of gastroenteritis. Brenner et al. (12) described three outbreaks of acute gastroenteritis in American tourists returning from the Orient in which it was isolated from some of the patients. However, the stool samples also contained other pathogens. In the first outbreak, involving 23 people, the symptoms were diarrhea (91%), abdominal cramps (79%), nausea (65%), and vomiting (39%). None of the patients had fever, and the onset of illness was 5–20 hours after consumption of implicated food. In the second outbreak, involving 24 people, 9 of the patients who had to be hospitalized had diarrhea (100%), abdominal cramps (100%), nausea (89%), and vomiting (78%). The illness lasted less than 24 hours. In the third outbreak, involving 67 people, symptoms included diarrhea (77%), nausea (37%), and abdominal cramps (26%). *V. furnissii* and *V. fluvialis* were isolated from a 1-month-old female who had bright red blood in her stool (27). The patient did not have fever, vomiting, or abdominal discomfort. During a cholera surveillance program carried out from 1994 to 1995 in Lima, Peru, *V. furnissii* was isolated from stools of 14 persons; no other pathogenic *Vibrio* spp. were recovered (38). The role played by the pathogen in the disease is not very clear since only 6 of these 14 persons had diarrhea. It is possible that the pathogen has low virulence and does not cause illness in all infected persons.

### **D. *Vibrio hollisae***

*V. hollisae* has been associated with sporadic cases of diarrheal illness. Fifteen of the 16 isolates originally described were of gastrointestinal origin. Morris et al. (39) described 9 cases in which only *V. hollisae* was isolated from the stool samples; there were no other enteric pathogens. All the patients had diarrhea and abdominal pains. The median duration of diarrhea was 1 day (range 4 hours to 13 days). Five patients had vomiting, and one patient had bloody diarrhea. Five of the patients had fever, and 8 of the 9 patients were hospitalized. The median duration of stay in the hospital was 5 days (range 2–9 days). The median age of the patients was 35 years; 6 of the patients were male and 3 were female. In one case infection led to very severe gastroenteritis that required exploratory surgery (40).

In rare instances *V. hollisae* has also been associated with bacteremia and septicemia. It has been isolated from blood in a patient with hepatic encephalopathy, bronchopneumonia, and cryptococcal sepsis (13). In addition, it has also been isolated from a patient who had a history of cirrhosis and splenectomy (41) and from another who had chronic active hepatitis, portal hypertension, and esophageal varices with gastrointestinal bleeding (42). In one case it was reported to cause both gastroenteritis and bacteremia (43).

### **E. *Vibrio alginolyticus***

*V. alginolyticus* is generally considered to be a pathogen of extraintestinal sites and has been implicated in infections of soft tissue, wound, eye, and ear. In a few cases it has also been implicated as a causative agent of gastroenteritis.

The role of *V. alginolyticus* in gastroenteritis is not well defined. A few reports have described the isolation of the organisms from clinical samples of patients with gastroenteritis. It was isolated

from “rice water” diarrheal stool of a female with acute enterocolitis and also from the trout roe that she had eaten (44). It has been isolated from an 8-month-old male child and an 18-year-old female suffering from acute gastroenteritis with symptoms such as watery diarrhea and vomiting that lasted 2 days (45). *V. alginolyticus* was the only organism isolated from the child; *V. alginolyticus*, *Escherichia coli*, and *Pseudomonas* spp. were isolated from the rectal swab of the 18-year-old female. Chapman (46) described the isolation of the organism from two cases of diarrhea in travelers returning from India and Nepal. However, in addition to this organism, both patients had either *Campylobacter jejuni* or *Shigella sonnei*, which are well-known enteric pathogens. *V. alginolyticus* was isolated from stools of an immunocompetent 75-year-old man who developed diarrhea during his stay in the hospital (47). Absence of viruses and parasites suggested that *V. alginolyticus* might have been the most probable cause of infection. The diarrhea was greenish in appearance and contained mucus and lasted 2 days. Stool culture performed 6 days after the diarrheal episode was negative for the pathogen. It is interesting to note that the patient did not have a history of recent travel, exposure to seawater, or consumption of raw seafood. Caccamese and Rastegar (48) reported the case of an immunocompromised 38-year-old man who had a 3-month history of diarrhea. He had about five episodes of watery nonbloody diarrhea per day and denied having made any recent trips to the sea or eating raw seafood. *V. alginolyticus* was the only organism identified in the stool culture. Of all the cases of gastroenteritis described here, *V. alginolyticus* was isolated as the only organism in four of them (44,45,47,48).

*V. alginolyticus* has been implicated as a causative agent of infection in various extraintestinal sites. The most common presentations of these infections are cellulitis, otitis media, otitis externa, and conjunctivitis, and bacteremia in immunocompromised hosts. Pien et al. (49) reviewed eight cases in which the pathogen was isolated from infections in the toe, shin, foot, scalp, and ear. It was the only bacterium isolated from four of the infections and was considered to be the most probable causative agent. It has been isolated from wound infections of patients who have had recent exposure to seawater (50–59). The patients had conditions such as wound infections in the leg, cellulitis of the leg and leg ulcer, ulcerated lesions, necrotizing fasciitis, superficial septicemic lesions, and open head injury. In a study in Western Australia, it was isolated from 20 of the 36 samples of infected superficial wounds that had come in contact with seawater (60). The pathogen has been reported to cause conjunctivitis (52,61,62) and infections of the ear (49,63,64). Its ability to cause bacteremia has been demonstrated by its isolation from blood of a burn patient (53), of a man diagnosed as having acute sphenoiditis (65), and of a patient who had osteogenic sarcoma (66). In one instance it was associated with peritonitis in a 20-year-old man who had end-stage renal failure and had changed his peritoneal dialysis fluid on the beach where he had been scuba diving (67). His symptoms included severe abdominal pain, fever, and signs of peritonism. The pathogen was also recovered from the dialysis fluid.

## **F. *Vibrio damsela***

*V. damsela* has been reported to cause skin ulcers and fatal disease in damselfish (15). It was isolated from a brown shark that died in captivity and was shown to be lethal when injected into spiny dogfish (19). Pederson et al. (68) isolated it from diseased fish rainbow trout, stingrays, and nurse shark in Denmark. Various reports describe the ability of the pathogen to cause infections in bottlenose dolphins, leatherback turtles, seabream, cultured sea bass, and cultured turbot (69–73).

In humans, *V. damsela* has been associated only with wound infections. Morris et al. (39) described the case histories of six patients who did not have any underlying disease but had wound infections from which the pathogen was isolated. Wounds in four of the six cases were erythematous, indurated, and had purulent discharge. None of the cases showed necrosis, fasciitis, or serious drainage. Wounds in five of the cases required surgical debridement. Three of the six patients had to be hospitalized. All the patients recovered completely. Two hemolytic variants of the pathogen were associated with a fatal wound infection in an alcoholic man who had an underlying history of pancreatitis and insulin dependency (74). A mild superficial wound infection progressed into marked edema

of the forearm and bullae formation on the hand. The patient underwent surgery but later died because of additional complications such as disseminated intravascular coagulation, acute renal failure, and hypercalcemia. Coffey et al. (75) reported the case of another diabetic who acquired an infection through a wound in a finger. The disease rapidly progressed to a swelling of the entire hand and arm. The arm had to be amputated at the shoulder. *V. damsela* could be isolated from both the wound and the blood in a case of fulminant septicemia in a 70-year-old man who had a history of mitral valve replacement and coronary artery bypass (76). The wound on the finger developed into a swelling of the entire arm that had erythema and hemorrhagic bullous lesions. Following surgical debridement, the patient suffered from oligouric renal failure, acidosis, jaundice, and disseminated intravascular coagulation and died on the third day of hospitalization. The pathogen was isolated from the blood and necrotic tissue of a 63-year-old male who had diabetes mellitus and alcoholic liver disease (77). However, in this case, infection was acquired through consumption of raw eels and not through a wound infection. Initial swelling and erythema on the arm appeared approximately 24 hours after consumption of the eel and led to the development of bullae on the lower arm and of necrosis, which then spread to the upper arm. The patient lost consciousness, exhibited signs of septic shock and intravascular hemolysis, and died 2.5 hours after arrival in a hospital. A 64-year-old man with a history of atherosclerotic heart disease and ventricular arrhythmias acquired a fatal infection through an injury on the hand (78). Examination of the swollen hand was remarkable for erythema and profound edema of the medial right hand. The hand was debrided and irrigated, but the patient died. Another case of fatal necrotizing fasciitis was described by Yuen et al. (79). The patient did not have any underlying condition and died after surgery.

### **G. *Vibrio metschnikovii***

*V. metschnikovii* rarely occurs in human infections. None of the strains studied by Lee et al. (16) included human isolates. All the cases associated with the pathogen are sporadic. It has been reported to cause bacteremia and in rare instances has been isolated from feces of patients with diarrhea.

The first significant clinical isolate was described in 1981 (80). It was isolated from a positive blood culture of an 82-year-old woman who had peritonitis and an inflamed gall bladder. Since the patient did not have a history of recent travel or of having eaten shellfish or crabs, the source of the pathogen could not be identified. The pathogen was also isolated from a blood culture of a 70-year-old patient who had liver cirrhosis, renal insufficiency, and diabetes (81). The patient did not have a history of recent travel or of having consumed seafood and died 5 days after admission. Blood culture of an 82-year-old lady who had septicemia, respiratory problems, and infected leg lesions yielded *V. metschnikovii* (81). Swab samples of the leg lesion revealed mixed flora that included *V. metschnikovii*. Another case of mixed bacteremia in an 83-year-old female who developed high fever, chills, and malaise after being admitted to a hospital for a suspected heart attack was reported by Hardarottir et al. (82). Blood cultures from the patient yielded *V. metschnikovii* and *Staphylococcus hominis*. The pathogen has also been isolated from midstream urine of an 80-year-old woman and from a routine urine culture of a 65-year-old man with chronic alcoholism, diabetes, and incontinence and urinary frequency (2).

*V. metschnikovii* was the only enteropathogen isolated from diarrheal feces of a 60-year-old woman who had diabetes mellitus and a hepatoma (83). Studies carried out in Peru indicate that it may be associated with diarrheal diseases in infants (84). The pathogen was isolated from five 1- to 20-month-old infants who had watery diarrhea; two infants had blood in their stools. None of the stool samples were screened for other enteric pathogens, and therefore, the role of *V. metschnikovii* in the disease is not very clear.

### **H. *Vibrio cincinnatiensis* and *Vibrio carchariae***

Both of these pathogens occur rarely in human infections. *V. cincinnatiensis* has been associated with bacteremia and meningitis in a 70-year-old male patient who had a 24-hour history of lethargy

and confusion (18,85). The patient did not have a history of rashes, diarrhea, or liver disease. The pathogen was recovered from the cerebrospinal fluid and blood. In one instance it has been isolated from stools of an immunocompromised patient suffering from diarrhea (86). It is not clear as to whether the pathogen played a role in the diarrheal disease.

Pavia et al. (87) described the isolation of *V. carchariae* as a pure culture from a wound infection in an 11-year-old girl who was attacked by a shark while wading in water off the South Carolina coast. The patient did not show any signs of extensive tissue invasion or systemic toxicity. The pathogen has been reported to cause gastroenteritis in cultured grouper fish (88). The disease, characterized by a swollen intestine containing yellow fluid, ultimately results in death of the fish.

## IV. EPIDEMIOLOGY

### A. Incidence of illness

#### 1. *Vibrio mimicus*

Even before the designation of sucrose-negative *V. cholerae* as *V. mimicus*, sporadic cases of gastroenteritis associated with the organism had been reported worldwide (10). The pathogen has been isolated from human stools in the United States, Bangladesh, Mexico, Guam, Philippines, the Orient, Costa Rica, India, Japan, Belgium, and Romania (10,21,22,89–91). Most of the illnesses are sporadic cases; however, a few outbreak-related cases have also been reported (20,22,92,93). Studies in Bangladesh show that all age groups of both sexes are susceptible to infection (94). However, Campos et al. (22) reported that the majority of patients infected with *V. mimicus* were males. In a survey in Florida (1981–1988), it was isolated from 16 of the 181 cases of gastroenteritis and 2 of the 91 cases of septicemia (95). Both the septicemia cases and 14 of the gastroenteritis cases were associated with consumption of raw oysters. In a 1989 survey of *Vibrio* infections on the Gulf Coast of the United States, *V. mimicus* was isolated from 4 of the 71 patients who had gastroenteritis (26). In more recent surveys of *Vibrio* infections reported to the Centers for Disease Control and Prevention (CDC), 11 of the 386 in 1997 and 10 of the 551 in 1998 were caused by *V. mimicus* (96). Nine of the 11 infections in 1997 and all 10 infections in 1998 were gastroenteritis cases.

#### 2. *Vibrio fluvialis*

Sporadic and outbreak-related cases have been reported from all over the world. The pathogen has been isolated from patients who had contracted the disease in Bahrain, Bangladesh, Egypt, India, Indonesia, Iran, Iraq, Jordan, Kenya, Philippines, Saudi Arabia, Spain, Tanzania, Tunisia, and the United States (11,27,29–34,95). In addition to adults, it also affects children (25,27,97). It was isolated from 12 patients during a 6-year period (1982–1988) in Florida (33). The pathogen was recovered from stools of 10 patients who had gastroenteritis. Eight of the 10 patients had consumed raw oysters (5 patients), shrimp (2 patients), or cooked fish (1 patient). *V. fluvialis* was isolated from 5.6% of the 125 cases of *Vibrio*-related gastroenteritis associated with consumption of raw oysters (95). In 1989 an investigation along the Gulf Coast of the United States showed that it was isolated from 7 of the 71 patients who had gastroenteritis (26). Three of the patients reported eating raw oysters, while a fourth had eaten shrimp prior to becoming ill. Three of the 7 patients had to be hospitalized. Recent data show that *V. fluvialis* was responsible for 17 of 386 and 27 of the 551 *Vibrio* infections reported to the CDC in 1997 and 1998, respectively (96). Nine infections in 1997 and 16 infections in 1998 were gastroenteritis cases; 5 cases in each year were associated with wound infections. *V. fluvialis* was isolated from an 81-year-old patient who had severe diarrhea and died after 5 days of illness (30). *V. fluvialis* and *V. mimicus* were isolated from the stools of a 30-year-old man who had ileitis and reported eating seafood a few days before his illness (31). Both *V. fluvialis* and *V. furnissii* were present in the stool sample of a 1-month-old female who had mild diarrhea and was passing bright red blood in her stool (27). The child had not been exposed to any shellfish or anyone who had eaten shellfish but had been fed formula mixed with tap water. *V.*

*fluviialis* was isolated from the stools of a 46-year-old man who had acute gastroenteritis which began 36 hours after consumption of raw oysters (32). It was the only pathogen isolated from the stool of a 54-year-old man who had severe diarrhea and vomiting and subsequently died of cardiopulmonary arrest (24). He had consumed raw oysters one day before the onset of symptoms. The pathogen has also been isolated from an AIDS patient who had diarrhea (34). Analysis of stools from 20 U.S. Peace Corps volunteers who came down with diarrhea in Thailand showed that one of the samples had *V. fluviialis* (35). Examination of stools of 76 patients with diarrhea following the recent floods in Bangladesh showed that *V. fluviialis* was present in some of the samples (98). During a 16-month cholera surveillance program in Lima, Peru, the pathogen was frequently isolated from cases of diarrhea (38).

### 3. *Vibrio furnissii*

In the first reported outbreak, 23 of the 42 elderly American tourists returning from the Orient developed gastroenteritis (12). Two of the people required hospitalization and one woman died. A shrimp and crab salad or a cocktail sauce or both were determined to be the cause of the illness. In another outbreak in tourists returning from Hong Kong, 24 of the 59 people developed the illness; 9 of these required hospitalization. A food vehicle responsible for the illness could not be determined. In a third outbreak involving 67 tourists returning from Tokyo, a common food vehicle could not be implicated. *V. furnissii* and *V. fluviialis* were isolated from blood-tinged stools of a 1-month-old female who had not been exposed to any shellfish or persons who ate shellfish (27). She had, however, been fed formula mixed with tap water. A common source of infection could not be established for the 14 persons in Peru who had the pathogen in their stools (38).

### 4. *Vibrio hollisae*

The original description of the disease included three cases in Florida, four in Maryland, and one each in Louisiana and Virginia (39). Six of the patients had eaten raw seafood, while one had eaten seafood that may not have been raw. A female who consumed two dozen oysters harvested from the Chesapeake Bay came down with severe gastroenteritis and had to be hospitalized (99). Her friend, who had consumed only six oysters, suffered from a milder illness. In one instance, a case of septicemia has been associated with the consumption of catfish harvested from the Mississippi River, which has ~0.5% salinity at locations 80 miles downstream (41). A food vehicle could not be identified in the case of a man who had bacteremia (42). A survey of 32 patients in the Chesapeake Bay area over a 15-year period (1974–1988) showed that 1 of the 40 isolates was *V. hollisae* (100). In a survey of *Vibrio* infections in 1989 on the Gulf Coast, Levine et al. (26) found that 9 of the 121 infections were caused by *V. hollisae*. Eight of these nine were cases of gastroenteritis, and one was a wound infection. Seafoods most commonly associated with the gastrointestinal infections included raw oysters, clams, crabs, and shrimp. A similar survey of *Vibrio* illnesses in Florida over an 8-year period showed that 32 of the 333 cases were caused by *V. hollisae* (95). Twenty of these 32 were associated with consumption of raw oysters; 17 of these were gastroenteritis, and 3 were septicemia cases. Recent data from the CDC indicate that the pathogen was associated with 9 of the 386 and 11 of the 551 infections in 1997 and 1998, respectively (96). Nineteen of these cases were gastroenteritis-related.

### 5. *Vibrio alginolyticus*

Illness caused by *V. alginolyticus* occurs worldwide. In a survey of *Vibrio* illness during 1981–1988 in Florida, Desenclos et al. (95) reported that the pathogen was isolated from 14 of the 333 cases. Eleven isolates were associated with wounds, 2 with gastroenteritis, 1 with septicemia, and one with other disease. In a similar survey of *Vibrio* infections on the Gulf Coast of the United States in 1989, it was isolated from 7 of the 121 infections (26). The 7 isolates were from wounds (5), ear infection (1) and gangrenous gallbladder (1). In Europe, Hornstrup and Gahrn-Hansen (101) reported that *V. alginolyticus* was found in 17 of the 30 extraintestinal *Vibrio* infections that occurred in a Danish county over a 6-year period. All the 17 patients had ear infections. Recent data compiled

by the CDC show that the pathogen accounted for 7 of the 386 infections reported in 1997; 2 were associated with gastroenteritis, 2 with wound infection, and 3 with other illnesses (96). Data for 1998 show that it accounted for 23 of the 551 infections; 2 were associated with gastroenteritis, 1 with septicemia, 8 with wound infections, and 12 with other types of infections.

#### 6. *Vibrio damsela*

Surveys of *Vibrio* illness in Florida during 1981–1988 and the Gulf Coast in 1989 found that *V. damsela* was responsible for only 1 of 52 and 1 of 29 wound infections, respectively (26,95). The pathogen was associated with 5 of the 386 and 3 of the 551 *Vibrio* infections reported to the CDC in 1997 and 1998, respectively (96). All 3 cases in 1998 and 2 of the cases in 1997 were associated with wound infections. Surprisingly, one case in 1997 was associated with gastroenteritis. Five of the 6 cases reported by Morris et al. (39) were patients who were injured in or near seawater or brackish water. Most infections were the result of injuries sustained while coming in contact with marine life (e.g., stingray bite, stepping on catfish barb, filleting fish, fish fin puncture, cleaning catfish) (39,74–76,78,79). A case of primary septicemia in a man with underlying diabetes and alcoholic liver disease was associated with consumption of raw eels (77). Eels have been reported to succumb to infections with *V. damsela* (102). Most of the victims have been males, and most had underlying conditions such as heart disease, alcoholic liver damage, diabetes, and pancreatitis. Infections were usually on the leg or the arms.

#### 7. *Vibrio metschnikovii*, *Vibrio cincinnatiensis*, and *Vibrio carchariae*

*V. metschnikovii* has been reported to cause bacteremia (80–82) and has been isolated from a 60-year-old woman and 11- to 20-month-old infants who had diarrhea (83,84). All the reported bacteremia cases have been in the elderly (70–84 years). None of the patients had any recent history of exposure to marine environment or consumption of seafood.

There have been only two reported isolations of *V. cincinnatiensis* from human clinical samples. It was first isolated from a 70-year-old man who had bacteremia and meningitis (18,85). The patient did not have a history of recent exposure to seawater, consumption of seafood, or recent travel. In addition, he did not have any diarrhea or underlying liver disease and was not receiving any medication at the time of admission to the hospital. The second isolate was from a stool sample of an immunocompromised elderly patient (86).

There has been only one reported illness caused by *V. carchariae*, and not much is known about the epidemiology of the pathogen (87).

## B. Natural reservoir

#### 1. *Vibrio mimicus*

*V. mimicus* is a typical marine *Vibrio* and, therefore, can be recovered from marine environments throughout the world. It has also been recovered from brackish and freshwater environments. Numerous reports describe its isolation from freshwater rivers, ponds and lakes (103–107), seawater (10,106–111), waternuts (10), turtle eggs (22,108), plankton (106), oysters (10,111), prawns (10,112), mussels (113), and fish (107,114). In a study by Wong et al. (115) it was present in 44% of oysters, 35% of hard clams, 50% of freshwater clams, 38% of shrimps, and 8% of crabs. Of the three sites sampled in the Atlantic coast of Brazil, only one site had *V. mimicus* in 17% of the mussels (113). The counts ranged from <3 to 9 per 100 g. In another study, the pathogen comprised 12% of the *Vibrio* isolates recovered from oysters originating from the southern coast of Brazil (116). The counts ranged from <3 to 40 per 100 g.

In Bangladeshi aquatic environments where the temperatures ranged from 21.5 to 35°C, *V. mimicus* could be isolated throughout the year (106). In contrast, a seasonal distribution was observed in waters of a temperate region of Japan where the temperatures ranged from 4 to 29°C. It was not recovered when the temperatures fell below 10°C. The highest counts in 100 mL of water were 900 and 15,000 colony-forming units (CFU) in Bangladesh and Japan, respectively. A similar sea-

sonal effect was also observed in a freshwater river (104). Plankton obtained from a river in Japan had counts as high as 60,000 CFU of *V. mimicus* (106).

## 2. *Vibrio fluvialis* and *Vibrio furnissii*

*V. fluvialis* has been recovered from marine environments and from brackish and freshwater environments throughout the world. Its presence in water bodies such as oceans, rivers, ponds, swamps, and estuaries has been described (11,111,117–121). It has also been isolated from sediments (118), crabs and prawns (11,115,118,120), oysters (115,122), clams (115), shrimp (115), seafish (123), and shark (124). Kelly and Stroh (122) found that 35% of the naturally harvested oysters from the Pacific Northwest had the pathogen. They also reported that it comprised 30% of the vibrios isolated from oysters. On the Atlantic coast of Brazil, 34–65% of mussels sampled at three different sites contained *V. fluvialis*; the counts ranged from <3 to 1100 per 100 g (113). It comprised 27% of the *Vibrio* isolates recovered from oysters originating from the southern coast of Brazil (116); the counts ranged from 3 to 150 per 100 g. The pathogen was isolated from 7% of the frozen shrimp imported from China, Ecuador, and Mexico (125). In a large-scale survey of foods in Taiwan, Wong et al. (115) found that *V. fluvialis* was present in oysters (69%), hard clams (65%), freshwater clams (79%), crabs (25%), and shrimps (6%).

*V. furnissii* has been isolated from brackish and estuarine waters and from feces of rabbits, pigs, and humans (11,12,27,38). In Brazil, 17% of the mussels at one of the three sites sampled on the Atlantic coast had the pathogen; counts ranged from <3 to 30 per 100 g (113). It comprised 19% of the isolates recovered from oysters originating from the southern coast of Brazil; the counts ranged from <3 to 40 per 100 g (116).

## 3. *Vibrio hollisae*

*V. hollisae* is a marine *Vibrio* but has not been isolated from many different kinds of environments. This is probably the result of the inability of many strains to grow on TCBS agar, which is widely used during isolation procedures. It has, however, been reported to be present in oysters (122) and coastal fish (126).

## 4. *Vibrio alginolyticus*

*V. alginolyticus* is very commonly found in the marine environment and occurs in human clinical specimens of patients suffering from gastroenteritis and wound, eye, and ear infections. The occurrence of the organism in seawater and seafood throughout the world was thoroughly reviewed by Joseph et al. (8). It is usually present in large numbers in seawater and is the most commonly isolated *Vibrio* from marine environments. In one of the first ecological studies done, Kempelmacher et al. (127) reported that the pathogen could be isolated from 3.5% of mussels, 6.8% of oysters, and 5.6% of water samples collected during January to March in the Netherlands. A much larger number (85%) of water samples collected from the Dutch coastal waters during the warmer months of August and September contained *V. alginolyticus* (128). In another study of marine and estuarine bathing waters of the Danish coast during the months of May to November, Larsen et al. (129) were able to isolate the pathogen from 8 and 16% of the marine and estuarine samples, respectively. In Indonesia, the pathogen was isolated more frequently than *V. parahaemolyticus* from seawater (130). The frequency of occurrence of the organism in seawater samples ranged from 0% in February and March to 100% in September and December. In Hong Kong it was the most frequently isolated *Vibrio* from coastal waters sampled at six different sites (131). In a study during the warmer months of April to May, it was isolated as the predominant *Vibrio* in the estuarine waters off the coast of Italy (132). In addition to its presence in Danish, Indonesian, and Hong Kong waters (127–131), it has been reported to be present in the United States, Alaskan, Japanese, and Italian waters (8,133–136). It has been isolated from a variety of fish, oysters, crabs, clams, shrimp, prawns, mussels, and lobsters. Molitoris et al. (130) were able to isolate the organism from mackerel, shrimp, squid, pomfret, trevally, milk fish, crab, and fusilier purchased from markets in Indonesia. Results of a survey of oysters, mussels, clam prawn, crab, rabbitfish, and grouper bought from local markets in

Hong Kong showed that *V. alginolyticus* was the most frequently isolated *Vibrio* species (137). A similar study done for foods in Taiwan showed that the pathogen was present in 69% of oysters, 60% of hard clams, 57% of freshwater clams, 70% of shrimps, and 67% of crabs (115). Almost all (92–100%) of the mussels harvested from three different locations in Brazil contained *V. alginolyticus* at concentrations of <3 to 24,000 per 100 g (113). The pathogen comprised 81% of all *Vibrio* isolates recovered from oysters originating from the southern coast of Brazil (116). The counts ranged from, 3 to 1500 per 100 g. In a study of raw shrimp imported from China, Ecuador, and Mexico, it comprised 27% of the isolates (125).

*V. alginolyticus* has also been isolated from organs and blood of a dolphin (138,139), teeth of white shark (124), moribund aquarium lobsters (140), and diseased kuruma and tiger prawns (141,142).

#### 5. *Vibrio damsela*

*V. damsela* has been reported to be isolated from the marine environment, marine fish, natural and cultivated oysters, and sewage (2,68,122,123).

#### 6. *Vibrio metschnikovii*, *Vibrio cincinnatiensis*, and *Vibrio carchariae*

*V. metschnikovii* has been isolated from river water, sewage, cockles, prawns, crabs, clams, lobsters, shrimps, fowl, and birds (16,17). There have been no reports describing the isolation of *V. cincinnatiensis* and *V. carchariae* from marine waters. However, *V. cincinnatiensis* has been recovered from stomachs of aborted bovine fetuses (86), and *V. carchariae* has been isolated from a brown shark and grouper fish (19,88).

### C. Transmission

The presence of vibrios in the marine environment suggests that consumption of a variety of seafoods, especially those that are raw or undercooked, can lead to infections. Moreover, exposure of body and wounds to seawater and marine life can result in serious, sometimes fatal, infections.

The presence of *V. mimicus* in foods such as oysters, clams, shrimp, prawns, crab, mussels, fish, and turtle eggs suggests that the pathogen may be present in other seafoods. Consumption of raw or undercooked seafood such as crawfish, raw oysters, shrimp, crabs, turtle eggs, and raw fish has led to gastrointestinal illness caused by *V. mimicus* (20,22,24,26,107). Foods such as oysters, shrimps, fish, and vegetarian meals have been implicated in sporadic and outbreak cases associated with *V. fluvialis* (26,29,31–34,95). Information obtained from one of the three outbreaks originally described and from the case of a 1-month-old infant suggest that seafood and water consumed by the patients may have been contaminated by *V. furnissii* (12,27). A common food vehicle, however, could not be determined for the 14 people in Peru who had *V. furnissii* in their feces (38). Foods such as raw oysters, raw clams, cockles, and catfish have been implicated in cases of gastroenteritis associated with *V. hollisae* (39–41,43,95,99,100).

Most of the infections caused by *V. alginolyticus* are associated with patients who have had recent exposure to a marine environment. Some wound infections were acquired through injuries sustained in or near seawater during recreational activities such as swimming, sailing, and diving (49–60). Eye and ear infections were probably acquired during swimming in seawater (63,64). Conjunctivitis acquired through an occupation as a fish cutter and through gardening where seashells were used as fertilizer have also been described (52,61).

*V. damsela* gains access to humans through an injury in or near a marine environment. In one case it was reported that a patient acquired infection through consumption of raw eel containing the pathogen (77). Its presence in seafish harvested for human consumption can also result in infections (123). The routes of transmission for *V. metschnikovii*, *V. cincinnatiensis*, and *V. carchariae* are not very clear. It is possible that *V. metschnikovii* may be acquired from river water, sewage, cockles, prawns, crabs, clams, lobsters, shrimps, fowl, and birds (16,17). *V. cincinnatiensis* has not been isolated from the environment. *V. carchariae* has been isolated from a shark (19), and the



only case associated with this pathogen is of a girl who was attacked by a shark while wading in water (87).

## V. ISOLATION AND IDENTIFICATION

Vibrios are facultatively anaerobic, grow in the presence of high levels of bile salts, grow well under alkaline conditions, and require  $\text{Na}^+$  for growth. Usually, incorporation of 0.5% NaCl in transport, enrichment, and isolation media is sufficient for supporting the survival and growth of vibrios. Higher concentrations (2–3%) can be incorporated into various media in order to make them more selective for certain vibrios. Isolation of vibrios from food samples usually involves an enrichment step. Enrichment is carried out in alkaline peptone water, and isolation is normally accomplished by plating the enrichment broth onto TCBS agar. The incorporation of sucrose helps in separating the vibrios into sucrose-positive and sucrose-negative species. *V. fluvialis*, *V. furnissii*, *V. alginolyticus*, *V. metschnikovii*, *V. cincinnatiensis*, and *V. carchariae* are sucrose-positive, while *V. mimicus*, *V. hollisae*, and *V. damsela* are sucrose-negative. *V. hollisae* and *V. damsela*, however, do not grow well on TCBS and may not be recovered from foods if other media are not used. In addition to TCBS agar, MacConkey agar and blood agar can be used for isolating these two pathogens.

Identification of the isolates is usually accomplished with the help of various biochemical media that are supplemented with 1% NaCl. Farmer and Hickman-Brenner (2) have described in great detail the various methods and the media normally used for identifying pathogenic vibrios. The various isolation and identification methods for foodborne vibrios are described by Elliot et al. (143).

An abbreviated list of growth characteristics and results of biochemical tests that can be used in identifying species that are not *V. cholerae*, *V. parahaemolyticus*, or *V. vulnificus* is shown in Table 1. Some of the major differences between the species are described here. Unlike other vibrios, *V. metschnikovii* does not exhibit any oxidase activity. *V. mimicus*, *V. alginolyticus*, *V. carchariae* and some strains of *V. metschnikovii*, *V. cincinnatiensis*, and *V. damsela* possess lysine decarboxylase activity, while only *V. mimicus* and some strains of *V. alginolyticus* are ornithine decarboxylase positive. *V. fluvialis*, *V. furnissii*, *V. damsela*, and some strains of *V. metschnikovii* are arginine dihydrolase positive. *V. hollisae* is the only *Vibrio* that is negative for all three enzymes, and *V. mimicus* is the only species that will grow without any additional NaCl. *V. furnissii* can be distinguished from *V. fluvialis* by its ability to produce gas from glucose.

## VI. PATHOGENICITY

### A. *Vibrio mimicus*

The clinical symptoms of illness are similar to those caused by *V. cholerae* and *V. parahaemolyticus*. This suggests that *V. mimicus* may produce virulence factors similar to those produced by the two pathogens. Investigators have demonstrated the production of an enterotoxin similar to cholera toxin and the presence of genes encoding the zonula occludens toxin (ZOT) and accessory cholera enterotoxin (ACE) produced by *V. cholerae*. It also produces heat-stable enterotoxins, a heat-stable hemolysin similar to the *V. parahaemolyticus* thermostable direct hemolysin (TDH), a heat-labile hemolysin similar to the *V. cholerae* hemolysin, a protease/hemagglutinin, and nonprotease hemagglutinins. It also has the ability to adhere to and colonize the mucosal surface of the small intestine of rabbits, adhere to formalin-fixed human intestinal mucosa, and produce a siderophore and pili.

#### 1. Enterotoxins

The ability of filtrates of culture broth to induce fluid accumulation in suckling mice provided the first evidence that *V. mimicus* produces enterotoxins (144). An enterotoxin that exhibited activity in rabbit ileal loops and Y-1 mouse adrenal cells was shown to be identical to cholera toxin

(145,146). The purified toxin has physicochemical and biological properties similar to cholera toxin (147). Both clinical and environmental isolates possess the ability to produce this toxin (89,105,148,149). Various ELISA methods employing ganglioside GM<sub>1</sub> and monoclonal antibodies specific for cholera toxin can be used for detecting the *V. mimicus* toxin (149–151). Some strains possess genes that encode ZOT and ACE of *V. cholerae* (152,153). A heat-stable factor that induces fluid accumulation in suckling mice was first demonstrated by Nishibuchi and Seidler (144). This toxin is identical to the heat-stable enterotoxin of non-O1 *V. cholerae* (NAG-ST) and is produced by environmental and clinical isolates of *V. mimicus* (105,149,153–155). The 17 amino acid residues of the purified toxin are identical to those of NAG-ST (156).

## 2. Hemolysins

In 1987 Honda et al. (157) reported that the pathogen produces a heat-labile as well as a heat-stable hemolysin. The heat-labile hemolysin is similar to the hemolysin of *V. cholerae* while the heat-stable hemolysin has strong homology to the TDH of *V. parahaemolyticus*. The heat-labile hemolysin is prevalent in clinical isolates, has a molecular weight of 63,000, belongs to the family of pore-forming hemolysins, and induces fluid accumulation in a ligated rabbit ileal loop (158–160). The ability of whole cells to elicit fluid accumulation is reduced but not eliminated when antihemolysin antibodies are also injected into the loop (160). These observations suggest that like the *V. cholerae* hemolysin, this hemolysin may be responsible for the dysentery-like diarrhea in human disease. The heat-stable hemolysin has a molecular weight of 22,000, an isoelectric point of 4.9, and immunologically cross-reacts with the TDH (161). In one study, 16 of the 17 clinical isolates screened were found to possess the TDH-like toxin gene (162). In contrast, only 1 of the 2 food isolates and none of the 29 environmental isolates were positive for the gene. In another study, 5 of the clinical isolates and none of the 3 environmental isolates produced this hemolysin (149).

## 3. Protease

Production of a soluble hemagglutinin was first described by Dotevall et al. (146). This hemagglutinin is a heat-labile metalloprotease that has a molecular weight of 31,000 and immunologically cross-reacts with the *V. cholerae* hemagglutinin/protease (163). The protease enhances vascular permeability and forms edema when injected into guinea pig and rat dorsal skin and consequently may play a role in extraintestinal infections caused by the pathogen (164). Only the crude, not the purified, *V. mimicus* protease preparation induces fluid accumulation in rabbit ileal loops (165). Also, the inability of crude preparation in which the proteolytic activity has been inactivated to induce fluid accumulation suggests that the protease may be an important virulence factor in the enterotoxigenicity of *V. mimicus*. The protease has also been postulated to play a role in nicking and activating cholera toxin and other toxins produced by the pathogen (163). In addition to its proteolytic function, the enzyme agglutinates chicken, sheep, bovine, rabbit, guinea pig, and human erythrocytes (163,166). In addition to this metalloprotease, *V. mimicus* is reported to produce another metalloprotease that has a higher molecular weight and has strong homology to the metalloprotease of *V. parahaemolyticus* (167). Its role in virulence has not been identified.

## 4. Other Virulence Factors

In diarrheal diseases, attachment to the mucosal surface and subsequent colonization are important determinants in the pathogenesis of the infection. Environmental isolates that did not show much activity in various animal models have been shown to be less capable of attaching and colonizing the small intestinal mucosa of rabbits; clinical isolates possessed both these properties (168). In another study, 96% of the 77 environmental isolates were capable of adhering to rabbit intestinal segments of the mucosa (169). Moreover, a strong relationship was observed between the ability of the pathogen to adhere to and to cause cell-mediated hemagglutination of rabbit erythrocytes. Clinical and environmental isolates have also been shown to adhere to HeLa cells in tissue culture (170). In addition to the protease, which has hemagglutination properties, the pathogen also produces two

other hemagglutinins that agglutinate rabbit erythrocytes (171,172). One of these is an outer membrane protein with a molecular weight of 39,000 while the other is a lipopolysaccharide. The outer membrane protein agglutinates rabbit, chicken, and guinea pig erythrocytes, while the lipopolysaccharide agglutinates only rabbit erythrocytes. Glycoproteins inhibit agglutination induced by both these hemagglutinins and eliminate the ability of the pathogen to attach to sections of rabbit intestinal mucosa (173). In addition, incubation of the outer membrane protein with the protease results in increased hemagglutination of rabbit, chicken, mouse, and guinea pig erythrocytes. These results suggest that all three hemagglutinins (protease, outer membrane protein, and lipopolysaccharide) play a role in the adherence of the pathogen to mucosal surfaces. The roles of these three virulence factors in adherence to human intestinal cell remains to be elucidated. In addition to these hemagglutinins, *V. mimicus* produces pili and a siderophore that is an aerobactin (174,175). Expression of pili, however, did not correlate with adherence to mucous layer and to epithelial cell surface. Also, the level of adherence in this study was much lower than that observed for *V. cholerae* O1. Low virulence of *V. mimicus* may be the result of its reduced ability to adhere to human intestinal cells.

## **B. *Vibrio fluvialis* and *Vibrio furnissii***

Clinical symptoms of gastroenteritis caused by *V. fluvialis* and *V. cholerae* are very similar. Consequently, the ability of *V. fluvialis* to cause diarrhea has been tested in various animal models originally designed for *V. cholerae*. Experiments aimed at optimizing the growth medium showed that production of the enterotoxin was dependent upon the culture medium used (144). Culture filtrates of clinical but not environmental isolates induced fluid accumulation in suckling mice. Whole cells were also shown to elicit fluid when fed to suckling mice (176,177) and in rabbit ileal loops (120,178). Lockwood et al. (179) described the production of a toxin that elongates Chinese hamster ovary (CHO) cells (similar to that observed for cholera toxin), a cytolysin active against rabbit erythrocytes, a nonhemolytic CHO cell-killing cytotoxin, and a protease. Further studies showed that partially purified preparations of elongation factor, cytolysin, and protease induced fluid accumulation in suckling mice (176). Studies with a partially purified cytotoxin indicate that it is heat-labile, inactivated by proteases, has an isoelectric point of 4.8, and has an apparent molecular weight of 12,200 (180). Chikahira and Hamada (178) showed that the CHO cell-killing factor and the CHO cell-elongation factor were produced by both clinical and environmental strains. Their studies also showed that the CHO cell-elongation factor could be neutralized by anticholera toxin serum. In addition, results of ELISAs suggested that almost two thirds of both the clinical and environmental isolates tested produced cholera toxin. Both clinical and human strains caused fluid accumulation in rabbit ileal loops; however, none of these strains elicited fluid accumulation in suckling mice. Studies done by Ahsan et al. (181) using 26 strains, however, showed that culture filtrates did not react with antibodies to cholera toxin when assayed in a GM<sub>1</sub> ELISA and in an Ouchterlony gel diffusion test. Hemolytic activity described by Lockwood et al. (179) has also been reported by other investigators. Almost 97% of isolates recovered from seafood and five of eight strains isolated from river water and prawns were hemolytic against sheep blood (115,120). Production of this heat-labile hemolysin is maximal in brain heart infusion broth (182). *V. fluvialis* has been shown to adhere to HeLa cells and to hemagglutinate guinea pig erythrocytes (170). The extracellular protease is elastolytic and can also hemagglutinate erythrocytes (183). Other putative virulence factors produced by *V. fluvialis* include a lipase, mucinase, chondritin sulfatase, hyaluronidase, lecithinase, DNase, and fibrinolysin (184). Production of siderophores has also been reported (185). However, the roles of most of the described factors remain to be elucidated.

*V. furnissii* is reported to produce siderophores and to have hemolytic, lipolytic, and proteolytic activities (185). Chikahira and Hamada (178) reported that most strains produced substances that were lethal to mice and possessed hemolytic activities. In addition, cell extracts of most strains exhibited cytotoxicity towards CHO cells. However, cell extract from only one strain elongated CHO cells. The elongation factor cross-reacted with cholera toxin. Shirai et al. (186) have shown that of all the different vibrios examined by DNA hybridization, only one of the three strains of

*V. furnissii* possessed the gene encoding the TDH-related hemolysin of *V. parahaemolyticus*. The significance of these virulence factors is not very clear, since even the role of the pathogen in causing a diarrheal disease is questionable.

### **C. *Vibrio hollisae***

The presence of a hemolysin similar to the TDH of *V. parahaemolyticus* was first demonstrated using TDH-specific gene probe (187). All 11 strains tested were positive for the gene. Both clinical and environmental isolates produce the hemolysin (188). The purified hemolysin has two subunits with a molecular weight of 23,200, is not as heat-stable as the TDH, and is lethal when injected into mice (189,190). It has 165 amino acid residues, and comparison of the amino acid sequence to that of TDH show that 23 of the amino acid residues in the hemolysin are different from those in TDH (191). Moreover, the sensitivity of chicken, sheep, and calf erythrocytes to the hemolysin is quite different from their sensitivity to TDH. Antibodies against the TDH react with the hemolysin (190). In addition, monoclonal antibodies raised against the TDH and the heat-labile hemolysin of *V. parahaemolyticus* (192) also react with the *V. hollisae* hemolysin (193).

Intragastric administration of *V. hollisae* cells into suckling mice elicits fluid accumulation (194). The pathogen produces an extracellular enterotoxin that elongates CHO cells in tissue culture. The purified enterotoxin has a molecular weight of 80,000, is heat-labile, and induces fluid accumulation in suckling mice (195). Elongation of the CHO cells is not accompanied by an increase in the levels of cyclic AMP, and the enterotoxin does not react with various gangliosides and antibodies to cholera toxin. These observations suggest that the enterotoxin is structurally and functionally different from other CHO cell–elongating toxins.

*V. hollisae* is able to adhere and invade cultured epithelial cells (196). Internalization involves eukaryotic and prokaryotic protein syntheses, microfilaments, microtubules, and receptor-mediated endocytosis. Internalized *V. hollisae* are able to multiply within the tissue culture cells. This ability of the pathogen to invade cells may explain the bacteremia and septicemia observed in some patients (13,41–43). *V. hollisae* produces a hydroxymate siderophore in response to low iron in the growth medium (174). The siderophore is an aerobactin and may play a role in the ability of the pathogen to cause infection.

### **D. *Vibrio alginolyticus***

Very little information is available about the pathogenic mechanisms of *V. alginolyticus*. Molitoris et al. (130) reported that all seven strains tested were lethal for mice when injected intraperitoneally. In addition, one of the five strains was positive for the Kanagawa phenomenon exhibited by *V. parahaemolyticus* (i.e., produced TDH-like hemolysin), and one of the eight strains tested elicited fluid accumulation in ligated rabbit ileal loops. One strain has been reported to produce lipase, lecithinase, chondroitin sulfatase, DNase, and hemolysin (184). The pathogen also produces various extracellular alkaline proteases and collagenase (197), but the roles of these proteolytic enzymes in infection are not clear. Of the 174 isolates recovered from seafood and aquacultured foods, 98% produced lipase, 78% produced protease, and 75% were hemolytic for sheep blood (115). A majority (80%) of the 35 isolates tested was cytotoxic for CHO cells. Recently, Barbieri et al. (132) reported that 19% of the strains isolated from water adhered to Hep-2 cells and exhibited cytotoxic activity against CHO cells. In addition, 7 of the 32 strains induced rounding of the CHO cells accompanied by a breakdown of the actin cytoskeleton.

### **E. *Vibrio damsela***

Grimes et al. (19) showed that culture supernatant of a broth culture was cytotoxic for Y1 mouse adrenal tumor cells in tissue culture. Kreger (198) reported that 17 of the 19 strains tested produced detectable amounts of a cytotoxin that was hemolytic against mouse erythrocytes. Moreover, 5 strains

that produced the largest amounts of the toxin had mouse LD<sub>50</sub> values that were 10- to 30-fold lower than those of 12 strains that produced small amounts of the toxin. Clarridge and Zigelboim-Daum (74) described the isolation of two phenotypes of the pathogen from the tissue of a patient with wound infection. Both of them exhibited different amount of hemolysis on rabbit blood agar. The purified cytolysin, termed damselysin, is heat-labile, has a molecular weight of 69,000 and an isoelectric point of 5.6, and is hemolytic for erythrocytes from mice, rat, rabbits, and damselfish (199,200). It is very lethal for mice and has an intraperitoneal median lethal dose of 1 µg/kg. Damselysin has been shown to be a unique phospholipase D that is active against phosphatidylcholine and phosphatidylethanolamine and also possesses hemolytic activity (201).

Using mice and turbotfish as experimental animals, Fouz et al. (202) showed that there was a correlation between the availability of iron in host fluids and the degree of virulence exhibited by *V. damsela*. Lethality of the organism was much greater in iron-overloaded animals.

#### **F. *Vibrio metschnikovii*, *Vibrio cincinnatiensis*, and *Vibrio carchariae***

A heat-labile cytolysin produced by a clinical isolate of *V. metschnikovii* lyses erythrocytes from calf, rabbit, guinea pig, mouse, human, sheep, chicken, and horse, elicits fluid accumulation in suckling mice, and increases vascular permeability in rabbit skin (83). It has a molecular weight of 50,000 and an isoelectric point of 5.1 and does not immunologically cross-react with TDH of *V. parahaemolyticus* and the hemolysin of *V. mimicus*. Although the cytolysin has properties that can enable it to act as an enterotoxin, it is not clear whether it plays a role in the pathogenicity of the organism.

*V. carchariae* has been reported to be weakly cytotoxic towards Y1 cells (19) and hemolytic against erythrocytes from sheep, rabbit, tilapia, and grouper (88). Not much is known about the pathogenic potential of *V. cincinnatiensis*.

### **VII. GENETIC FACTORS INVOLVED IN VIRULENCE**

#### **A. *Vibrio mimicus***

##### *I. Enterotoxins*

Early studies aimed at determining the molecular epidemiology of *V. mimicus* using a cholera toxin gene (*ctx*) probe showed that all three strains isolated from stools and water at different times possessed the gene (109). However, the toxin gene pattern and the chromosomal digest pattern were quite different from the pattern observed for *V. cholerae* O1 strains; the three *V. mimicus* strains had identical patterns. It has been shown that nontoxigenic *V. mimicus* may have become toxigenic by acquiring the filamentous bacteriophage CTXΦ, which encodes cholera toxin in *V. cholerae* (203). Such converted strains were shown to produce cholera toxin in vitro and to cause diarrhea in the removable intestinal tie-adult rabbit diarrhea model. In another study, five *V. mimicus* strains that were positive or negative for the *ctx* gene were probed for the presence of zonula occludens toxin gene (*zot*) by polymerase chain reaction using primers based on the *V. cholerae* *zot* gene (152). Three strains were positive for the *zot* gene, but only one of these had the *ctx* gene. This result suggests that ZOT may play an important role in enterotoxigenicity of the pathogen. Shi et al. (153) screened 51 clinical strains isolated in Bangladesh, India, the United States, and Japan for the presence of *ctx*, *zot*, *ace* (accessory cholera enterotoxin), and *st* (heat-stable enterotoxin) genes. Four strains were positive for the *ace* gene, and three of these also had the *ctx* gene, but only one of the *ctx*<sup>+</sup> strains had the *zot* gene. The fourth strain had the *zot* gene but was *ctx*<sup>-</sup>. Nine strains had the *st* gene but were negative for the other three genes. The presence of different combinations of these genes in different strains indicates that many virulence factors may be participating in pathogenesis of the disease.

## 2. Hemolysin

The chromosomal gene encoding the TDH-like hemolysin of *V. mimicus* has been cloned and the sequence compared to the *tdh* gene of *V. parahaemolyticus* (204,205). The nucleotide sequences in the coding regions of both the hemolysins had very strong homology to each other. Primers based on the *tdh* gene have been utilized in screening clinical and environmental strains for the presence of TDH-like hemolysin. The gene was present in 16 of the 17 (162) and 5 of the 10 (149) clinical strains examined; it was detected in only 1 of the 31 (162) and none of the 3 (149) environmental strains tested. The structural gene encoding the heat-labile hemolysin has also been cloned and sequenced (206,207). The gene has an open reading frame consisting of 2232 nucleotides that codes for a protein of 744 amino acids. The molecular weights as predicted from the sequence are 83,059 and 83,903, but the molecular weight of the secreted hemolysin is 63,000. The molecular weight of the mature protein is lower because 151 amino acids are cleaved off during processing (207). The nucleotide sequence of the gene has a very strong homology (76–81%) to the gene encoding the El Tor hemolysin of *V. cholerae*.

## 3. Protease

The gene encoding a metalloprotease similar to that produced by *V. parahaemolyticus* has been cloned and sequenced (167). The gene contains 1884 nucleotides and codes for a polypeptide of 628 amino acids. The predicted molecular weight is 71,275 and the sequence has a 68.5% homology with the *V. parahaemolyticus* protease gene. The role of this protease in virulence has not been studied.

## B. *Vibrio hollisae*

Initial experiments using four synthetic probes based on different regions of the *V. parahaemolyticus* TDH structural gene had shown that *V. hollisae* strains hybridized with all the probes in a colony hybridization experiment (187). The *V. hollisae* gene encoding the hemolysin was subsequently shown to contain a 567-base-pair open reading frame that has ~93% homology to that of the *tdh* gene of *V. parahaemolyticus* (208). Moreover, strain-to-strain variation of the *V. hollisae* hemolysin gene is reported to exist. Additional studies using nucleic acid hybridization and polymerase chain reaction assays showed that the *tdh* gene of *V. hollisae* was not very closely related to the *tdh* genes of some other non-*V. hollisae* vibrios (209).

## C. *Vibrio damsela*

The gene encoding damselysin was cloned and expressed in *E. coli* (210). Using a DNA probe, colony hybridization experiments showed that strains that produce very little hemolytic activity when grown in liquid medium do not react with the probe. These strains may be producing a different hemolysin. In addition, none of the 318 different strains belonging to eight different *Vibrio* species had sequences homologous to the damselysin gene. Southern blots of *V. damsela* chromosomal DNA digests showed that only one copy of the gene is present in the strain. It was also shown that the gene is not plasmid-encoded.

## VIII. CONTROL MEASURES

Pathogenic vibrios are ubiquitous in estuarine waters and therefore will be present as contaminants in seafood harvested from these waters. The counts are especially high in the warmer months when the higher temperatures promote the growth of the pathogens. In addition, storage of harvested seafood at inadequate temperatures can lead to further increases in the numbers. This growth may be accompanied by the production of various cell-associated and extracellular toxins, enzymes, etc. Consumption of seafoods contaminated with high numbers of these pathogens can lead to gastroin-

testinal disease. In addition, exposure of wounds to either seawater or seafood, especially during processing, can lead to various forms of, sometimes fatal, wound infections.

Information regarding the minimum dose of these vibrios that is required to cause a gastrointestinal infection is not available. Therefore, no recommendations have been made regarding the acceptable limits of these organisms in foods. In general, proper refrigeration of harvested seafood in order to minimize the numbers of vibrios, cooking seafoods thoroughly, and taking precautions when handling seafood can minimize the chances of acquiring an infection. Attempts should also be made by processors and consumers to prevent cross-contamination of cooked food with raw seafood. Seafoods that are to be consumed raw should be harvested in seasons when the temperature of the water is cold. Fresh and cooked seafood should be stored at temperatures (60°C or greater or 4°C or lower) that do not promote growth of vibrios (211). Care should be exercised in ensuring that injured body parts, especially in people with underlying conditions, are not exposed to seawater, raw seafoods, and other marine life harboring these pathogens. Infection of wounds acquired during recreational activities in seawater and during seafood processing may be difficult to prevent.

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# 18

## ***Yersinia* Species**

**Roy M. Robins-Browne**

*Murdoch Children's Research Institute and University of Melbourne, Parkville,  
Victoria, Australia*

**Elizabeth L. Hartland**

*Monash University, Clayton, Victoria, Australia*

### **I. BACKGROUND**

In 1883, Malassez and Vignal reported a disease in guinea pigs they called *tuberculose zoogloeique* (1). Their description matched that of an epizootic tuberculosis-like disease which Eberth subsequently termed pseudotuberculosis (2). From 1889, the suspected causative of this condition was assigned a series of name changes that reflected its uncertain taxonomic status. These included *Bacillus pseudotuberculosis rodentium* (3) and *Bacillus parapestis* (4), the latter reflecting the biological similarity of this organism to the plague bacillus. In 1944, van Loghen indicated that *Pasteurella pseudotuberculosis*, as it was then known, and the closely related *Pasteurella pestis* were sufficiently distinct from the pasteurellae of hemorrhagic septicemia to warrant their own generic status (5). He proposed the name *Yersinia* after Alexandre Yersin, who first described the plague bacillus and had named it in honor of Louis Pasteur.

Whereas *Yersinia pseudotuberculosis* first attracted attention as a pathogen of animals, *Yersinia enterocolitica* was initially associated with human infection; more than 50 years after the description of *tuberculose zoogloeique*, in 1934 McIver and Pike identified a novel bacterium in pus from the facial skin of a New York farm worker (6). They named this bacterium *Flavobacterium pseudomallei* Whitmore, but in retrospect it seems likely that this was *Y. enterocolitica*. Five years later Schleifstein and Coleman recorded the similarity of McIver and Pike's isolate, and four others they had obtained, to *Pasteurella (Yersinia) pseudotuberculosis* (7). Later they proposed the name *Bacterium enterocoliticum* for this organism (8).

Today, the genus *Yersinia* comprises 11 species within the family Enterobacteriaceae (Table 1). The genus includes three well-characterized pathogens of mammals, one of fish, and several other species whose etiological role in disease is uncertain (for a review of the latter, see Ref. 10). The four known pathogenic species are *Yersinia pestis*, the causative agent of bubonic and pneumonic plague (the black death), *Y. pseudotuberculosis*, a rodent pathogen that occasionally causes intestinal infection, mesenteric lymphadenitis, septicemia, and immune-mediated diseases in humans, *Yersinia ruckeri*, a cause of enteric redmouth disease in salmonids and other freshwater fish, and *Y. enterocolitica*, a versatile intestinal pathogen. When *Y. enterocolitica* first emerged as a human pathogen it was considered an oddity, but now it is by far the most prevalent *Yersinia* species among humans. On the other hand, *Y. pseudotuberculosis*, which was once quite common in Europe, is now comparatively rare (11).

*Y. pestis* is transmitted to its host via the bites of fleas or respiratory aerosols, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are foodborne pathogens that enter the body through the gastrointestinal tract. Given that the role of the other *Yersinia* species in disease is uncertain, this chapter will focus on *Y. enterocolitica* and *Y. pseudotuberculosis*.



**TABLE 1** Some Biochemical Tests Used to Differentiate *Yersinia* Species

Test	<i>Y. aldovae</i>	<i>Y. bercovieri</i>	<i>Y. enterocolitica</i>		<i>Y. frederiksenii</i>	<i>Y. intermedia</i>	<i>Y. kristensenii</i>	<i>Y. mollaretii</i>	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	<i>Y. rohdei</i>	<i>Y. ruckeri</i>
			Biovars 1–4	Biovar 5								
Indole	– <sup>a</sup>	–	d	–	+	+	d	–	–	–	–	–
Voges-Proskauer	+	–	+	+ <sup>c</sup>	d	+	–	–	–	–	–	–
Citrate (Simmons)	d	–	–	–	d	+	–	–	–	–	+	–
L-ornithine	+	+	+	–	+	+	+	+	–	–	+	+
Mucate, acid	d	+	–	–	d	d	–	+	–	–	–	–
Pyrazinamidase	+	+	d	–	+	+	+ <sup>c</sup>	+	–	– <sup>c</sup>	+	ND <sup>b</sup>
Sucrose	–	+	+	d	+	+	–	+	–	–	+	–
Cellobiose	–	+	+	+	+	+	+	+	–	–	+	–
L-rhamnose	+	–	–	–	+	+	–	–	–	+	–	–
Melibiose	–	–	–	–	–	+	–	–	d	+	d	–
L-sorbose	–	–	d	d	+	+	+	+	–	–	ND	ND
L-fucose	d	+	d	–	+	d	d	–	ND	–	ND	ND

<sup>a</sup> +, positive; –, negative; d, different reactions.

<sup>b</sup> ND, not determined.

<sup>c</sup> Some reactions may be delayed or weakly positive.

Source: Adapted from Ref. 9.

## II. CHARACTERISTICS

As members of the family Enterobacteriaceae, yersiniae are gram-negative, oxidase-negative, rod-shaped, nonsporing, facultative anaerobes that ferment glucose. They are smaller than most other enterobacteria and, when grown at 37°C, often appear as coccobacilli in stained smears. Yersiniae share between 10 and 30% DNA homology with other genera in the Enterobacteriaceae (12). *Y. enterocolitica* itself is only approximately 50% related to *Y. pseudotuberculosis* and *Y. pestis*, whereas the last two species are more than 90% related to each other. Indeed, recent genetic analysis suggests that *Y. pestis* is a clone of *Y. pseudotuberculosis* which evolved some 1,500–20,000 years ago, shortly before the first known pandemics of human plague (13).

*Y. pseudotuberculosis* is a relatively homogeneous species, which is subdivided into serotypes according to its lipopolysaccharide (O-) antigens. Currently, 11 major serotypes, designated 1–11 (or I–XI), have been reported (14,15). Serotypes 1 and 2 are further subdivided into three subgroups, A, B, and C; serotypes 4 and 5 are each divided into subgroups A and B (14,15).

*Y. enterocolitica* is far more heterogeneous than *Y. pseudotuberculosis*, being divisible into a large number of subgroups according to biochemical activity and lipopolysaccharide O-antigens (Tables 2 and 3). Biotyping is based on the ability of *Y. enterocolitica* to metabolize selected organic substrates and provides a convenient means to classify members of this species into subtypes of different clinical and epidemiological significance (Tables 2 and 3) (16). Most primary pathogenic strains of humans and domestic animals occur within biovars 1B, 2, 3, 4, and 5, whereas *Y. enterocolitica* strains of biovar 1A are commonly obtained from terrestrial and freshwater ecosystems. For this reason, the latter are often referred to as “environmental” strains, although some biovar 1A strains also appear to be responsible for human disease (17).

Not all isolates of *Y. enterocolitica* obtained from the environment or unprocessed foods can be assigned to a biovar. These strains lack the characteristic virulence determinants of biovars 1B through 5 (see below) and may represent novel subtypes or even new *Yersinia* species (18,19).

To date, at least 30 distinct lipopolysaccharide O-antigens have been identified in *Y. enterocolitica* (Table 3) (20). The usefulness of serotyping is limited, however, due to the presence of cross-reacting O-antigens in *Y. enterocolitica* strains of varying pathological and epidemiological significance and the fact that the overwhelming majority of human infections are due to strains of serotype O:3. In addition, some bacteria that were originally allocated O-serotypes of *Y. enterocolitica* were later reclassified as separate species (20). At least 18 flagellar (H-) antigens of *Y. enterocolitica*, designated by lowercase letters (a,b; b,c; b,c,e,f,k; m, etc.), have been also identified. Although there is little overlap between the H-antigens of *Y. enterocolitica sensu stricto* and those of

**TABLE 2** Biotyping Scheme of *Y. enterocolitica*

Test	Biovar					
	1A	1B	2	3	4	5
Lipase (Tween hydrolysis)	+	+	–	–	–	–
Esculin hydrolysis	d	–	–	–	–	–
Indole production	+	+	(+)	–	–	–
D-Xylose fermentation	+	+	+	+	–	d
Voges-Proskauer reaction	+	+	+	+	+	(+)
Trehalose fermentation	+	+	+	+	+	–
Nitrate reduction	+	+	+	+	+	–
Pyrazinamidase	+	–	–	–	–	–
β-D-Glucosidase	+	–	–	–	–	–
Proline peptidase	d	–	–	–	–	–

+, positive; (+) delayed positive; –, negative; d, different reactions.

**TABLE 3** Relationship Between O-Serotype and Pathogenicity of *Y. enterocolitica* and Related Species

Species	Serotype(s)
<i>Y. enterocolitica</i>	
biovar 1A	O:4; O:5; O:6,30; O6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57; NT
biovar 1B	O:4,32 <sup>a</sup> ; O:8 <sup>a</sup> ; O:13a,13b <sup>a</sup> ; O:16; O:18 <sup>a</sup> ; O:20 <sup>a</sup> ; O:21 <sup>a</sup> ; O:25; O:41,42; NT
biovar 2	O:5,27 <sup>a</sup> ; O:9 <sup>a</sup> ; O:27
biovar 3	O:1,2,3 <sup>a</sup> ; O:3 <sup>a</sup> ; O:5,27 <sup>a</sup>
biovar 4	O:3 <sup>a</sup>
biovar 5	O:2,3 <sup>a</sup>
<i>Y. bercovieri</i>	O:8; O:10; O:58,16; NT
<i>Y. frederiksenii</i>	O:3; O:16; O:35; O:38; O:44; NT
<i>Y. intermedia</i>	O:17; O:21,46; O:35; O:37; O:40; O:48; O:52; O:55; NT
<i>Y. kristensenii</i>	O:11; O:12,25; O:12,26; O:16; O:16,29; O:28,50; O:46; O:52; O:59; O:61; NT
<i>Y. mollaretii</i>	O:3; O:6,30; O:7,13; O:59; O:62,22; NT

NT, not typeable.

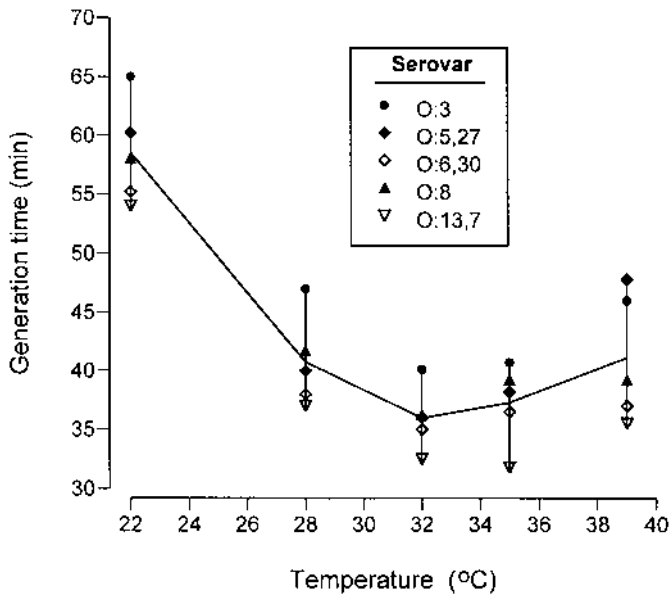
<sup>a</sup> Serotypes that include pYV-bearing strains.

the related species, antigenic characterization of isolates by complete O- and H-serotyping is seldom attempted (20).

Other schemes for subtyping *Yersinia* species include bacteriophage typing, multienzyme electrophoresis, and the demonstration of restriction fragment length polymorphisms of chromosomal and plasmid DNA (21). These techniques can be used to facilitate epidemiological investigations of outbreaks or to trace the source of sporadic infections.

*Y. enterocolitica* is unusual among pathogenic enterobacteria in being psychrotrophic, as evidenced by its ability to replicate at temperatures below 4°C. The doubling time at the optimum growth temperature (approximately 28–30°C) is around 34 minutes, which increases to 1 hour at 22°C, 5 hours at 7°C, and approximately 40 hours at 1°C (Fig. 1) (22). *Y. enterocolitica* readily withstands freezing and can survive in frozen foods for extended periods even after repeated freezing and thawing (23). Studies of the ability of *Y. enterocolitica* to survive and grow in artificially contaminated foods under various conditions of storage have shown it generally survives better at room temperature and refrigeration temperatures than at intermediate temperatures. *Y. enterocolitica* persists longer in cooked foods than in raw foods, probably due to an increased availability of nutrients in cooked foods and the fact that the presence of other psychrotrophic bacteria, including nonpathogenic strains of *Y. enterocolitica*, in unprocessed food may restrict bacterial growth (22). The number of viable *Y. enterocolitica* may increase more than a million-fold on cooked beef or pork within 24 hours at 25°C or within 10 days at 7°C (24). Growth rates are slower on raw beef and pork. *Y. enterocolitica* can grow at refrigeration temperatures in vacuum-packed meat, boiled eggs, boiled fish, pasteurized liquid eggs, pasteurized whole milk, cottage cheese, and tofu (soybean curd) (22,25). Proliferation also occurs in refrigerated seafoods, such as oysters, raw shrimp, and cooked crab meat, but at a slower rate than in pork or beef (26). Bacteria may also persist for extended periods in refrigerated vegetables and cottage cheese, particularly in the presence of chicken meat (27). The psychrotrophic nature of *Y. enterocolitica* also poses problems for the blood transfusion industry, chiefly because of its ability to proliferate and release endotoxin in blood products stored at 4°C without manifestly altering their appearance (28).

*Y. enterocolitica* and *Y. pseudotuberculosis* are able to grow over a pH range from approximately pH 4–10, with an optimum pH of around 7.6 (29). They tolerate alkaline conditions extremely well, but their acid tolerance is less pronounced and depends on the acidulant used, the environmental



**FIGURE 1** Generation time at different temperatures of *Y. enterocolitica* strains of five different serotypes, grown in 4% tryptone, 1% mannitol salt broth, pH 7.6. (Adapted from Ref. 22.)

temperature, the composition of the medium, and the growth phase of the bacteria (30). Acid tolerance of *Y. enterocolitica* is enhanced by the production of urease, which hydrolyzes urea to release ammonia that elevates the cytoplasmic pH (31,32).

*Y. enterocolitica* and *Y. pseudotuberculosis* are susceptible to heat and are readily destroyed by pasteurization at 71.8°C for 18 seconds or 62.8°C for 30 minutes (23,33). Exposure of surface-contaminated meat to hot water (80°C) for 10–20 seconds reduces bacterial viability by at least 99.9% (34). *Y. enterocolitica* is also readily inactivated by ionizing and ultraviolet irradiation (35,36) and by sodium nitrate and nitrite when added to food (37). It displays relative resistance to these salts in solution, however, and can also tolerate NaCl at concentrations of up to 5% (37,38). *Y. enterocolitica* is generally susceptible to organic acids, such as lactic and acetic acids, and to chlorine (39). However, some resistance to chlorine is displayed by bacteria grown under conditions that approximate natural aquatic environments or when they are cocultivated with predatory aquatic protozoa (40,41).

Sutherland and coworkers have formulated models to predict the influence of temperature, pH, and the concentrations of sodium chloride and lactic acid on the growth and survival of *Y. enterocolitica* in foods and found that they correlated well with published data for meat and milk products (42,43). Bhaduri et al. have devised a model to assess the likely safety of foods contaminated with *Y. enterocolitica* as a result of manufacturing problems or storage abuse (44). Nevertheless, mathematical models should not be relied upon to predict food safety, because they may overestimate bacterial numbers in some circumstances and underestimate them in others (45).

### III. DISEASES

Infections with *Y. enterocolitica* and *Y. pseudotuberculosis* overlap considerably in their clinical manifestations (Table 4). Symptomatic infection with either species typically results in nonspecific,

**TABLE 4** Clinical Manifestations of Infections with *Y. enterocolitica* and *Y. pseudotuberculosis*

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*Common manifestations*

- Diarrhea (“gastroenteritis”), especially in young children
- Enterocolitis
- Pseudoappendicitis syndrome due to terminal ileitis, acute mesenteric lymphadenitis
- Pharyngitis
- Postinfection autoimmune sequelae
  - Arthritis, especially associated with HLA-B27
  - Erythema nodosum
  - Uveitis, associated with HLA-B27
  - Glomerulonephritis (uncommon)
  - Myocarditis (uncommon)
  - Thyroiditis (uncertain)

*Less common manifestations*

- Septicemia
  - Visceral abscesses (e.g., in liver, spleen, lung)
  - Skin infection: pustules, wound infection, pyomyositis, etc.
  - Pneumonia
  - Endocarditis
  - Osteomyelitis
  - Peritonitis
  - Meningitis
  - Intussusception
  - Eye infections: conjunctivitis, panophthalmitis
- 

self-limiting diarrhea, but may lead to a variety of suppurative and autoimmune complications, the risk of which is determined partly by host factors, in particular age and underlying immune status.

## **A. Acute Infection**

*Y. enterocolitica* and *Y. pseudotuberculosis* enter the gastrointestinal tract after ingestion in contaminated food or water. The median infective dose for humans is not known, but is likely to exceed  $10^4$  colony-forming units. Gastric acid appears to be a significant barrier to infection with *Y. enterocolitica*, and in individuals with gastric hypoacidity the infectious dose may be lower (31,46).

Most symptomatic infections with *Y. enterocolitica* occur in children, especially in those less than 5 years of age. In these patients, yersiniosis presents as diarrhea, often accompanied by low-grade fever and abdominal pain (47,48). The character of the diarrhea varies from watery to mucoid. A small proportion of children (generally <10%) have frankly bloody stools. Children with *Y. enterocolitica*-induced diarrhea often complain of abdominal pain and headache. Sore throat is a frequent accompaniment and may dominate the clinical picture in older patients (49). The illness typically lasts from a few days to 3 weeks, although some patients develop chronic enterocolitis, which may persist for several months (50). Occasionally, acute enteritis progresses to intestinal ulceration and perforation or to ileocolic intussusception, toxic megacolon, or mesenteric vein thrombosis (51). On rare occasions, patients may present with peritonitis in the absence of intestinal perforation (52).

In children older than 5 years and in adolescents, acute yersiniosis often presents as a pseudo-appendicular syndrome due to acute inflammation of the terminal ileum or the mesenteric lymph nodes. The usual features of this syndrome are abdominal pain and tenderness localized to the right lower quadrant. These symptoms are usually accompanied by fever, with little or no diarrhea. The

importance of this form of the disease lies in its close resemblance to appendicitis (51). Of those patients with this syndrome who undergo laparotomy, approximately 60–80% have terminal ileitis, with or without mesenteric adenitis, and a normal or slightly inflamed appendix (53,54). *Y. enterocolitica* may be cultured from the distal ileum and the mesenteric lymph nodes. The pseudoappendicular syndrome appears to be more frequent in patients infected with the relatively more virulent strains of *Y. enterocolitica*, notably strains of biovar 1B. *Y. enterocolitica* is rarely found in patients with true appendicitis (54).

Although *Y. enterocolitica* is seldom isolated from extraintestinal sites, there appears to be no tissue in which it will not grow. In adults, pharyngitis, sometimes with cervical lymphadenitis, may dominate the clinical presentation (49). Focal disease, in the absence of obvious bacteremia, may present as cellulitis, subcutaneous abscess, pyomyositis, suppurative lymphadenitis, septic arthritis, osteomyelitis, urinary tract infection, renal abscess, sinusitis, pneumonia, lung abscess, or empyema (51).

Bacteremia is a rare complication of infection, except in patients who are immunocompromised or in an iron-overloaded state (46). Factors that predispose to the development of *Yersinia* bacteremia include immunosuppression, blood dyscrasias, malnutrition, chronic renal failure, cirrhosis, alcoholism, diabetes mellitus, and acute and chronic iron overload states, particularly when managed by chelation therapy with desferrioxamine B (51). Bacteremic dissemination of *Y. enterocolitica* may lead to various manifestations, including splenic, hepatic and lung abscesses, catheter-associated infections, osteomyelitis, panophthalmitis, endocarditis, mycotic aneurysm, and meningitis (51). *Yersinia* bacteremia is reported to have a case fatality rate of between 30 and 60%.

Bacteremia may also result from direct inoculation of *Y. enterocolitica* into the circulation during blood transfusion (55,56). Indeed, *Y. enterocolitica* is the single most important cause of fatal bacteremia following transfusion with packed red blood cells or platelets (55). Patients infused with contaminated blood may develop symptoms of a severe transfusion reaction minutes to hours after exposure, depending on the number of bacteria and the amount of endotoxin administered with the blood (28). The varieties of *Y. enterocolitica* responsible for transfusion-acquired yersiniosis are the same serobiovars as those associated with enteric infections. The probable source of these infections are blood donors with low-grade, subclinical bacteremia. A small number of bacteria in donated blood will increase during storage at refrigeration temperatures without manifestly altering the appearance of the blood (57).

## **B. Autoimmune Complications**

Although most episodes of yersiniosis remit spontaneously without long-term sequelae, infections with *Y. enterocolitica* are noteworthy for the large variety of immunological complications, such as reactive arthritis, erythema nodosum, uveitis, glomerulonephritis, carditis, and thyroiditis, which have been reported to follow acute infection (51). Of these, reactive arthritis is the most widely recognized (58–60). This manifestation of infection is infrequent before the age of 10 years and occurs most often in Scandinavian countries where serotype O:3 strains and the human leukocyte antigen, HLA-B27, are especially prevalent. Men and women are effected equally. Arthritis typically follows the onset of diarrhea or the pseudoappendicular syndrome by 1–2 weeks with a range of from 1 to 38 days. The joints most commonly involved are the knees, ankles, toes, tarsal joints, fingers, wrists, and elbows. Synovial fluid from affected joints contains large numbers of inflammatory cells, principally polymorphonuclear leukocytes, and is invariably sterile, although it generally contains bacterial antigens (61). The duration of arthritis is typically less than 3 months, and the long-term prognosis in terms of joint destruction is excellent, although some patients may have symptoms that persist for several years (60,62). Many patients with arthritis also have extra-articular symptoms, including urethritis, uveitis, and erythema nodosum (59).

*Y. enterocolitica*-induced erythema nodosum occurs predominantly in women and is not associated with HLA-B27. Other autoimmune complications of yersiniosis, including Reiter's syndrome, uveitis, acute proliferative glomerulonephritis, collagenous colitis, and rheumatic-like carditis, have

been reported, mostly from Scandinavian countries (63). Yersiniosis has also been linked to various thyroid disorders, including Graves' disease hyperthyroidism, nontoxic goiter, and Hashimoto's thyroiditis, although the causative role of yersiniae in these conditions is uncertain (64). In Japan, *Y. pseudotuberculosis* has been implicated in the etiology of Kawasaki's disease (65).

## IV. EPIDEMIOLOGY

### A. Geographic Distribution

Infections with *Y. enterocolitica* and *Y. pseudotuberculosis* occur throughout the world, but their distribution is uneven. Most human infections with *Y. enterocolitica* occur in cool climate countries of the northern hemisphere: Belgium, Germany, Holland, Hungary, the Scandinavian countries, Canada, and Japan (66). In these countries, *Y. enterocolitica* ranks alongside *Campylobacter jejuni* and *Salmonella* species as one of the three most frequent causes of acute bacterial enteritis in children. Yersiniosis is uncommon in tropical or developing countries. The relatively low optimum growth temperature of *Y. enterocolitica* may account in part for the higher incidence of yersiniosis in temperate regions and the tendency for infections to peak during late autumn and winter in countries where yersiniosis is endemic.

Most infections with *Y. enterocolitica* (up to 90% in some countries) are caused by biovar 4 strains of serotype O:3, which have a worldwide distribution. Other serobiovars may be associated with particular geographic areas (67), but not as closely as was once thought (68). For example, biovar 1B strains were said to be confined to North America, but they are now known to occur more widely. Serotype O:9 biovar 2 is relatively common in northern Europe, where it accounts for between 11 and 30% human cases (11). In Europe, most human isolates of *Y. pseudotuberculosis* belong to O-serotypes 1, 2, and 3, whereas serotype 4B is common in Japan (65).

### B. Reservoirs

Infections with *Yersinia* species are zoonoses. As expected, those subgroups of *Y. enterocolitica* and *Y. pseudotuberculosis* that commonly occur in humans also occur in domestic animals, whereas those that are infrequent in humans generally reside in wild rodents.

*Y. enterocolitica* and *Y. pseudotuberculosis* can occupy a broad range of environments and have been isolated from the intestinal tract of many different mammalian species (65,66). *Y. enterocolitica* has also been obtained from birds, frogs, fish, flies, fleas, crabs, and oysters (51).

Foods that may harbor *Y. enterocolitica* include pork, beef, lamb, poultry, and dairy products, notably milk, cream, and ice cream (22,25,69). *Y. enterocolitica* is also commonly found in a variety of terrestrial and freshwater ecosystems, including soil, vegetation, lakes, rivers, wells, and streams, and can persist for extended periods in soil, vegetation, streams, lakes, wells, and spring water, particularly at low environmental temperatures (69,70).

Many environmental isolates of *Y. enterocolitica* lack markers of bacterial virulence and are of uncertain significance for human or animal health (71). In contrast to *Y. enterocolitica*, *Y. pseudotuberculosis* is seldom isolated from food, water, or the environment, unless these are contaminated with fecal matter. When such contamination occurs, water may be an important source of infection (72).

Although *Y. enterocolitica* has been recovered from a variety of wild and domesticated animals, pigs are the only animal species from which *Y. enterocolitica* of biovar 4 serotype O:3 (the variety most commonly associated with human disease) has been isolated with any degree of frequency (69). Pigs may also carry *Y. enterocolitica* of serotypes O:9 and O:5,27, particularly in regions where human infections with these varieties are comparatively common. In countries with a high incidence of human yersiniosis, *Y. enterocolitica* is commonly isolated from pigs at slaughterhouses (73–75). The tissue most frequently culture positive at slaughter is the tonsils, which appears to be the preferred site of *Y. enterocolitica* infection in pigs. Other tissues that frequently yield yersiniae

**TABLE 5** Some Reported Foodborne Outbreaks of Infections with *Y. enterocolitica*

Location	Year	Month	No. of cases	Serotype	Source	Ref.
Canada	1976	April	138	O:5,27	Raw milk (?)	84
New York	1976	Sept	38	O:8	Chocolate-flavored milk	85
Japan	1980	April	1051	O:3	Milk	86
New York	1981	July	159	O:8	Powdered milk/chow mein	87
Washington	1981	Dec	50	O:8	Tofu/spring water	88
Pennsylvania	1982	Feb	16	O:8	Bean sprouts/well water	51
Southern US	1982	June	172	O:13a,13b	Milk (?)	89
Hungary	1983	Dec	8	O:3	Pork "cheese" (sausage)	90
Georgia	1989	Nov	15	O:3	Pork chitterlings	82

(?), bacteria were not isolated from the incriminated source.

include tongue, cecum, rectum, feces, and gut-associated lymphoid tissue. *Y. enterocolitica* is seldom isolated from meat offered for retail sale, however, apart from pork tongue (22,69), although standard methods of bacterial isolation and detection may underestimate the true incidence of contamination (76).

Although some domesticated farm animals, notably sheep, cattle, and deer, may suffer symptoms as a result of infection with *Y. enterocolitica* or *Y. pseudotuberculosis* (77,78), in most cases the biovars and serotypes of these bacteria differ from those responsible for human infection, indicating a lack of transmission of these particular bacteria between animals and humans. By contrast, individual isolates of *Y. enterocolitica* from pigs and humans are indistinguishable from each other in terms of serotype, biovar, restriction fragment length polymorphism of chromosomal and plasmid DNA, and carriage of virulence determinants (69). Further evidence that pigs are a significant reservoir of human infections is provided by epidemiological studies pointing to the ingestion of raw or undercooked pork as a major risk factor for the acquisition of yersiniosis (79,80). Infection also occurs after handling of contaminated chitterlings (pig intestine), particularly by children (81–83).

Several outbreaks of *Y. enterocolitica* infection have been linked to the consumption of contaminated cows' milk (Table 5), but cattle do not appear to be a important reservoir of these bacteria. In these outbreaks, milk may have been contaminated with pig or human feces during or after processing (91,92). Contamination of pasteurized milk appears to pose a greater threat of infection than raw milk, probably because *Y. enterocolitica* outgrows other fecal microorganisms during storage at refrigeration temperatures more readily when competing psychrotrophic microflora have been eliminated (92).

Food animals are seldom infected with biovar 1B strains of *Y. enterocolitica*, the reservoir of which remains unknown (22). The relatively low incidence of human yersiniosis caused by these strains, despite their comparatively high virulence, points to a lack of significant contact between their reservoir and humans. As yersiniae of this biovar are primary pathogens of rodents, it is conceivable that rats or mice are the natural reservoir of these strains (93).

Outbreaks of infection with *Y. pseudotuberculosis* in humans are uncommon, although they have been documented in a variety of domesticated or caged animals, including horses, goats, deer, and monkeys. In two well-documented outbreaks of *Y. pseudotuberculosis* infection of humans, no source was identified (94,95). In some outbreaks, untreated drinking water contaminated with the feces of wild animals was incriminated (72).

## V. ISOLATION AND IDENTIFICATION

The isolation of *Y. enterocolitica* from food and clinical samples is often complicated by the low number of yersiniae relative to other bacteria. Accordingly, isolation usually requires enrichment



followed by plating on selective and indicator media and the characterization of colonies that display typical morphology.

## A. Enrichment

Enrichment of food samples for *Y. enterocolitica* generally involves the use of low-temperature incubation. Incubating samples in phosphate-buffered saline at 4°C for 2 weeks markedly increases the likelihood of recovering *Y. enterocolitica*, but better results are obtained with broths containing sorbitol and peptone, particularly when buffered at a pH of 8–8.5 (96–98). The unusual tolerance of both *Y. enterocolitica* and *Y. pseudotuberculosis* to alkaline conditions can be exploited by pre-treating enrichment cultures for a few seconds in 0.5% potassium hydroxide (99) or by incubating the bacteria for 1–3 days in alkaline phosphate buffer at 25°C (100). A variety of agents, including bile, selenite, Irgasan, and ticarcillin, can be added to liquid media to improve their selectivity. Some of these supplements may enhance the growth of specific serotypes of *Y. enterocolitica* while retarding that of others.

## B. Plating Media

*Y. enterocolitica* is highly resistant to bile salts and grows readily on most enteric media, including MacConkey, desoxycholate, Salmonella-Shigella, eosin methylene blue, Hektoen-Enteric, and xylose-lysine-desoxycholate (XLD) agar (66). At 37°C, however, *Y. enterocolitica* grows more slowly than most other enterobacteria, and consequently may be overgrown in samples that contain large numbers of contaminating microorganisms. A simple strategy to overcome this problem is to plate the sample on standard enteric media, such as MacConkey, desoxycholate, or Salmonella-Shigella agar, and to incubate the cultures at 25°C for 48 hours. On these media, *Y. enterocolitica* appears as lactose-negative or weakly lactose-positive colonies, 1–2 mm in diameter. On XLD and Hektoen-Enteric agar, *Y. enterocolitica* may be mistaken for coliforms because it ferments sucrose.

Several media have been devised for the selective isolation of *Y. enterocolitica*, of which cefsulodin-Irgasan-novobiocin (CIN) agar is of proven effectiveness for clinical specimens as well as samples of food and water (101,102). Growth of *Y. pseudotuberculosis* is inhibited on CIN agar, which should not be used for the isolation of this species (101). When cultured on CIN agar for 18–20 hours at 32°C, *Y. enterocolitica* yields colonies 0.5–1 mm in diameter, which are smooth and slightly raised, with a distinctive red “bull’s-eye” and a clear border. Confirmation of presumptive colonies as *Y. enterocolitica* is achieved by following standard identification procedures, including the assays listed in [Table 1](#) (103). The API20E identification system is a sensitive and specific alternative to in-house biochemical tests (104,105). If required, confirmed isolates can be assigned to a biovar to facilitate evaluation of their epidemiological and pathological significance. For reliable assessment of the pathogenic potential, however, it is preferable to demonstrate the presence of the *Yersinia* virulence plasmid (pYV) and/or chromosomal virulence-associated genes (106).

Formerly, virulent strains of *Y. enterocolitica* were identified by their pathogenicity for animals or their ability to invade tissue culture cells in large numbers (107–109). Today, these tests have been superseded by in vitro surrogate assays, such as the demonstration of so-called “calcium dependency” or an ability to bind Congo red or crystal violet (110–112). “Calcium dependency” refers to the growth restriction of strains of *Y. enterocolitica* and *Y. pseudotuberculosis*, which carry the virulence plasmid, pYV, on media containing low concentrations of calcium at 37°C. Media that reveal Congo red binding and calcium dependency concurrently can be used for the primary isolation of *Y. enterocolitica* to permit early tentative identification of pYV-bearing strains (113,114). More direct evidence of the presence of virulence-associated genes can be obtained by the ability of isolates to hybridize with labeled DNA probes or by PCR amplification of selected pYV-borne genes, such as *virF* or *yadA*, or chromosomal genes, such as *ail* and *yst*, which are more or less

**TABLE 6** PCR Primers Used for the Specific Detection of Classical Pathogenic *Yersinia* Species

Primers	Target gene	Ref.
5'-GAACTCGATGATAACTGGG-3'	<i>ail</i>	119
5'-GCAATTCAACCCACTTCAA-3'		
5'-TCATGGCAGAACAGCAGTCAG-3'	<i>virF</i>	120
5'-ACTCATCTTACCATTAAGAAG-3'		
5'-TAAGATCAGTGTCTCTGCGGCA-3' <sup>a</sup>	<i>yadA</i>	121
5'-TAGTTATTTGCGATCCCTAGCAC-3' <sup>a</sup>		
5'-AATGCTGTCTTCATTTGGAGCA-3'	<i>yst</i>	116
5'-GAAGTCAGTAGTGATTGGGAT-3'		

<sup>a</sup> Used in the first round of amplifications in a nested PCR.

restricted to highly invasive strains (Table 6) (106,115–118). PCR can also be used for the rapid detection of potentially pathogenic strains of *Y. enterocolitica* in food or water (122,123). Because of the inherent instability of pYV particularly in cultures held at 37°C (124), caution is needed in interpreting negative assays for the presence of pYV-borne determinants.

## VI. PATHOGENICITY

*Y. enterocolitica* and *Y. pseudotuberculosis* are paradigms of invasive enteric pathogens whose virulence determinants have been the subject of intensive investigation (125), but not all strains of *Y. enterocolitica* are equally virulent (Table 7). *Y. pseudotuberculosis* and the classical pathogenic strains of biovars of *Y. enterocolitica*, namely biovars 1B, 2, 3, 4, and 5, possess a multitude of interactive virulence determinants, including a chromosomally encoded invasin and a ~70-kb virulence plasmid, termed pYV (plasmid for *Yersinia* virulence) (125). In addition, *Y. pseudotuberculosis* and biovar 1B strains of *Y. enterocolitica* carry a high pathogenicity island, which is associated with enhanced virulence for mice and probably humans. All pYV-bearing clones of *Y. enterocolitica* have the capacity to invade epithelial cells in large numbers in vitro—a feature that distinguishes them from clones that never carry pYV (17,106). Surprisingly, however, this highly invasive phenotype is not specified by genes within pYV and is maximally expressed by bacteria from which pYV has been cured.

Until recently, weakly invasive, pYV-negative strains of *Y. enterocolitica*, most of which belong to biovar 1A, were regarded as avirulent, because they never carry pYV or any of the other well-characterized virulence-associated genes of this species (see below). There is now persuasive epidemiological evidence, however, showing that at least some of these strains may cause gastrointestinal symptoms clinically indistinguishable from those due to pYV-bearing strains (126,127). This is supported by the laboratory demonstration that biovar 1A strains fall into two categories, one of

**TABLE 7** Characteristics of Pathogenic Subgroups of *Y. enterocolitica*

Subgroup	Capacity to invade epithelial cells in vitro	Biovars	Virulence-associated determinants
Classical	High (lower if pYV present)	1B, 2, 3, 4, 5	Invasin, Ail, Myf, heat-stable enterotoxin (Yst), virulence plasmid (pYV); high-pathogenicity island (biovar 1B only)
Atypical	Low to moderate	1A	Unknown

which includes strains obtained from symptomatic patients that have the ability to penetrate epithelial cells in moderate numbers and to resist killing by macrophages to a significantly greater extent than strains of the same biovar isolated from nonclinical sources (17,128). Because the mechanisms by which these biovar 1A strains cause disease are unknown, the remainder of this section focuses chiefly on the virulence determinants of the classical pathogenic, i.e., pYV-bearing, highly invasive strains of *Y. enterocolitica*.

Examination of surgical specimens from patients with yersiniosis reveals that *Y. enterocolitica* is an invasive pathogen that displays a tropism for lymphoid tissue (129,130). The distal ileum, in particular the gut-associated lymphoid tissue, bears the brunt of the infection, although adjacent regions of the intestine and the mesenteric lymph nodes are also frequently involved.

As investigations in volunteers are precluded by the risk of autoimmune sequelae, most information regarding the pathogenesis of yersiniosis in vivo has been obtained from animal models, in particular mice and rabbits (131–133). Although these animals are not the natural hosts of the serotypes of *Y. enterocolitica* that commonly infect humans, they have provided important insights into the likely pathogenesis of human disease. Nevertheless, some data derived from animal studies should be interpreted with caution, particularly where death is used as the endpoint of infection, since this is not the usual outcome of human infection (134).

After oral inoculation of mice with a virulent strain of serotype O:8, biovar 1B, most bacteria remain within the intestinal lumen, while a small number adhere to the mucosal epithelium, showing no particular preference for any cell type (135). However, invasion of the epithelial cells takes place almost exclusively through M cells (135). The latter are specialized epithelial cells that overlie intestinal lymphoid follicles (Peyer's patches), where they play a major role in antigen sampling (136,137). Studies in experimentally infected rabbits and pigs have shown that after penetrating the epithelium, *Y. enterocolitica* traverses the basement membrane to reach the gut-associated lymphoid tissue and the lamina propria, where it causes localized tissue destruction leading to the formation of microabscesses (132,138). The bacteria may also spread through the lamina propria to adjacent villi and via the lymph to more remote sites. The hallmark lesion of yersiniosis in the intestine comprises microcolonies of bacteria surrounded by granulocytic and mononuclear inflammatory cells. These lesions occur chiefly within intestinal crypts but may extend as far as the crypt-villus junction. *Y. enterocolitica* often spreads via the lymph to the draining mesenteric lymph nodes, where it may also cause the formation of microabscesses. If the bacteria circumvent the lymph nodes to enter the blood stream, they can disseminate to any organ but continue to show a tropism for lymphoid tissue by preferentially localizing in the reticuloendothelial tissues of the liver and spleen. Although *Y. enterocolitica* and *Y. pseudotuberculosis* are classified as facultative intracellular pathogens, because of their innate resistance to killing by macrophages (139), most of the bacteria observed in histological sections are located extracellularly (140,141). Nevertheless, macrophages containing viable bacteria are likely to play an important role in the dissemination of yersiniae throughout the body (138,142).

## VII. GENETIC FACTORS IN VIRULENCE

### A. Chromosomal Virulence Determinants

#### 1. *Invasin*

All classical pathogenic strains of *Y. enterocolitica* produce a 91 kDa surface-expressed protein, termed *invasin*. This outer membrane protein was first identified in *Y. pseudotuberculosis* as a 102 kDa protein product of the chromosomal *inv* gene (143). Despite the difference in size of *invasins* from *Y. enterocolitica* and *Y. pseudotuberculosis*, the two proteins are functionally highly related. When introduced into an innocuous laboratory strain of *E. coli*, such as *E. coli* K-12, *inv* imbues the recipient with the ability to penetrate mammalian cells, including epithelial cells and macrophages (139,144). Analysis of *invasin* proteins has shown that the amino terminus is inserted in the bacterial

outer membrane, and the carboxyl terminus is exposed on the surface, where it specifies the binding of invasins to host cell integrins. The latter are heterodimeric transmembrane proteins that act as receptors for fibronectin, laminin, and related host proteins and communicate extracellular signals to the cytoskeleton (145). The affinity of invasins for some integrins is much greater than that of fibronectin. When invasins bind to these integrins, it causes the latter to cluster and initiate a sequence of events, including the activation of focal adhesion kinase. This results in reorganization of the host cell cytoskeleton and internalization of the bacteria. The internalization process is propagated entirely by the host cell, because killed bacteria and even latex particles coated with invasins are internalized in the same way as living bacteria (143). Inhibitors of actin polymerization and tyrosine kinases block invasion-mediated invasion, indicating that uptake of *Y. enterocolitica* by eukaryotic cells requires both an intact cytoskeleton and signal transduction pathways involving tyrosine phosphorylation (146,147).

Although DNA sequences homologous to *inv* occur in all *Yersinia* species (except *Y. ruckeri*), this gene is functional only in *Y. pseudotuberculosis* and the classical pathogenic varieties of *Y. enterocolitica*, suggesting that invasins play an important role in virulence (148). The absence of functional invasins from *Y. pestis* is readily explained by the fact that this species usually enters its hosts by direct inoculation into subcutaneous tissues via a flea bite, circumventing the need to penetrate mucous membranes. Although *inv* mutants of *Y. enterocolitica* and *Y. pseudotuberculosis* show a pronounced reduction in their ability to invade epithelial cells in vitro, their virulence for orally inoculated mice is barely affected (134). Nevertheless, *inv* appears to play a role in gastrointestinal tract colonization, as *inv* mutants of *Y. enterocolitica* and *Y. pseudotuberculosis* show diminished translocation into Peyer's patches when compared with wild-type strains (134).

## 2. *Ail*

Classic pathogenic strains of *Y. enterocolitica* produce an outer membrane protein, unrelated to invasins, which also confers invasive ability on *E. coli*. This 17 kDa peptide is specified by a chromosomal *ail* (attachment-invasion) locus, so called because it mediates bacterial attachment to some cultured epithelial cell lines and invasion of others (148). In concert with YadA, a pYV-encoded protein, Ail, may also allow yersiniae to resist complement-mediated lysis by interfering with the binding of complement proteins to the bacterial surface (149). The *ail* gene is found only in classical pathogenic varieties of *Y. enterocolitica* and in vitro is optimally expressed at 37°C [unlike *inv*, which is optimally expressed at 25°C, unless the pH is lowered to 5.5 (150)]. Despite this clear association with virulence, Wachtel et al. (151) reported that an *ail* mutant of *Y. enterocolitica* retained full virulence for perorally inoculated mice, indicating that Ail is not required to establish infection or even to cause systemic infection in these animals. The possibility that Ail makes some contribution to virulence, however, cannot be entirely ruled out by these findings, because mice may not be well suited to demonstrating certain aspects of *Yersinia* virulence, such as the role of anticomplement factors in disease.

## 3. Heat-Stable Enterotoxins

When first isolated from clinical material, most strains of *Y. enterocolitica* secrete a heat-stable enterotoxin, known as Yst, which is reactive in infant mice (152). Yst is encoded by the chromosomal *yst* gene and is made up of 30 amino acids with a carboxyl terminus that is highly homologous to heat-stable enterotoxins from enterotoxigenic *E. coli* and *Vibrio cholerae*. These polypeptides share a common mechanism of action, which involves binding to and activation of cell-associated guanylate cyclase and elevation of intracellular concentrations of cyclic GMP (153). This in turn causes perturbation of fluid and electrolyte transport in intestinal absorptive cells, which may result in diarrhea.

Despite the similarity of Yst to known virulence factors and the fact that its production is by and large restricted to the classical pathogenic biovars of *Y. enterocolitica* (154), the contribution of Yst to the pathogenesis of diarrhea is uncertain. Doubts regarding its role in virulence stem from the observations that (a) Yst is generally not detectable in bacterial cultures incubated at temperatures above 30°C, (b) production of Yst has not been demonstrated in vivo, and (c) strains of *Y. entero-*

*colitica* that have spontaneously lost the ability to produce Yst retain full virulence for experimental animals (138). On the other hand, a *yst* mutant of a serotype O:9 of *Y. enterocolitica* caused milder diarrhea in infant rabbits than the wild type (155). Moreover, the fact that *yst* (and *inv*) are not normally expressed at 37°C in vitro may indicate that the laboratory conditions used to study expression of these genes do not reflect those to which the bacteria are exposed in host tissues.

Although *Yersinia* enterotoxins are typically produced over a narrow temperature range around 25°C, some strains elaborate these toxins at temperatures ranging from 4 to 37°C (156,157). Since these toxins are relatively acid stable, they could conceivably resist inactivation by stomach acid, and thus cause food poisoning, if they were ingested preformed in food. In artificially inoculated foods, however, *Yersinia* enterotoxins are synthesized optimally at 25°C during the stationary phase of bacterial growth (158). Thus the storage conditions required for their production in food would generally result in severe spoilage, making the possibility of Yst ingestion unlikely.

After repeated passage or prolonged storage, Yst-secreting strains of *Y. enterocolitica* frequently become toxin negative. This phenomenon is not caused by mutation of the *yst* gene, but is due to silencing of this gene by YmoA (*Yersinia* modulator). The latter is an 8 kDa, histone-like protein, which downregulates gene expression in yersiniae by altering DNA topology.

Toxins that resemble Yst in terms of heat stability and reactivity in infant mice, but with different structure, molecular weight, and/or mechanism of action, have been detected in various *Yersinia* species, including biovar 1A strains of *Y. enterocolitica* and “avirulent” *Yersinia* species, such as *Y. bercovieri* and *Y. mollareti* (157,159). As these bacteria are occasionally obtained from patients with diarrhea, the contribution of these toxins to virulence cannot be discounted. Some of these toxins have been characterized and the genes encoding their production have been cloned and sequenced, thus enabling their detection by DNA hybridization or PCR.

#### 4. *Myf* Fibrillae and the pH6 Antigen

Many enteric pathogens carry distinctive colonization factors on their surface, which mediate their adherence to specific sites on the intestinal epithelium. In noninvasive, enterotoxin-secreting bacteria, such as enterotoxigenic *E. coli*, these factors frequently take the form of surface fimbriae, which allow the bacteria to bind to the mucosal surface and deliver their toxins close to epithelial cells while resisting removal by peristalsis (160). In enteroinvasive bacteria, surface adhesins may augment virulence by allowing the bacteria to home in on cells, such as M-cells, which they preferentially invade (137). Although the key intestinal colonization factors of *Y. enterocolitica* appear to be invasins and YadA (see below), some strains of *Y. enterocolitica* also produce a fimbrial adhesin, named Myf (for mucoid *Yersinia* fibrillae), because it bestows a mucoid appearance on the bacterial colonies that expresses it (161). Myf are narrow flexible fimbriae that resemble CS3, an essential colonization factor of some strains of enterotoxigenic *E. coli*. MyfA, the major structural subunit of Myf, is 44% identical at the DNA level to the so-called pH6 antigen of *Y. pseudotuberculosis* and *Y. pestis*, which also has a fibrillar structure and mediates thermoinducible binding of *Y. pseudotuberculosis* to tissue culture cells (162).

The *myf* operon in *Y. enterocolitica* comprises three genes, *myfA*, *myfB*, and *myfC*, corresponding to *psaA*, *psaB*, and *psaC* in *Y. pseudotuberculosis* (161,163). *myfA* encodes the major 21 kDa pilus subunit, while *myfB* and *myfC* appear to be involved in fimbrial assembly (161). Two regulatory genes, *myfE* and *myfF* (*psaE* and *psaF*), are located upstream of *myfA*. Like *ail* and *yst*, *myf* occurs predominantly in *Y. enterocolitica* strains of the classical pathogenic biovars commonly associated with disease. In vitro, *myf* and *psa* are optimally expressed at 37°C in stationary phase and low pH (164). The pH 6 antigen of *Y. pestis* is synthesized within the acidic phagolysosomes of macrophages and may play a role in the interaction between bacteria and phagocytic cells, although it evidently does not contribute to bacterial survival in these cells (162). Its main role in virulence may relate to its ability to facilitate binding of bacteria to intestinal mucus before they make contact with epithelial cells (165). Although Myf seems likely to contribute to the colonizing ability of *Y. enterocolitica*, direct proof of this is lacking.

## 5. Lipopolysaccharide

As with other gram-negative bacteria, yersiniae may be classified as smooth or rough depending on the amount of O-side chain polysaccharide attached to the core region of cell wall lipopolysaccharide. Synthesis of the O-side chain by *Y. enterocolitica* is specified by the chromosomal *rfb* locus and is regulated by temperature, such that colonies are smooth when grown at temperatures below 30°C, but rough at 37°C (166). *Y. enterocolitica* strains of serotypes O:3 and O:8 that carry a mutation in the *rfb* locus display reduced virulence for mice, indicating that smooth lipopolysaccharide is required for the full expression of virulence (166). Its mechanism of action may involve maintenance of membrane integrity and enhancing resistance of *Y. enterocolitica* to bactericidal peptides in host tissues.

## 6. Flagella

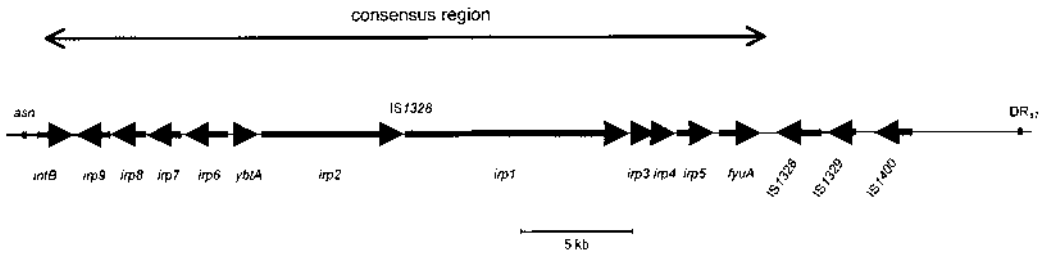
*Y. enterocolitica* and *Y. pseudotuberculosis* are motile when grown at 25°C, but not at 37°C. Although flagellar proteins are evidently synthesized *in vivo* as evidenced by the antibody response of patients with systemic yersiniosis to these antigens, motility does not contribute to the virulence of *Y. enterocolitica* in mice (167).

## 7. Iron Acquisition and the High Pathogenicity Island

Iron is an essential micronutrient of most bacteria. Despite the nutrient-rich environment provided to bacteria by mammalian tissues, the availability of iron in the extracellular milieu of some sites is generally severely limited (168). This is because most of the iron in these locations is bound to high-affinity transport glycoproteins such as transferrin and lactoferrin or is incorporated into organic molecules such as hemoglobin. Several species of pathogenic bacteria produce low molecular weight, high-affinity iron chelators known as siderophores (169,170). These compounds are secreted by the bacteria into the surrounding medium, where they bind ferric iron. The resultant ferri-siderophore complex then binds to specific receptors on the bacterial surface and is taken up by the cell. The observation that patients suffering from iron overload show increased susceptibility to severe infections with *Y. enterocolitica* suggested that the availability of iron in tissues may determine the outcome of yersiniosis (171).

Investigation of the relationship of *Y. enterocolitica* to iron has revealed that most strains do not produce high-affinity siderophores, thus accounting for their reliance on abnormally high concentrations of iron for growth in tissues (172,173). Interestingly, however, biovar 1B strains of *Y. enterocolitica* and wild-type strains of *Y. pseudotuberculosis* carry genes for the biosynthesis, transport, and regulation of a 482 dalton, catechol-containing siderophore, known as yersiniabactin. The approximately 40 kb *ybt* locus that contains these genes has a higher G+C content (57.5 mol%) than that of the host chromosome, is flanked on one side by an *asn* tRNA gene, and carries the gene for a putative integrase (Fig. 2). These features, which are typical of a pathogenicity island, have led to the *ybt* locus also being known as the *Yersinia* high-pathogenicity island. The designation “high” alludes to the observation that bacteria that carry this locus are significantly more virulent for mice infected perorally (median lethal dose < 10<sup>3</sup> cfu) than pYV-bearing strains of other biovars (median lethal dose typically > 10<sup>6</sup> cfu).

The complete nucleotide sequence of the high-pathogenicity island of a serotype O:8 strain of *Y. enterocolitica* has been determined (174). It contains 22 open reading frames within 43.4 kb, approximately 30.5 kb of which are conserved between *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis* (Fig. 2). The functions of several genes in this locus are still to be elucidated, but it is evident that the synthesis of yersiniabactin requires *irp* (iron-regulated protein) genes *irp1-irp5*, and possibly *irp9*, as well as *ybtA*, which encodes an Ara-C like regulator. The products of *irp6*, *irp7*, and *irp8* may be involved in the uptake of the ferri-yersiniabactin complex. The major receptor for this complex is a 65 kDa outer membrane protein receptor, FyuA, which also serves as a receptor for pesticin, a bacteriocin produced by *Y. pestis*. Accordingly, highly virulent strains of *Y. enterocolitica* and *Y. pseudotuberculosis* display susceptibility to pesticin (175).



**FIGURE 2** Structure of the high-pathogenicity island of *Y. enterocolitica* O:8 strain, WA-C. Arrows indicate the positions of the open reading frames and the direction of transcription. The region that is conserved in *Y. pestis* and *Y. pseudotuberculosis* is indicated by a double-headed arrow. (Adapted from Ref. 174.)

Transport of ferri-yersiniabactin complexes across the cell wall of *Y. enterocolitica* resembles the analogous pathway in *E. coli*, in that it is an energy-dependent process that requires the TonB protein. TonB, FyuA, and yersiniabactin-deficient mutants of *Y. enterocolitica* all show reduced virulence for mice, presumably because of their limited capacity to acquire sufficient iron to grow in tissues (169).

Although biovars of *Y. enterocolitica* other than 1B do not produce yersiniabactin, they can acquire iron from a number of sources, including ferri-siderophore complexes, in which the siderophore, such as desferrioxamine B, was synthesized by another microorganism (169,173). The receptor for ferrioxamine in *Y. enterocolitica* is FoxA, a 76 kDa outer membrane protein, which shares 33% amino acid homology with FhuA, the ferrichrome receptor of *E. coli* (176). The ability of *Y. enterocolitica* to acquire iron via ferrioxamine may have important clinical implications, because desferrioxamine B is used therapeutically to reduce iron overload in patients with hemosiderosis and other forms of iron intoxication. When administered to patients, desferrioxamine B forms a ferri-siderophore complex, which *Y. enterocolitica* utilizes as a growth factor (177). Accordingly, if patients undergoing iron chelation therapy with desferrioxamine B become infected with *Y. enterocolitica*, the bacteria may be able to proliferate in tissues where, under normal circumstances, poor availability of iron would limit their growth. Desferrioxamine B may also impair host immunity (178).

### 8. Phospholipase

Some isolates of *Y. enterocolitica* are hemolytic due to the production of phospholipase. A strain of *Y. enterocolitica* in which the *yplA* gene encoding this enzyme was deleted showed diminished virulence for perorally inoculated mice (179). The *yplA* mutant induced less inflammation and necrosis in intestinal and lymphoid tissues than the wild type, suggesting that phospholipase A contributes to microabscess formation by *Y. enterocolitica*.

### 9. Urease

All enteric pathogens must negotiate the acid barrier of the stomach to cause disease. In *Y. enterocolitica*, acid tolerance relies on the production of urease, which catalyzes the release of ammonia from urea and allows the bacteria to resist pH levels as low as 2.5 (31,32). Urease also contributes to the survival of *Y. enterocolitica* in host tissues, but the mechanism by which this occurs is not known (180). Although *Y. pseudotuberculosis* also produces urease, it has a relatively low specific activity and evidently does not contribute to acid tolerance in this species (181).

## B. The Virulence Plasmid (pYV)

All fully virulent, highly invasive strains of *Y. enterocolitica* and *Y. pseudotuberculosis* carry a ~70 kb plasmid, termed pYV (plasmid for *Yersinia* virulence), which is highly conserved among these

**TABLE 8** Overview of Major pYV-Encoded Virulence Determinants of *Y. enterocolitica*

Determinant(s)	Role in virulence
Yops	
Effector Yops	
YopE, YopT	Interference with phagocytosis by disrupting actin filaments
YopH	Interference with phagocytosis by dephosphorylating focal adhesion kinase, etc.
YopM	Not known
YopO (YpkA) <sup>a</sup>	Disruption of cytoskeletal actin
YopP (YopJ) <sup>a</sup>	Reduces inflammation by suppressing TNF- $\alpha$ ; induces macrophage apoptosis by suppressing host kinases
YopB, YopD, LcrV	Yop translocation; possible antihost effects and some regulation of <i>yop</i> gene expression
Syc proteins	
SycE, H, D, N, T	Yop chaperones
Ysc complex (product of <i>virC</i> , <i>virG</i> , <i>virA</i> , <i>virB</i> )	Type III secretory apparatus for Yops
YadA	Surface adhesin; reduces opsonization by interfering with binding of complement proteins
VirF	Regulation of expression of <i>yop</i> , <i>virC</i> , and <i>yadA</i> genes

<sup>a</sup> Designation in *Y. pseudotuberculosis*.

species. pYV functions as an antihost genome, which permits bacteria that carry it to resist phagocytosis and complement-mediated lysis, thus allowing them to proliferate extracellularly in tissues (for a review, see Ref. 182) (Table 8). *Yersiniae* that carry pYV exhibit a distinctive in vitro phenotype known as “calcium dependency” or “the low calcium response,” so called because it manifests when pYV-bearing bacteria are grown in media containing low concentrations of Ca<sup>2+</sup>. The principal features of this response are the cessation of bacterial growth after one or two generations and the appearance of at least 12 new proteins (Yops) on the bacterial surface or in the culture medium.

These proteins are termed Yops because they were once thought to be outer membrane proteins. They are now known to be secreted by the bacteria via the type III secretory pathway. Although they are highly conserved between *Yersinia* species, there is little homology between the individual Yops of a single species. The 12 Yops encoded by pYV are characterized by their common mode of secretion and their regulation by a pYV-encoded regulator, known as VirF.

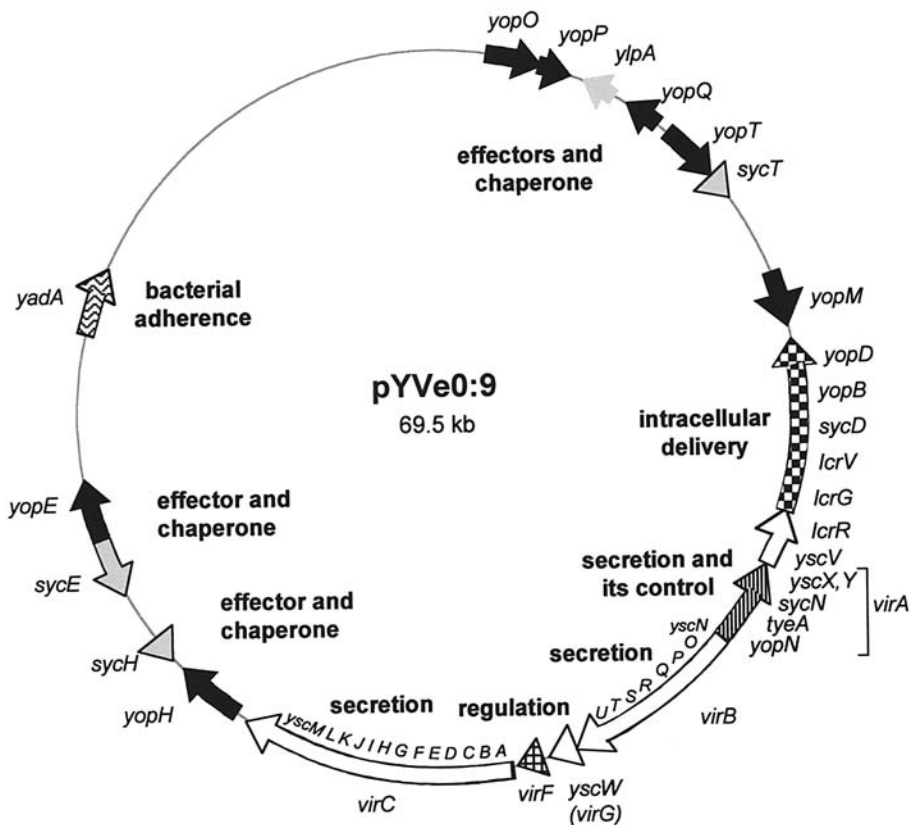
The genes carried on pYV include those for (a) an outer membrane protein adhesin, YadA, (b) a secretion apparatus (Ysc), which transports Yops across the *Yersinia* inner and outer membranes via the type III secretory pathway, (c) at least six distinct antihost, effector Yops, (d) a translocation apparatus comprising certain Yops, which the effector Yops require to gain access to the host cell cytosol, and (e) factors for the regulation of Yop biosynthesis, secretion, and translocation.

Genes for the effector Yops are scattered around pYV, while those required for Yop secretion and translocation are clustered together (Fig. 3). Homologs of several of these genes occur in *Pseudomonas aeruginosa* (184,185). pYV also encodes YlpA, a 29 kDa lipoprotein related to the TraT protein of plasmids in various enterobacteria and, in *Y. enterocolitica* strains of biovars 2–5 (but not biovar 1B or *Y. pseudotuberculosis*), an operon that specifies resistance to arsenic (186).

### 1. *YadA*: A pYV-Encoded Adhesin

YadA, formerly known as Yop1 or P1, is a 44–47 kDa outer membrane protein that contains a typical 25-amino-acid signal sequence indicating that it transported across the bacterial membrane





**FIGURE 3** Map of the virulence plasmid, pYVe, of *Y. enterocolitica* serotype O:9 showing the location and direction of transcription (arrows) of the genes encoding: (a) YadA; (b) YlpA; (c) Yops B, D, E, H, M, N, O, P, Q, T and LcrV; (d) specific Yop chaperones, Syc D, E, H and T; (e) secretion elements, Vir A, B, C, G; and the regulatory element, VirF. (Adapted from Ref. 183.)

via the general (type II) secretory pathway. Individual YadA monomers aggregate in solution to form oligomers with an apparent molecular mass of around 200 kDa. Early studies of the morphology of YadA suggested that it polymerized to form fibrils on the bacterial surface (187), but more recent work has shown that YadA comprises lollipop-shaped oligomers, which envelop the entire outer membrane as a densely packed array (188).

YadA mediates bacterial adhesion to intestinal mucus and to certain extracellular matrix proteins, including collagen, laminin, and cellular fibronectin (189). These proteins in turn may bind to  $\beta_1$  integrins, including  $\alpha_1\beta_1$ ,  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ , and  $\alpha_5\beta_1$ , on epithelial cells and stimulate bacterial internalization by endocytosis in a manner similar to that mediated by invasins. YadA may also promote bacterial invasion by binding to integrins directly (190,191).

Apart from its role as an adhesin and invasin, YadA also contributes to virulence by conveying resistance to complement-mediated opsonization. It achieves this by binding factor H, thereby reducing deposition of C3b on the bacterial surface (192). As a consequence, YadA is associated with the ability of *Y. enterocolitica* to resist complement-mediated lysis and phagocytosis and to inhibit the respiratory burst of polymorphonuclear leukocytes, all of which require the bacteria to be preopsonized (192,193). Given the pluripotential capacity of YadA to increase the likelihood of bacterial survival in host tissues, it is not surprising that YadA mutants of *Y. enterocolitica* show sig-

nificantly reduced virulence for mice (194,195). This is in contrast to *YadA* mutants of *Y. pseudotuberculosis*, which show no attenuation, and *Y. pestis*, which is extremely virulent but is naturally defective in *YadA* production due to a single-base-pair deletion, resulting in a shift of the reading frame of the gene (196,197).

## 2. *VirF*

Synthesis of *YadA* is regulated at the transcriptional level by temperature, but not by the concentration of  $\text{Ca}^{2+}$ . This is in contrast to *ylpA*, the *yop* genes, and the *virC* operon (see below), which are regulated both by temperature and  $\text{Ca}^{2+}$ . Expression of *yadA* is controlled by *VirF*, a pYV-encoded, DNA-binding protein of the AraC family (198). Other members of this family include *VirF* from *Shigella flexneri*, *Rns* from enterotoxigenic *E. coli*, and *ExsA* from *Pseudomonas aeruginosa*, all of which are involved in the regulation of expression of virulence in their respective bacteria. *VirF* plays a central role in the virulence of *Y. enterocolitica* by controlling the transcriptional activation of *yadA*, *ylpA*, all of the *yop* genes, and the *virC* operon. Because these genes are co-regulated, they have been named the Yop regulon (199). Transcription of *virF* itself is regulated by temperature (but not by  $\text{Ca}^{2+}$ ), probably as a result of combined effects of *YmoA* and the influence of elevated temperature on DNA supercoiling (200).

## 3. *Yop Secretion*

The inner and outer membranes of gram-negative bacteria are major barriers to protein export. The term “secretion” is used to denote the active transport of proteins from the cytoplasm across these membranes. Secretion of Yops occurs via the type III secretory pathway in which protein transport is governed by an amino terminal sequence. In contrast to proteins exported via the type II or general secretory pathway (which also depends upon the amino terminal sequence), type III secreted proteins show no resemblance to each other and are not cleaved during export (for reviews, see Refs. 201 and 202). Another distinguishing feature of type III secretion is the presence of structurally conserved chaperone proteins, which bind specifically to individual secreted proteins and guide them to the secretion apparatus while preventing their premature interaction with other proteins. Chaperone proteins for Yops are denoted by the prefix *Syc* (for specific Yop chaperone) and include *SycE* (for *YopE*), *SycH* (for *YopH*), *SycD* (for *YopB* and *YopD*), *SycN* (for *YopN*), and *SycT* (for *YopT*). The genes encoding these chaperones are located on pYV close to the corresponding *yop* gene (Fig. 3). Although they share no significant homology with each other, all Yop chaperones identified to date are low molecular weight (14–19 kDa) proteins, with a C-terminal amphipathic  $\alpha$ -helix and a pI of around 4.5 (182).

The N-terminal signal required to secrete a Yop generally resides within the first 20 amino acid residues of the protein. Nevertheless, Yop secretion domains show no similarity to each other with respect to amino acid sequence, hydrophathy profile, distribution of charged structures, or predicted secondary structure (182). Studies by Anderson and Schneewind (203,204) involving mutagenesis of the signal sequences of *YopE* and *YopN*, and frameshift mutations that completely altered the peptide sequence, suggested that the signal to Yop secretion lies in their messenger RNA rather than the peptide sequence per se. Subsequent work from the same laboratory, however, identified a second secretion signal in *YopE* that corresponds to the *SycE* binding site (205,206). Thus it appears that there are two distinct signals for the secretion of *YopE*: one dependent on the nucleotide sequence at the 5'-end of the messenger RNA, the other determined by the *SycE*/*YopE* complex. Similar processes seem likely to govern the secretion of *YopH* and *YopT*. For Yops without specific chaperones, however, it is not known if secretion is determined by their amino or 5' signal.

## 4. *The Ysc Secretion Apparatus*

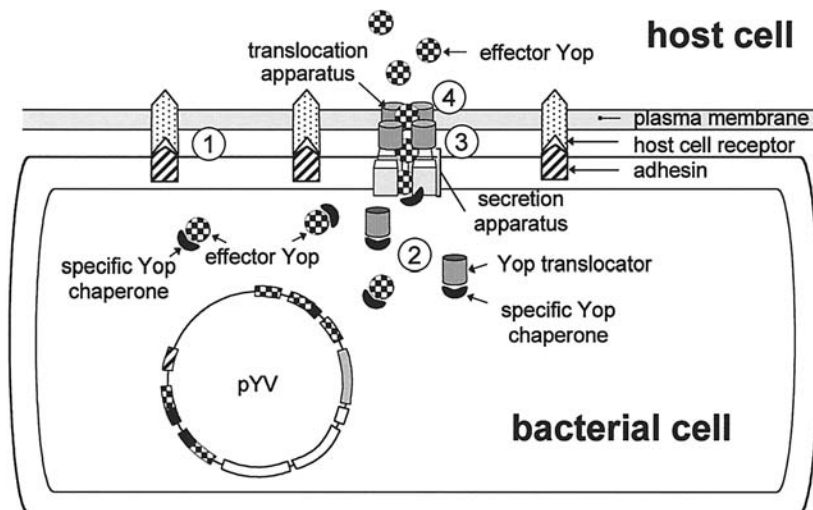
The *Ysc* secretion apparatus is a paradigm of all type III secretory machines (182). The 29 genes encoding this apparatus are contained within four contiguous loci, called *virC* (comprising *yscABCDEFGHIJKLM*), *virG* (which encodes *YscW*), *virA* (encoding *YopN*, *TyeA*, *SycN*, *YscX*, *YscY*, *YscV*, and *YscR/LcrD*), and *virB* (comprising *yscNOPQRSTU*) (Fig. 3) (183). Almost all

of these genes have homologs in the type III secretory machines of other enteric pathogens, such as *Salmonella* and *Shigella* species, and enterohemorrhagic and enteropathogenic strains of *Escherichia coli* (201). Many Ysc proteins have not been characterized, and their contribution to Yop secretion is obscure. Nevertheless, some components of Ysc have been localized to the bacterial cell envelope, where they are envisaged to form a channel that spans the inner and outer membranes (182).

### 5. Yop Translocation

Although the type III secretory pathway appears to have evolved from the process used to assemble flagella, many type III systems are used by bacteria to inject (translocate) proteins into the cytosol of eukaryotic cells and thus to facilitate pathogenesis (201). Yops are classified into those that are translocated into host cells to exert an antihost action (effector Yops) and those that are primarily involved in the translocation process. There is evidence, however, that some Yops required for translocation may also act as antihost effectors.

The transport of Yops from the bacterial cytoplasm (via Ysc) into the host cell cytosol (via the translocation apparatus) is envisaged to occur in one step from bacteria that are closely bound to the host cell (via invasins and/or YadA) (Fig. 4) (207). Two of the proteins required for translocation are YopB (42 kDa) and YopD (33 kDa,) both of which have hydrophobic domains suggesting that they could interact directly with host cell membranes (208,209). YopB resembles members of the RTX toxin family and can evidently form a pore in the plasma membrane of eukaryotic cells (209). YopD associates with YopB and may contribute to the formation of the putative pore (209). YopB and YopD are encoded by the *lcrGV-sycD-yopBD* operon, which also encodes LcrV, LcrG, and SycD, the chaperone for YopB and YopD. LcrV, also known as the V antigen, is a versatile



**FIGURE 4** Depiction of the translocation of effector Yops across the eukaryotic cell membrane. Once bacteria have established contact with the host cell (1), pYV-encoded effector Yops are synthesized together with their chaperones, translocators (2), and the components required for their secretion (3). Yops are secreted by the bacteria (3), and then transported across the eukaryotic plasma membrane by the translocators, YopB and YopD (4). In the diagram, effector Yops are represented by checkered balls and the translocators by shaded cylinders. The Ysc secretion apparatus is shown as open cubes and the Yop chaperones as solid crescents. Striped arrows represent bacterial adhesins and dotted arrows depict eukaryotic cell receptors. The genes encoding the bacterial proteins are depicted on pYV with the corresponding pattern and shading. (Adapted from Ref. 125.)

Yop, which acts as an essential component of the translocation apparatus, a regulator of Yop expression, and possibly as an effector Yop by inhibiting neutrophil chemotaxis and cytokine production (210,211). Its key role in virulence is evidenced by the fact that antibodies to LcrV can protect mice from infection with *Y. pestis* (212). Another protein that appears to contribute to the translocation process is YopQ (known as YopK in *Y. pseudotuberculosis*), the gene for which lies outside the *lcrGV-sycD-yopBD* operon (Fig. 3). Although LcrG does not appear to be a component of the translocation apparatus per se, it acts in concert with YopN and TyeA to control Yop release in vitro and may also mediate binding between the Syc/translocation channel and a heparin-like receptor on host cells (213,214).

#### 6. Mechanism of Action of Effector Yops

YopE is a 25 kDa protein that is translocated into host cells and contributes to the ability of *Y. enterocolitica* to resist phagocytosis. Direct microinjection of YopE into mammalian cells disrupts actin microfilaments and leads to cytotoxic changes (215). YopE does not act on actin directly, and its target in host cells is not known. YopT is a 35 kDa protein that exerts a similar effect on actin microfilaments to that of YopE (216). Its intracellular target appears to be a Rho GTPase, known as RhoA (217).

YopH, a 51 kDa protein, is a potent protein tyrosine phosphatase. Protein tyrosine phosphorylation is a key element of signal transduction in host cells that affects many fundamental processes, including phagocytosis and cell division. YopH dephosphorylates several phosphotyrosine residues, including those on FAK (focal adhesion kinase) and the focal adhesion proteins paxillin and p130<sup>cas</sup> (218). Focal adhesions are sites where integrin receptors act as a transmembrane bridge between extracellular matrix proteins and intracellular signaling proteins. The fact that autophosphorylation of FAK is directly involved in invasion-mediated uptake of yersiniae by epithelial cells (219) provides an explanation for the antiphagocytic effects of YopH.

YopM is a 41 kDa protein that contains a succession of 12 repeated structures that resemble leucine-rich repeat motifs (220). For this reason it shows weak homology to a large number of proteins. YopM is translocated into cells and traffics to the nucleus by means of a vesicle-associated, microtubule-dependent pathway (221), but its precise antihost function is not known.

YopO is an 82 kDa protein that is known as YpkA (*Yersinia* protein kinase A) in *Y. pseudotuberculosis*. YopO catalyzes autophosphorylation on serine, suggesting that it may play a regulatory role in *Yersinia* (222), but YopO could also act on host cells to undermine signal transduction and protein phosphorylation pathways that are part of the physiological response to infection. This possibility is supported by the observation that YpkA is targeted to the inner aspect of the plasma membrane of eukaryotic cells, where it would be ideally positioned to influence signal transduction (223).

YopP (known as YopJ in *Y. pseudotuberculosis*) is a 32 kDa protein that is encoded by the same operon as YopO/YpkA. YopP interferes with the mitogen-activated protein (MAP) kinase activities of c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (224). Its inhibitory action on these enzymes is linked to the ability of YopP to induce apoptosis in macrophages. YopP also acts on various cell types to inhibit release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a proinflammatory cytokine, which plays a central role in inflammatory and immune responses (225). This effect of YopP may be due to an inhibitory action on NF- $\kappa$ B.

#### 7. Antihost Action of pYV

When pYV-cured strains of *Y. enterocolitica* are incubated with host epithelial cells or phagocytes, they typically penetrate them in large numbers without causing significant cytopathology. By contrast, pYV-bearing bacteria generally remain extracellular but exert a range of cytopathic effects that are attributable to the action of effector Yops. Some of these effects can be ascribed to one particular Yop, while others are due to a number of Yops acting collaboratively.

As macrophages and polymorphonuclear leukocytes are major participants in the first line of defense against invading microbes, the effects of *Y. enterocolitica* on these cells are of particular

interest in explaining the contribution of pYV to virulence. *Y. enterocolitica* can reduce inflammatory responses by inhibiting the release and/or production of several pro-inflammatory cytokines, including TNF- $\alpha$ , gamma-interferon (IFN- $\gamma$ ), and Il-8 (225–227). When pYV-positive strains of *Y. enterocolitica* are incubated with macrophages in vitro, they resist phagocytosis, inhibit the respiratory burst, interfere with the release of TNF- $\alpha$  and IFN- $\gamma$ , and induce apoptosis (182). If the bacteria are preopsonized by antibodies, resistance to phagocytosis is mediated largely by YadA, whereas unopsonized bacteria resist ingestion by phagocytes due to the action of YopH, possibly acting in concert with YopE and YopT. These same Yops are involved in inhibition of the respiratory burst, whereas the inhibition of cytokine release and the induction of apoptosis are primarily due to YopP/YopJ, which thus may contribute to the establishment of bacterial infection by eliminating macrophages and retarding inflammatory responses (228). Although plasmid-cured strains of *Y. enterocolitica* exhibit none of this anti-macrophage activity and are readily phagocytosed by these cells, they are able to resist killing, possibly due to the action of HtrA (also known as DegP), a chromosomally encoded, heat shock-induced serine protease (229).

pYV-bearing strains of *Y. enterocolitica* resist ingestion and killing by polymorphonuclear leukocytes, whereas plasmid-cured strains are killed (193). The key proteins involved in this process are YadA, which interferes with opsonization, and YopH and YopE, which inhibit the respiratory burst and retard phagocytosis (192). Should pYV-bearing bacteria be ingested by polymorphonuclear leukocytes, they are able to resist killing due to their reduced susceptibility to the antimicrobial peptides produced by these cells. This effect is attributable to YadA and smooth lipopolysaccharide (230).

## VIII. CONTROL

At present, prevention of yersiniosis relies chiefly on good hygienic practices, especially with regard to food preparation. Since pork products are the most frequently identified source of human infections with *Y. enterocolitica* serotypes O:3 and O:9, measures to reduce contamination and improve hygiene during all stages of pig and pork processing should reduce infection with these bacteria (69). Pigs infected with *Y. enterocolitica* are asymptomatic, making the detection of infection during routine meat inspection impracticable. However, serological testing of herds can identify infected animals, which can then be separated from seronegative herds to reduce the overall rate of infection (231). As contamination of meat usually occurs during slaughtering, particular attention should be paid to critical control points, such as excision of the tongue, pharynx, and tonsils, the deboning of head meat, and the removal of the intestine (69). Raw chitterlings should be handled with caution and not at all by children (82). Bacterial numbers in contaminated meat can be reduced by gamma irradiation, scalding, and thorough cooking (34,80,232). Contamination of milk can be controlled by adequate pasteurization (33). Since *Y. enterocolitica* is able to grow at temperatures approaching 0°C, chilling of food should not be viewed as an effective control measure.

Measures to reduce the risk of transmission of *Y. enterocolitica* by blood transfusion include excluding blood donors with a recent history of gastrointestinal illness, screening of blood products for *Yersinia* species by PCR, and specific strategies to minimize bacterial numbers by filtration, removal of buffy coat or storage of blood at 0°C (233–236).

Although natural infection with *Y. enterocolitica* and *Y. pseudotuberculosis* affords immunity to reinfection, not only with the autologous strain but to some extent with other *Yersinia* species, there is no licensed vaccine for these species (237,238). Indeed, a vaccine for humans seems unlikely given the relatively low incidence of infections with these bacteria and the possibility that immunization may induce the autoimmune complications that vaccination is partly intended to prevent. On the other hand, immunization of food animals, in particular pigs, to reduce bacterial load is a worthwhile goal. Approaches to vaccine development include the development of living attenuated vaccines or recombinant protective antigens, in particular, LcrV (212,239,240). Although some experi-

mental vaccines have proved effective in laboratory animals, their usefulness for reducing the extent of bacterial colonization in food animals is not known.

## IX. SUMMARY AND CONCLUSIONS

*Y. enterocolitica* and *Y. pseudotuberculosis* are versatile foodborne pathogens with a remarkable ability to adapt to a wide range of environments within and outside their hosts. These species typically access their hosts in food or water in which they will have grown to stationary phase at ambient temperature. Under these circumstances *Y. enterocolitica* expresses factors such as urease, flagella, and smooth lipopolysaccharide, which facilitate its passage through the stomach and the mucus layer of the small intestine. Bacteria in this state may also carry Myf fibrillae and invasins, which may promote adherence to and penetration of the dome epithelium overlying the Peyer's patches. The higher infectivity of *Y. enterocolitica* when grown at ambient temperature compared with that grown at 37°C may account for the small number of reports of human to human transmission of yersiniosis (241).

Once *Y. enterocolitica* begins to replicate in the intestine at 37°C, lipopolysaccharide becomes rough, exposing Ail and YadA on the bacterial surface. These factors may promote further invasion while protecting the bacteria from complement-mediated opsonization. When *Y. pseudotuberculosis* and *Y. enterocolitica* make contact with host cells in lymphoid tissue, they are stimulated to synthesize, secrete, and translocate Yops, notably the effector Yops E, T, H, O and P, which further frustrate the efforts of phagocytes to ingest and remove them. Further bacterial replication may lead to tissue damage and the formation of microabscesses. If *Y. pseudotuberculosis* or strains of *Y. enterocolitica* that bear the *Yersinia* high-pathogenicity island penetrate sites where iron supplies are growth limiting, they may produce yersiniabactin so that replication can proceed. Eventually the cycle is completed when the bacteria rupture through microabscesses in intestinal crypts to reenter the intestine and regain access to the environment. This well-defined life cycle of *Y. pseudotuberculosis* and *Y. enterocolitica* with its distinctive temperature-induced phases is reminiscent of the flea-rat-flea cycle of *Y. pestis*.

Although much remains to be learned about *Y. pseudotuberculosis* and *Y. enterocolitica*, investigations into the pathogenesis of yersiniosis to date have provided fascinating new insights into bacterial pathogenesis as a whole and its genetic control. *Y. enterocolitica* and *Y. pseudotuberculosis* were the first invasive human pathogens in which plasmid-mediated virulence was documented, from which internalins (invasin, Ail and YadA) were cloned and characterized, in which the relationship between iron limitation and ferri-siderophore uptake assumed clinical significance, and in which type III protein secretion was identified. Future research in this area will no doubt lead to new and unexpected discoveries of bacterial strategies to evade host immunity that will further advance our understanding of the interface between microbes and the animal world.

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## Aeromonas Species

Karel Krovacek

SLU (Swedish University of Agricultural Sciences), Uppsala, Sweden

Ahmed Faris

Simonsbo, Bälinge, Sweden

### I. BACKGROUND AND CHARACTERISTICS

The first report on *Aeromonas* dates back to 1890 when Zimmerman isolated this organism from tap water and named it *Bacillus punctatus* (1). One year later in 1891, Sanarelli reported the isolation of *Bacillus hydrophilus fuscus* (2). This article also represents the first report on the pathogenicity of *Aeromonas* demonstrating that *A. hydrophila* produced septicemia and other disease symptoms after reinoculation into poikilothermic (cold-blooded) and homeothermic (warm-blooded) animals. Kluver and van Niel proposed in 1936 (3) that the genus *Aeromonas* should include the gram-negative rod-shaped fermentative bacteria, which are facultative anaerobes, oxidase and catalase positive, and resistant to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropyl pteridine). For members of the genus, the minimal growth temperature is 0–5°C, and the maximum, 38–41°C, although some species fail to grow at 37°C. According to *Bergey's Manual of Determinative Bacteriology* (4), the genus *Aeromonas* is still included in the family Vibrionaceae together with genera *Vibrio*, *Plesiomonas*, *Photobacterium*, and *Enhydrobacter*.

The positive oxidase reaction easily differentiates *Aeromonas* from Enterobacteriaceae. The resistance of *Aeromonas* to vibriostatic agent O/129 and its inability to grow in peptone water containing 6% NaCl distinguishes it from *Vibrio* spp. Characteristics that differentiate *Aeromonas* from *Plesiomonas* include decarboxylation of ornithine and fermentation of inositol, which are both positive for *Plesiomonas*. In becoming more complex, the taxonomy of the genus *Aeromonas* still includes some confusion, resulting in several proposals for clarification. For example, some scientists propose moving *Aeromonas* from the family Vibrionaceae and creating a new family Aeromonadaeaceae (5). However, the genus as yet is well separated in two groups. The first group consists of psychrophilic and nonmotile *Aeromonas salmonicida* with three subspecies: *A. salmonicida* subspecies *salmonicida*, *A. salmonicida* subspecies *achromogenes*, and *A. salmonicida* subspecies *masoucida*. These nonmotile *Aeromonas* spp., which are unable to grow at 37°C, are extremely important fish pathogens (not human pathogens) causing furunculosis in wild and cultured fish (6,7). The second group consists of motile mesophilic aeromonads. Most laboratories identify these mesophilic aeromonads as *A. hydrophila*, *A. caviae*, and *A. sobria*, using biochemical tests for phenotypic classification based on identification criteria set up by Popoff in 1984 (8).

The genetically diverse genus *Aeromonas* is composed of 14 DNA hybridization groups (HGs), referred to as genom-species, that correspond to the following phenospecies: *A. hydrophila* (HG 1), *A. caviae* (HG 4), *A. veronii* biotype *sobria* (HG 8), *A. veronii* biotype *veronii* (HG 10), *A. jandaei* (HG 9), *A. schubertii* (HG 12), *A. salmonicida* (HG 3), *A. sobria* (HG 7), *A. media* (HG 5), *A. eucrenophila* (HG 6), *A. trota* (HG 13), *A. allosaccharophila*, *A. encheleia* (HG 11), *A. bestiarum*

(HG 2), and *A. popoffi*. Taxonomically, the majority of human fecal strains (>85%) belong to the DNA groups 1, 4, and 8 (*A. hydrophila*, *A. caviae*, and *A. veronii* biotype *sobria*). In addition, strains of *A. media* (HG5), *A. veronii* biotype *veronii* (HG10), *A. jandeyi* (HG9), *A. trota* (HG14), and *A. shubertii* (HG12) have also been recovered from human clinical material (9).

## II. ECOLOGY AND FOODSTUFFS

Aeromonads are ubiquitous organisms, readily isolated from a variety of sources. Although considered generally aquatic organisms, they have also been recovered from different animals, plants, and food products of animal origin.

### A. Water

Aeromonads have been isolated from various aquatic environments, which include not only fresh and marine (saline) water systems, but also chlorinated and unchlorinated drinking water–distribution systems, and bottled uncarbonated mineral drinking water (10–16). Genetic clones of *Aeromonas* spp. can colonize spring and domestic well water over long periods (17).

### B. Food

Although seafood, such as shellfish, oysters, and fish, are common sources of this microorganism, *Aeromonas* spp. have also been isolated from a number of food products of animal origin such as beef, chicken, pork, lamb, and raw milk, and from vegetables such as broccoli, celery, spinach, and alfalfa sprouts, which can harbor them in significant amounts (18–25).

### C. Humans

In humans, *Aeromonas* spp. have been isolated from stools of a small percentage of healthy individuals, and occasionally from skin and sputum samples (26).

### D. Animals

*Aeromonas* spp. have been isolated from cold-blooded animals such as fish, frogs, snakes, alligators, and turtles and from warm-blooded animals such as dolphins, cattle, horses and dogs (2,5–7,27).

### E. Sewage

Although human beings are minor contributors of these pathogens in the environment, aeromonads that are shed by infected humans can nevertheless multiply to significant numbers in sewage systems before discharge of sewage into receiving waters (28). Thus, sewage effluent is yet another potent source of aeromonads in the environment, in addition to the spread of these pathogens in the environment through wastewater sprinkler irrigation (29).

## III. DISEASES

Some *Aeromonas* spp. may act as primary as well as opportunistic human pathogens. The most common human infections caused by *Aeromonas* spp. are gastrointestinal, extraintestinal, and wound infections. Furthermore, *Aeromonas* infections may lead to fatal consequences as a result of disseminated disease in patients with neoplastic illnesses, strongly immunocompromised individuals, and patients with hepatobiliary diseases (26).

## A. Gastrointestinal Infections

Worldwide, gastroenteritis caused by *Aeromonas* spp. is most common in children (particularly those <2 years old), in adults older than 50 years, and in immunocompromised persons (26,30–35). Two types of gastroenteritis have been attributed to *Aeromonas* spp., namely, a cholera-like illness, characterized by watery diarrhea and mild fever, and a dysentery-like illness, characterized by blood and mucus in the stools. Although *Aeromonas* gastroenteritis is usually mild and self-limiting, there are severe cases of both types of diarrheal illnesses (26).

## B. Extraintestinal Infections

Cases of endocarditis, urinary tract infections, pulmonary infections, arthritis, and osteomyelitis involving *Aeromonas* spp. occur (9,26,36–38). In patients of various age groups, *Aeromonas hydrophila* and *Aeromonas veronii*, isolated from cerebral spinal fluid (CSF) and blood, have been connected with meningitis. Enhanced patient susceptibility might result from various underlying illnesses, such as liver disease and head trauma (39). Several *Aeromonas*-linked respiratory tract diseases, ocular infections, and peritonitis have occurred, and recently, *Aeromonas veronii* biotype *sobria* has been implicated as the cause of septic arthritis (39,40).

## C. Septicemia

*Aeromonas* sepsis usually occurs in immunocompromised patients or in hosts with hepatobiliary diseases and cirrhosis, but rare episodes of *Aeromonas* sepsis have occurred in nonimmunocompromised hosts (9,39,41–44). The fatality rate in certain groups of patients with *Aeromonas* sepsis may be up to 60% depending on underlying illnesses (9,39).

## D. Wound and Skin Infections

Wound infections caused by *Aeromonas* spp. are often associated with aquatic accidents and constitute the largest group of *Aeromonas*-associated wound infections (26,45–47). Other non-aquatic-related *Aeromonas* infections involve cutaneous lesions, such as cellulitis, impetigo, and wound abscess (9,26).

## E. Hemolytic Uremic Syndrome

Several cases of hemolytic uremic syndrome (HUS), manifesting itself as bloody diarrhea followed by abdominal pains in association with the presence of *Aeromonas hydrophila* in HUS patients, have recently been described (39). Although *Aeromonas*-associated HUS is rare, *A. hydrophila* is now regarded as an emerging diarrhea-associated HUS pathogen.

## IV. ANTIMICROBIAL SUSCEPTIBILITY

Most *Aeromonas* spp. are susceptible to a number of antibiotics, such as tetracyclines, trimethoprim-sulfamethoxazole, aminoglycosides, cephalosporins, and the quinolones. However, emerging antibiotic-resistance properties of aeromonads directed towards these antibiotics are being pinpointed (48). *Aeromonas schubertii*, *A. jandaei*, and *A. veronii* biotype *veronii*, each associated with human infections or obtained from human clinical samples, have been found to exhibit various levels of antibiotic resistance towards ampicillin, ampicillin-sulbactam, cefazolin, imipenem, piperacillin, and ticarcillin (49). The production of chromosome-mediated  $\beta$ -lactamases by *Aeromonas* spp. threatens therapy against *Aeromonas* infections, and therefore public health (50). Even aeromonads isolated from sewage possess a transferable tetracycline R-plasmid that can easily spread to clusters of *Aeromonas* spp. found in humans (51).

## V. EPIDEMIOLOGY

Although *Aeromonas* has been isolated from different types of foods and drinking water, only a few instances have directly linked *Aeromonas* spp. to foodborne diseases. Seafoods, predominantly implicated in these outbreaks, have been consumed either raw or after inadequate heating. In Sweden recently, a group of 22 (81.5%) individuals experienced severe acute diarrhea, abdominal pains, fever, and vomiting 20–34 hours after consuming *landgång*, a typical Swedish food containing shrimp with mayonnaise, liver pâté, ham, sausage, and legume salad. Of the remaining five healthy persons, who the next day consumed the leftover food, two became ill with similar symptoms. More than 10 million *A. hydrophila* organisms per gram of food were found in the leftover shrimp with mayonnaise, liver pâté, smoked sausage, and boiled ham. The isolated *A. hydrophila*, which produced  $\beta$ -hemolysin, cytotoxin, cytotoxic toxin, and enterotoxin, could also adhere to and invade human intestinal tissue culture cells (Henle 407) (52). A few other reports directly link *A. hydrophila* (found in the egg salad and shrimp or other foods consumed by patients of various age groups) with diarrhea episodes and indicate that food was the most likely vehicle of infection (53–56).

## VI. ISOLATION AND IDENTIFICATION

### A. Isolation

For detection of *Aeromonas* spp. in foods, several media have been proposed. For quantitative estimation of aeromonads, the best seems to be phenol red broth-based starch ampicillin agar (SAA) (25°C incubation temperature), with ampicillin as the inhibitory agent and starch and phenol red, the differential agents. However, Rippey and Cabelli agar (incubated at 25–30°C), composed of ampicillin, deoxycholate ethanol (inhibitory agent), and trehalose (differential agent), has been effective for isolation of environmental strains, whereas clinical strains are suitably cultured on inositol-brilliant green bile salts (IGB) at 35–37°C. Ampicillin dextrin agar containing ampicillin sodium deoxycholate (inhibitory agent) and dextrin (differential agent) has also been successful for isolation of environmental strains at 30°C. For detection of clinical isolates when incubated at 35–37°C, alkaline peptone water (pH 8.5) has been used. A good combination of agar media uses an enrichment step in alkaline peptone water at 28°C for acquiring satisfactory isolation of mesophilic *Aeromonas* spp. from meat, fish, and shellfish, followed by streaking onto sheep blood agar supplemented with 30 mg/L ampicillin (ASBA) and bile salts–irgasan–brilliant green agar (BIBG) at 35°C (16,57).

### B. Identification

The Aerokey II identification scheme has been used to identify clinical and environmental *Aeromonas* strains, such as *A. veronii* biovar *veronii*, *A. schubertii*, *A. jandaei*, *A. trota*, *A. caviae*, *A. hydrophila*, and *A. veronii* biovar *sobria* (58). The Aerokey II biotyping method combined with Abbot's method seems to be superior to API 20 NE and Biolog System (59).

Serological typing of motile *Aeromonas* spp. (according to the scheme of Sakazaki and Shimada) can differentiate *Aeromonas* species into their O-antigen groups (60). The most predominant serogroups for clinical and environmental strains are O:11, O:16, and O:34 (60–62).

Molecular biology techniques, such as rDNA restriction pattern, restriction fragment length polymorphism (RFPL), pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR), and random amplified polymorphic DNA (RAPD) analysis, have been successfully used to identify *Aeromonas* spp. (63–65). Another technique, gas-liquid chromatography of cell wall fatty acid methyl esters (FAME), has been used to identify strains of water and food aeromonads (66).

## VII. PATHOGENESIS AND GENETIC FACTORS INVOLVED IN VIRULENCE

Aeromonads are known to produce many different putative virulence factors such as extracellular toxins, e.g., cytotoxins and enterotoxins. Furthermore, some strains of *Aeromonas* are able to adhere

to and invade epithelial cells. However, these properties may be found among clinical isolates as well as among isolates from other sources, including healthy humans, and are thus not clearly correlated to pathogenicity to humans. So far, no true virulence factors have been identified among *Aeromonas* spp. that can be used to define pathogenicity in this genus.

## A. Enterotoxins

Generally published reports described two different types of *A. hydrophila* enterotoxins, namely a cytotoxic enterotoxin and cytotoxic/cytolytic enterotoxin. In addition, several investigators reported that certain *A. hydrophila* strains produced cholera toxin cross-reactive factor (CTC).

### 1. Cytotoxic Enterotoxin

Cytotoxic enterotoxin is defined by (a) rounding of adrenal cells (Y-1) without death, (b) stimulation of cyclic AMP synthesis and steroid secretion by adrenal cells, and (c) stimulation of fluid accumulation in the ligated rabbit ileal loop (LRIL) (67). The molecular weight of the cytotoxic enterotoxin was estimated to be 15,000 daltons and the biological activities were reported to be stable after treatment at 56°C for 10 minutes. In addition, some investigators reported cloning of the gene for *A. hydrophila* cytotoxic enterotoxin into *E. coli* (68,69). The clone produced enterotoxin that caused elongation of the Chinese hamster ovary cells (CHO-K1) and fluid accumulation in the LRIL. Moreover, the cytotoxic enterotoxin produced by *A. hydrophila* was distinct from cholera toxin and *E. coli* heat-labile toxin and showed no antigenic relationship between these toxins (68,70). On the other hand, true cytotoxic reaction or steroidogenesis in adrenal cells (Y-1) caused by *A. hydrophila* were not observed by other investigators (71,72).

### 2. Cytotoxic Enterotoxin

Cytotoxic enterotoxin causes destruction and/or death of tissue culture cells, as well as LRIL fluid accumulation (67). Preliminary evidence for *A. hydrophila* cytotoxic enterotoxin was presented by a number of investigators who correlated cytotoxic, hemolytic, and enterotoxic activities of *Aeromonas* isolates (71,73–78). Further evidence for a cytotoxic enterotoxin was described by Asao et al. (74), who purified an *A. hydrophila* hemolysin that was cytotoxic to Vero cells and caused fluid accumulation in the LRIL test. The molecular weight of the so-called Asao toxin/hemolysin had a molecular weight of 50,000 daltons and was inactivated by treatment at 56°C for 5 minutes. It was also demonstrated that the purified Asao toxin was  $\beta$ -hemolysin and that antisera to the purified  $\beta$ -hemolysin completely neutralized the LRIL activity of culture filtrates of  $\beta$ -hemolytic *Aeromonas* isolates (79).

### 3. Cholera Toxin Cross-Reactive Factor

Several investigators reported that certain *A. hydrophila* strains produced a factor that cross-reacted serologically with antiserum to *V. cholerae* toxin (68,80–83). It seems that this capability is not common, as demonstrated by some investigators who found that only 8 of 179 (4.5%) *A. hydrophila* isolates possessed the CTC (80). However, this cytotoxic CTC enterotoxin from human diarrhea strains of *A. hydrophila* has been purified (82,83). The cytotoxic enterotoxin was lethal to mice, gave an enterotoxin-positive LRIL response, and caused membrane damage on CHO cells, indicating cytotoxic activity and hemolytic activity to rabbit erythrocytes (82,83).

## B. Adhesive and Invasive Ability

### 1. Adhesive Ability

Some clinical and environmental *Aeromonas* spp. strains are known to attach to Hep-2, Caco-2, and intestinal human 407 tissue culture cells, and are also known to adsorb to plastic and metal material of water-distribution system pipes (84–88). Some *Aeromonas* strains are also able to adhere to human erythrocytes and to buccal epithelial cells, fish mucus, and fish tissue culture cells (89,90).

Adhesion of *Aeromonas* spp. to such varied substratums, including those of planktonic copepods, reflects an ecologically significant wide range of adhesion possibilities (91). Most work on *Aeromonas* adhesion has concentrated on pilus adhesions. The pili on *Aeromonas* strains belong to one of two morphological types: short, rigid (S/R) pili or long, wavy, flexible pili (L/W). The S/R pili are numerous for bacterial cells and are commonly seen in environmental isolates. The S/R pili, thought to be involved in colonization, are 7–10 nm in diameter, weigh 17 kDa, and do not cause hemagglutination. The L/W pili, present in both clinical and environmental isolates, and deemed important virulence determinants, are fewer per bacterial cell (1–60 per cell), and have a diameter of 4–7 nm, an N-terminal sequence of 21 kDa, and can form bundles or filamentous networks, and may also be involved in colonization. Experimental production of pili depends on the type of medium and incubation temperature. Food and environmental strains produce more pili when grown in liquid medium at 20°C or less. The sequence of the structural *fxp* gene for a flexible pilus is known and can be found on a 7.6 kb plasmid, encoding the 4 kDa “mini pilin” (92–96).

Other cell surface adhesins such as nonfimbrial outer membrane proteins (OMPs) have also been described on *Aeromonas* spp. (97,98). It has been reported that *Aeromonas* strains produce a great number of cell surface hemagglutinins/lectins, which may facilitate trapping of bacteria in the mucins and may also permit interaction with enterocytes (97,98). In addition, it has been described that *Aeromonas* spp. isolated from clinical sources commonly bind to various collagens exposed to damaged tissue (99). Yet other surface characteristics, such as the presence of a surface array protein (S-layer) or the type of lipopolysaccharide (LPS), have been used to classify *Aeromonas* spp. into different categories on the basis of virulence (97). It seems that serotype O:11 strains possessing an S-layer appear to be of highly virulent strains isolated from humans as well as from fish (97).

## 2. Invasive Ability

There are very few studies of this aspect of *Aeromonas* spp. pathogenicity (100–105). However, the ability to invade epithelial cells by *Aeromonas* can be associated with their ability to cause dysentery like diarrhea (101). It has also been reported that 14–36% of *Aeromonas* strains isolated from animals and from the environment were able to invade Hep-2 (human carcinoma larynx) tissue culture cells (102). In this report the invasive strains were particularly *A. sobria*. On the other hand, some investigators reported the invasive potential of 24 clinical *A. caviae* strains isolated from the Sheffield Children’s Hospital (G. Britain) (104). These investigators described that the majority of the *A. caviae* strains tested possessed little or no invasive ability measured using the tissue culture models with Hep-2 and Caco-2 tissue culture cells.

## VIII. CONTROL MEASURES

The frequent presence of *Aeromonas* in many types of foods is a significant problem for food safety. Since *Aeromonas* spp. appear to be ubiquitous in water and various food products, consumption of raw or improperly cooked food can be hazardous to consumers. Moreover, even though most *Aeromonas* spp. have the optimal growth characteristics of mesophiles in foods, many of them can also grow and produce toxins at low temperatures (106–112). Heat treatment and cooking effectively eliminate them from foods. Some *Aeromonas* species grow in mayonnaise-based salads having a pH of 5.8. They generally do not grow in NaCl at concentrations higher than 5%, although a few strains can grow at 6% NaCl (110). A decrease in oxygen level fails to completely inhibit their growth, which may explain why they have been isolated from vacuum-packed foods (111).

However, liquid smoke produced from birch, aspen, and Douglas fir seems to inhibit their growth (113). In addition, both ascorbic and sorbic acids have growth-inhibitory effects on *Aeromonas* spp. (114,115). Although some *Aeromonas* species are resistant to chlorine, a minute of exposure to sodium hypochlorite (5 ppm) will destroy others (116). In food, *Aeromonas* spp. can also be destroyed by doses of irradiation (125–150 krad) (116). Plant extracts such as eugenol (clove extract)

can inhibit *Aeromonas hydrophila* and can be useful food additives, serving to eliminate or retard bacterial growth in, for example, cooked ready-to-eat meat and other foods (117). Many plant extracts, classified as GRAS (generally recognized as safe), possess antimicrobial effects. Extracts like plant essential oils, food flavoring compounds, and menthol should be more thoroughly investigated for their effectiveness in inhibiting growth of *Aeromonas* spp. in various foods. Basil methyl chavicol (BMC) seems to be bactericidal to *A. hydrophila* (118). Another natural product, chitosan, known to inhibit growth of *Aeromonas* spp. and other pathogens, extends the shelf life of oysters at 5°C for up to 7 days (119). Finally, the increasing number of reports on *Aeromonas* spp. as human-, food-, and waterborne organisms mean that further epidemiological studies together with new taxonomic data on genospecies and on aeromonad pathogenicity are needed to elucidate the public health significance of these pathogens in foods and drinking water.

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# 20

## *Plesiomonas shigelloides*

Karel Krovacek

*SLU (Swedish University of Agricultural Sciences), Uppsala, Sweden*

### I. BACKGROUND

*Plesiomonas shigelloides*, another newcomer among the expanding group of known water- and foodborne pathogens, is increasingly regarded an emerging significant enteric pathogen and is implicated in both intestinal and extraintestinal infections in humans. The genus *Plesiomonas*, with only the single species *Plesiomonas shigelloides*, is placed in the family Vibrionaceae. However, plesiomonads are closely related to members of the families Enterobacteriaceae and Pseudomonadaceae.

### II. CHARACTERISTICS

*P. shigelloides* (formerly Paracolon C 27, *Fergusonia shigelloides*, *Pseudomonas shigelloides*, or *Aeromonas shigelloides*) was first isolated in 1947 by Ferguson and Henderson. They are a gram-negative, non-spore- and capsule-forming bacilli, which are facultatively anaerobic and oxidase positive. The temperature range for growth of *P. shigelloides* is 8–44°C, and optimal growth occurs in the 37–38°C range. NaCl tolerance varies from 0 to 5%, and pH tolerance from 4.0 to 9.0. Most strains are susceptible to O/1299, and most are motile by means of lophotrichous or monotrichous polar flagella (1).

### III. ECOLOGY AND FOODSTUFFS

*Plesiomonas* is an ubiquitous microorganism and can be isolated from freshwater (rivers, streams, ponds, lakes, etc.) and estuarine water; from cold-blooded animals, such as freshwater fish, shellfish, snakes, and toads; and from warm-blooded animals, such as goats, swine, cats, dogs, and monkeys (2–6). *Plesiomonas* may also be present in unsanitary water, including that used as drinking water, and in recreational water, aquarium water, and tropical fish tanks. Freshwater fish and shellfish, including oysters, have been implicated in *P. shigelloides* food poisoning. However, the most common route of transmission of the pathogen in sporadic or epidemic cases is by ingestion of contaminated water, raw fish, and shellfish (7–12).

### IV. DISEASES

Although all people are susceptible to infection by *Plesiomonas*, infants, children, and immunocompromised people are more likely to experience protracted illness and complication. There are three major clinical presentations of *P. shigelloides* gastroenteritis: a secretory-watery type of diarrhea, an invasive dysenteric form (the most common presentation of *Plesiomonas* gastroenteritis), and a subacute or chronic disease. *Plesiomonas* gastroenteritis is usually a self-limiting disease with fever,

abdominal pain, nausea, diarrhea, or vomiting. Duration of the illness in healthy individuals may be 1–7 days. The infectious dose is presumed to be very high, at least one million organisms (10,13–16).

Although *Plesiomonas* extraintestinal infections are uncommon in the literature, septicemia, septic arthritis, meningitis, cholecystitis, and cellulitis are reported. Extraintestinal complications such as septicemia and death have been described for patients who are immunocompromised (cancer, blood disorders, and hepatobiliary disease) (10,14–21). *P. shigelloides* shows susceptibility patterns similar to those of *Aeromonas*, but susceptibility to ampicillin is more frequent in *Aeromonas* spp. (22,23).

## V. EPIDEMIOLOGY

Most human *Plesiomonas* strains have been isolated from stool samples of diarrheic patients from subtropical and tropical areas, such as Africa, Cuba, Thailand, and Peru (24–27). However, there are reports of isolation of *P. shigelloides* from human patients from cold-climate areas, such as northern Europe (Sweden and Finland) (20,28). In addition, in Japan there have been reports of outbreaks resulting from contaminated water, in Zaire, from consumption of freshwater fish; in the United States, from contaminated raw oysters and shellfish; and in Holland, from freshwater bathing, where there have been gastroenteritis outbreaks (4,8,12–14,29). Recent studies from Japan and Hong Kong show that the number of *P. shigelloides* cases increases each year and that this organism is a ubiquitous enteric pathogen in this area (30–32).

## VI. ISOLATION AND IDENTIFICATION

*Plesiomonas* are easily cultivated using culture media that are readily available in bacteriological laboratories. On blood agar, after 24 hours of incubation at 37°C, shiny and opaque colonies with raised centers are observed. Minimal criteria for the identification of *P. shigelloides* are as follows: motility + (85%), oxidase + (100%), araginine dihydrolase + (93%), ornithine decarboxylase + (100%), inositol fermentation + (100%), gas from glucose – (100%). The API 20E system (API, La Balme Les Grottes, Vercieu, France) can be used for biochemical identification of *P. shigelloides* (5,15,33–36). Recently, *Plesiomonas* strains, isolated from various sources, have been detected using PCR based on 23S rRNA (37).

*P. shigelloides* strains can be distinguished serologically, even though they are morphologically and biochemically almost identical (two published antigenic schemes separate strains based on somatic and flagellar antigens). There are now more than 100 serotypes (38–43). Some O groups also give cross-agglutination with antigens from *Shigella* or *Aeromonas*, and such strains need to be subjected to the positive oxidase test. Interestingly, some investigators speculated if protection against shigellosis could be induced by natural infection with *P. shigelloides* (44).

## VII. PATHOGENICITY

Controversy about the enteropathogenicity of *P. shigelloides* continues. The ingested *P. shigelloides* organism does not always cause illness in the host animal, but may instead reside temporarily as a transient, noninfectious member of the intestinal flora. *Plesiomonas* has been isolated from the stools of patients with diarrhea but is also sometimes isolated from stools of healthy individuals (0.2–3.2% of population) (10,13,15,25,45). However, besides being reported to have enterotoxin activity, *Plesiomonas* is suspected of being toxigenic, adhesive, and invasive (46–51). Both a heat-labile and a heat-stable toxin have been described (15,46). Some investigators also reported production of cholera-like toxin activity by *P. shigelloides* (49). Current evidence suggests that the exact mechanism of *Plesiomonas* enteropathogenicity is not fully elucidated and that more than one mech-

anism may be required to cause diarrhea in humans (52). However, predominant isolation of *Plesiomonas* from stools of patients with diarrhea results in its assumed significance as an enteric (intestinal) pathogen.

## VIII. CONTROL

Like *Aeromonas* spp., *Plesiomonas* appear to be ubiquitous in water. Thus, we assume that *Plesiomonas* contaminates natural water unless the water is adequately treated. We also assume that consumption of raw or improperly cooked food, such as fish and shellfish, is hazardous to consumers. Therefore, as a precaution, we must cook natural water-related foods and must avoid water contamination of cooked and processed food. *P. shigelloides* is heat-sensitive and readily destroyed by heating at 60°C for 30 minutes. However, food processing and refrigeration must take into consideration that some *Plesiomonas* strains are able to grow at pH 4 and at a temperature of 8°C. It is also possible that some strains of *Plesiomonas*, like *Aeromonas* spp., might even be able to grow in refrigerated foods. Thus, increased awareness of *P. shigelloides* as a potential human pathogen, together with improved monitoring of these organisms in food- or waterborne infections, can be expected to clarify the importance of *Plesiomonas* in human illness and the biological role it plays as an emerging food- and waterborne pathogen.

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# 21

## ***Streptococcus* Species**

**Barry M. Gray**

*University of Illinois College of Medicine at Peoria, Peoria, Illinois, U.S.A.*

**Sylvie Rodriguez Arnavielhe**

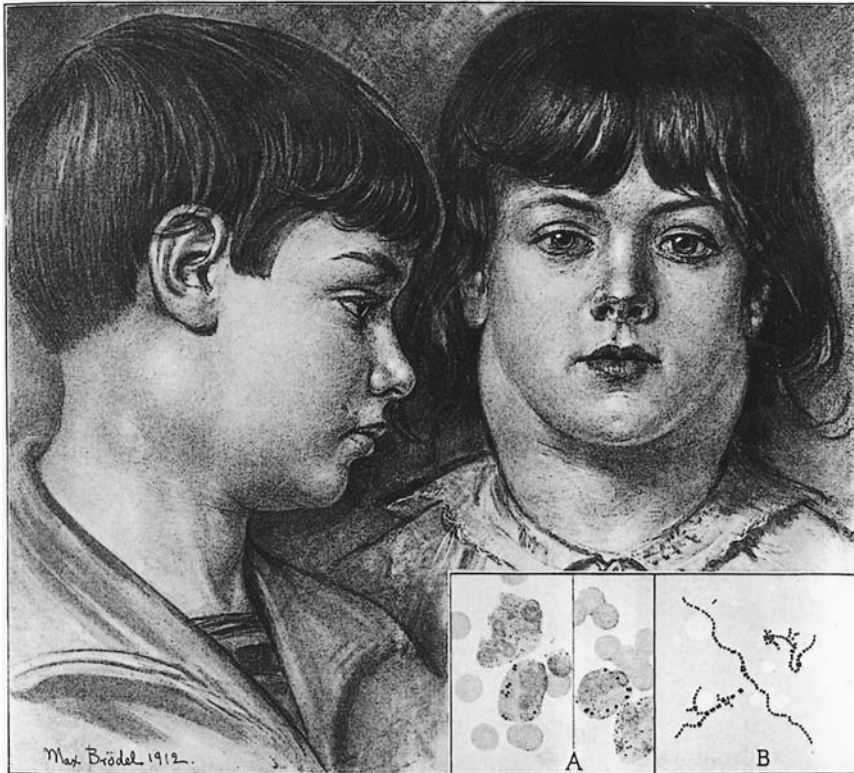
*Aventis-Pharma, Romainville, France*

### **I. BACKGROUND**

The genus *Streptococcus* includes some of the most important organisms encountered in medicine and in the food industry (1). A small number of species cause disease in humans, some are associated with disease in animals, and a few have been domesticated for use in culture of yogurt, buttermilk, and certain cheeses. While no longer a major cause of foodborne illness, streptococci were among the first to be recognized as pathogens transmitted by milk and prepared foodstuffs. Widespread milkborne epidemics of scarlet fever and “septic sore throat” occurred in England in the late nineteenth century and in the United States in 1911–1915 (2,3). In the Baltimore epidemic of 1911, children especially were afflicted by disease of unusual severity with marked enlargement of cervical lymph nodes, as beautifully illustrated in a figure from Hamburger’s report (Fig. 1) (4). Investigations of major epidemics in Norway (5), Boston (6), and Chicago (7) established an unequivocal association between streptococci and milk (8,9). These and later outbreaks were responsible for prompting major improvements in the dairy industry, especially the growing use and popular acceptance of pasteurization (10–13). There have been no large-scale milkborne epidemics in the last half-century, but streptococci continue to cause sporadic outbreaks of food-associated illnesses. Current estimates, extrapolated from surveillance data from the U.S. Centers for Disease Control and Prevention (CDC), suggest that streptococci may account for about 50,000 cases of foodborne disease per year in the United States, about 0.4% of all cases of food-related illness (14).

Streptococci received their descriptive name from the Greek words *streptos*, meaning twisted chain, and *kokkos*, meaning grain or seed, from Billroth, who observed the organisms microscopically in infected wounds in 1874 (15). Pasteur recovered similar organisms a few years later, and Ogston isolated “chain-cocci” from surgical abscesses in 1881 and used them in animal experiments of pathogenicity (16). Early species names often reflected disease associations: *Streptococcus scarlatinae* (scarlet fever), *S. erysipelatis* (erysipelas), *S. puerperalis* (childbed fever), *S. pyogenes* (pus-forming), *S. equi* (strangles in horses), *S. bovis* (cattle), and so forth.

By the early 1900s streptococci were firmly associated with the more spectacular clinical infections, such as scarlet fever, erysipelas, and puerperal fever, but there was yet no system of identification adequate for epidemiological or pathological studies. Some of the first detailed investigations of streptococci were done on isolates from the Chicago milk epidemic of 1911 by Davis, who called his organisms *Streptococcus epidemicus* (8,17,18). Three decades later this strain was formally identified as group A streptococcus, T type 13 (19,20). Smith and Brown coined the terms describing



**FIGURE 1** Illustration from Hamburger's report on the Baltimore milkborne epidemic of 1912. Children exhibited the cervical adenitis of septic sore throat; insets show streptococci within phagocytes (A) and streptococci growing in chains (B). Hamburger remarked, "When bilaterally prominent the little ones looked like victims of Hodgkin's disease!" (From Ref. 4.)

hemolysis of streptococci grown on blood agar as alpha (green) or beta (clear) in their study of organisms isolated during the 1913 and 1914 milk epidemics in Massachusetts (21).

Persons with scarlet fever were routinely quarantined as a public health measure. Although scarlet fever was routinely accompanied by severe pharyngitis, it was regarded as a different disease from that described as the "septic sore throat" seen in food- and milkborne outbreaks. In 1918 Shultz and Carleton provided the first direct evidence for the streptococcal etiology of scarlet fever, and in 1924 George and Gladys Dick showed that erythrogenic toxins (now called streptococcal pyrogenic exotoxins, SPE) were responsible for the scarlatiniform rash (22). Griffith developed a system for typing group A streptococci, including many scarlet fever strains, based on the agglutination pattern of the T protein antigens (23). Meanwhile, Lancefield developed the M protein typing system (24) and later the system of grouping the different streptococcal species by their "group" antigens (25), designated A, B, C, D, E, F, G, etc. These technical advances, still in use today, led to more exacting clinical and epidemiological studies and to more enlightened approaches to streptococcal disease. In reporting a foodborne epidemic in an army camp in 1942, Bloomfield and Rantz (26) wrote, "One may raise the question of whether the use of the term 'scarlet fever' with the implication of a fundamental difference from acute 'tonsillitis' does not serve to maintain confusion and diagnostic quibbling. The entire group should perhaps be referred to as streptococcal sore throat (with or without associated scarlatin rash), and the same rules for isolation and quarantine should hold for all.

Health departments that quarantine ‘scarlet fever’ but not ‘tonsillitis’ show complete lack of understanding of the subject.”

We now take for granted a safe milk supply and regard the occasional foodborne streptococcal outbreak as a nuisance to be cured with a course of antibiotics. The streptococci are nevertheless resourceful and dangerous pathogens, capable of causing death as well as serious, expensive disruptions in institutions and social gatherings. Most of these can be prevented by attention to routine sanitary food practices and prompt identification and treatment of food handlers with sore throat or skin infection.

## II. CHARACTERISTICS

Streptococci are spherical gram-positive bacteria that characteristically grow in pairs or chains, depending on the species and growth conditions. They are nonmotile, non-spore-forming, and catalase-negative. Most of the streptococci under discussion grow aerobically and are facultative anaerobes. They prefer enriched culture media, such as blood agar, and they grow well in complex foodstuffs, such as mixed salads with egg, meat, or seafood, as well as milk and cheese.

Of the many streptococcal groups and species, only streptococci of groups A, C, and G have been regularly associated with foodborne disease. First among these is the group A streptococcus. Organisms of other groups, generally identified by the Lancefield group carbohydrate reactions, are technically classified in several species and subspecies on the basis of DNA relatedness (27). Acute infections are similar with all of these streptococci, but only group A causes rheumatic fever. Species causing food-related disease include the following:

Group A (*S. pyogenes*), the major human pathogen, occasionally infecting cows and other animals through contact with infected human caretakers

Group C (*S. equi* subspecies *zoëpidemicus*), a species mainly associated with infections in horses and cattle, occasionally causing human disease via direct contact or transmission in unpasteurized milk

Group C (*S. dysgalactiae* subspecies *equisimilis*, group C), a subspecies mainly associated with pharyngitis in humans and seen in foodborne epidemics

Group G (*S. dysgalactiae* subspecies *equisimilis*, group G), another subspecies associated with foodborne pharyngitis outbreaks

Group L (*S. dysgalactiae* subspecies *equisimilis*, group L), a subspecies common to animals and occurring in human skin infections among poultry and meat workers

*Streptococcus iniae* (no group designation), an aquatic species occasionally causing skin and other infections in fish handlers

Other streptococci are important in human disease and may possibly be transmitted in food but are not associated with foodborne outbreaks. The group B streptococcus causes severe acute disease in newborn infants and puerperal and other infections in adults, but it does not cause pharyngitis or foodborne illness. Bovine group B strains are a major cause of mastitis in dairy cows, but they appear to be distinct from strains associated with human disease. Enterococci, formerly included among the streptococci, have been accorded a separate genus, *Enterococcus*. These are human and animal commensals and occasional nosocomial pathogens with possible links to the food chain (28), but they have never been convincingly implicated in acute foodborne disease.

## III. DISEASES

Streptococci cause a variety of diseases in humans, the most common of which is pharyngitis (1,29–31). Acute sore throat is by far the most frequent presentation of foodborne streptococcal illness, although any complication can occur in patients who do not receive timely medical attention. Skin

infections may result from direct inoculation or from transmission of organisms harbored in the throat of the patient or a contact. The major diseases caused by the group A streptococcus are listed in Table 1. Other hemolytic streptococci, especially groups C and G, are associated with foodborne illness, including throat and skin infections. Group L streptococci have been reported in epidemics of “skin sepsis” in meat packers, along with occasional infections caused by groups B, C, E, and G. *Streptococcus iniae* is associated with occupational contact with aquacultured fish. There are occasional reports of outbreaks characterized by gastrointestinal symptoms, with acute nausea, vom-

**TABLE 1** Diseases Associated with Group A *Streptococcus*

	Comments
Suppurative infections	
Sore throat	Pharyngitis, often referred to as “septic sore throat” in reports of milkborne disease
Scarlet fever	Pharyngitis associated with a scarlatiniform rash caused by pyrogenic exotoxin A, B, or C
Lymphadenitis	Infection of the lymph nodes, typically the anterior cervical nodes, when associated with pharyngitis; may occur in the axillae or inguinal nodes due to skin infections with lymph drainage into those areas
Impetigo	Superficial skin infection, often referred to as “skin sepsis” in occupational infections
Cellulitis	Infection of skin and immediate underlying tissue with edema and erythema
Erysipelas	A form of cellulitis with extensive lymphatic involvement characterized by raised erythematous lesions with sharply demarcated borders
Fasciitis	Deep infection of subcutaneous tissues with thrombosis and destruction of fascia, and variable associated cellulitis
Focal infections	Without systemic involvement: otitis media, mastoiditis, sinusitis, retropharyngeal abscess, perianal cellulitis, vaginitis, omphalitis Usually with systemic involvement: orbital cellulitis, septic arthritis, osteomyelitis, pneumonia, empyema, fasciitis, myositis
Systemic infection	Bacteremia, puerperal sepsis, peritonitis, pericarditis, meningitis
Streptococcal toxic shock	Acute severe infection with hypotension and multisystem involvement, usually associated with bacteremia or cellulitis/fasciitis, and at least two of the following: renal impairment, disseminated intravascular coagulation, hepatic abnormalities, adult respiratory distress syndrome, scarlet fever rash, soft tissue necrosis
Nonsuppurative sequelae	
Acute rheumatic fever (ARF)	Inflammatory disease following untreated pharyngitis, characterized by fever, inflammation of joints, heart, subcutaneous tissue, and central nervous system; corresponding major manifestations include migratory polyarthritis, carditis, subcutaneous nodules, and chorea
Acute glomerulonephritis (AGN)	Kidney disease characterized by inflammation of the glomeruli resulting in hypertension, edema, hematuria, and proteinuria, associated with either pharyngitis or impetigo

Source: Refs. 1,29–31.

iting, and diarrhea caused by “green” streptococci (32), but for the most part group A, C, and G streptococci remain the key players.

## **A. Streptococcal Sore Throat**

The signs and symptoms of streptococcal pharyngitis are pain on swallowing, tender cervical lymph nodes, fever, malaise, and headache (33–35). The oropharynx is erythematous; the tonsils are swollen and often have a whitish exudate; the anterior cervical lymph nodes are usually swollen and tender. Nausea, vomiting, and abdominal pain are occasionally seen in children but are less common in adults. Muscle weakness, fainting, and prostration are sometimes seen acutely. There are no particular features that distinguish foodborne from other forms of streptococcal pharyngitis. Some outbreaks are characterized by gastrointestinal symptoms (nausea, vomiting, diarrhea) in about a quarter of affected persons (36–38). In other outbreaks gastrointestinal symptoms are entirely absent (39–41), and most studies do not mention symptoms other than those usually associated with sore throat. There are no particular features that distinguish pharyngitis in outbreaks associated with group C or G streptococci. With milk or food disease, symptoms may begin as early as 6–12 hours, and the first cases are usually recognized by the second or third day, with the majority of patients becoming symptomatic by the third or fourth day.

Because the clinical presentation of patients with streptococcal pharyngitis may be indistinguishable from those with viral and other infections, a throat culture or rapid antigen test should be done to confirm or exclude a streptococcal etiology (33,34) (see Sec. V). Pharyngitis suspected of being part of an outbreak should always be diagnosed by conventional culture in order to recover the live organisms, which may be needed for specialized identification methods or epidemiological studies.

Antibiotic treatment reduces symptoms, minimizes spread to other persons, and reduces the likelihood of both acute and nonsuppurative complications (33,35). Treatment usually consists of penicillin (orally for 10 days or single intramuscular injection of benzathine penicillin G). Erythromycin is recommended for penicillin-allergic patients. Second-line antibiotics include amoxicillin, clindamycin, and oral cephalosporins. Although symptoms subside within several days or a week even without treatment, it is very important to complete the full course of antibiotics to prevent complications (33). Untreated patients may be infectious for several weeks. Treated patients are considered contagious for 24 hours after beginning appropriate antibiotic therapy and may return to school or work after that time (42).

## **B. Scarlet Fever**

Scarlet fever (scarlatina) (30) may occur when a streptococcal infection, usually pharyngitis, is caused by a strain that elaborates a “streptococcal pyrogenic exotoxin,” such as SPE-A, B, or C, in a person who does not already have antibodies to the specific exotoxin type. The classic scarlet fever rash is fine, erythematous, and has a “sandpaper” texture and blanching upon pressure. The rash may cover almost the entire body but is often prominent only in the groin and axillae. The tongue may be bright red with a “strawberry” appearance. Within a week or so after the acute infection, there is usually some desquamation of the skin, especially on hands, feet, and the tips of fingers and toes.

## **C. Impetigo**

Impetigo is the most common and most superficial skin infection (43,44). Lesions begin with a small pustule surrounded by reddened skin; they quickly fill with pus, break down, and form a thick honey-like crust. The lesions seen in food handlers and meat packers usually result from small lacerations from knives or bones and often develop some degree of cellulitis in surrounding soft tissues. There are no particular features that distinguish group A streptococcal infections from those

**TABLE 2** Early Studies of Epidemic Scarlet Fever and Septic Sore Throat Due to Milk Infections

Year	Location	Total cases	Comments	Ref.
1867	Penrith, England	—	An epidemic of scarlet fever thought to be spread by milk	65
1885–1888	England	—	“Hendon cow disease” investigated and debated. Bacteriological studies by Klein suggested <i>Micrococcus scarlatinae</i> as the etiological agent, but Crookshank and the Agriculture Department disputed this, blaming the outbreak on cowpox.	3,70
1881–1908	England	—	18 outbreaks of milkborne sore throat are summarized in the appendix. Savage reviewed the Hendon cow investigations, concluding that Klein was working with material from initial bacterial infections limited to a small number of cows, but that later investigations revealed only cowpox, as described by Crookshank. Savage also demonstrated that bovine udder infections were not harmful to man by twice trying to infect his own throat with “ <i>Streptococcus mastitidis</i> ” from cases of cow mastitis.	2
1908	Norway	548	This epidemic at Christiania was quickly traced to a particular dairy and to one cow with a diseased udder. Streptococci recovered from the cow appeared identical to streptococci recovered from patients with sore throat during the epidemic.	5
1911	Boston	964	Two related epidemics of tonsillitis or septic sore throat were investigated and traced one dairy, which ironically had long been “a pioneer in the work of dairy inspection and in the marketing of clean milk.” 48 deaths were recorded. No definitive bacteriological studies were done. This outbreak prompted the introduction of routine pasteurization in the Boston area. Winslow also reviewed 16 similar epidemics from the British literature, 1875–1905. The strain was later identified a group A, T type 13 (19).	6

1911	Chicago	10,000	Of 622 cases studied, 87% used milk from the same dairy. At the dairy 5% of cows had mastitis, and there was a coincident epidemic of sore throats among employees. A virulent <i>Streptococcus</i> was isolated from a cow with chronic mastitis and from a girl on the same farm who had sore throat and arthritis. There were 19 deaths. Records from the dairy revealed failure of pasteurization a few days preceding the largest peaks of the outbreak. The streptococci were studied in detail by Davis (17). Years later the isolates were confirmed to be group A <i>Streptococcus</i> , T type 13 (19).	7
1912	Baltimore	92	This is one of the best studied outbreaks, with excellent epidemiological data, clinical descriptions of individual cases, illustrations, bacteriology, and analysis of the literature. 35 households involved over a 6-week period were all served by the same dairy; this was followed by a prosodemic of secondary cases unrelated to milk exposure. Deaths in 11 children and 5 adults were attributed to peritonitis, "sepsis," and erysipelas.	4
1907–1915	Massachusetts	5,259	These chronological reports describe milkborne typhoid, septic sore throat, scarlet fever, and diphtheria recorded by the Massachusetts Dept. of Public Health. The decrease in frequency and number of outbreaks was correlated to the increase in proportion of milk pasteurized, which rose from 34% of the state's production in 1919 to 90% by 1931. Despite more cases reported during 1933–1940, these were associated with only three outbreaks, all associated with raw milk, and including only 2 deaths.	13,66, 71–74
1915–1918	(statewide surveillance	1,007		
1919–1923	program)	171		
1924–1925		56		
1930–1932		249		
1933–1940		422		

**TABLE 3** Milkborne Epidemics of Streptococcal Infections of Defined Etiology

	Location	Total cases	Organism identified	Comments	Ref.
1928	Massachusetts	950	<i>S. epidemicus</i>	After a decade with few significant outbreaks, due largely to pasteurization and better dairy practices, this epidemic with 48 deaths led local boards of health in Massachusetts to require pasteurization and certification of all milk sold.	11
1935	Chelmsford, England	1600	Group A T2 <sup>a</sup>	This epidemic of scarlet fever, due to <i>S. pyogenes</i> type 2 (Griffith), was traced to raw milk from a dairy where 8/17 employees had sore throat with the same organism. The milk was contaminated by the employees, whereas cultures from the only cow with mastitis grew only bovine streptococci. There were 51 cases of "arthritis." 2 cases of nephritis, and a total of 6 deaths.	48
1936	Doncaster, England	1343	Group A T2 <sup>a</sup>	This outbreak of scarlet fever and septic sore throat was traced to a dairy where a single cow had a injured teat that was infected by the milker who had been changing the dressing on his child's infected ear. <i>S. pyogenes</i> type 2 was recovered from the child's ear, the milker's throat, the cow's teat, and from the throats of all patients from whom swabs had been submitted for culture. Complications included 23 cases of arthritis, 1 nephritis; 2 deaths were recorded. Cases not associated with milk occurred in a "seasonal" outbreak of scarlet fever due to types 1 and 5. The milkborne outbreak was arrested by isolating the cow and pasteurizing the milk.	49
1934–1936	New York State	1529	Group A	Three outbreaks of scarlet fever and four of septic sore throat in rural New York State all occurred in villages of <6000 people, and all were associated with raw milk or cream. In most	75



1936	Denmark	92	Group A	instances mastitis in cows was preceded by illness in the milkers. In one instance the milk had been bottled and capped by hand by a person with acute sore throat. All cases were confirmed to be due to a hemolytic <i>Streptococcus</i> identified as Lancefield group A. Skin sensitivity tests for scarlet fever toxin (Dick test) were done on over 8000 persons in the affected counties. 24 deaths were attributed to these epidemics. This well-studied outbreak originated in one cow probably infected by the milkers on a small dairy. The isolated did not type with Griffith's sera, and attempts to raise a specific antiserum were unsuccessful. The outbreak was stopped by pasteurization. No deaths were recorded; 2 cases of "arthralgia" were noted; no nephritis was seen.	50
1935–1942	New York State		Group A (various T types) <sup>a</sup>	Nine outbreaks (1680 cases) were all associated with raw milk; nearly all were traced to infected cows. Type 3 was found in 3 outbreaks, others types were T2, T11–12, T15–17, T19, and T27. One epidemic studied in great detail was traced to a single cow (infected by a milkhand) out of over 6000 cows supplying milk to a cheese plant.	58
1911–1942	(various)		Group A (T types) <sup>a</sup>	The history and bacteriology of 29 epidemics were reviewed. The organisms were reexamined, confirmed to be group A streptococci, and T typed. The author noted in particular the associations of T3 and T11 with scarlet fever, T13 with a distinctive outbreak of septic sore throat, and the related T types T11, 12, and 27 with sore throat in 8 outbreaks. These types were much more common than others because of their ability to invade the bovine udder.	19,20
1969	Romania	85	Group C	This unusual epidemic was due to <i>S. zooepidemicus</i> , which was identified in the milk of 3 cows with mastitis, in the throats of 4 asymptomatic dairy workers, and from 85 patients. A third of those infected later developed acute glomerulonephritis.	52

**TABLE 3** Continued

	Location	Total cases	Organism identified	Comments	Ref.
1983	New Mexico	16	Group C	Invasive disease associated with unpasteurized cheese from a home dairy, with 15 bacteremic illnesses and 2 deaths, including cases of pneumonia, meningitis, endocarditis, and pericarditis.	46
1983–1984	England	12	Group C	This outbreak was associated with raw milk from a single dairy farm. There were 12 cases of meningitis or endocarditis, with 8 deaths (some not primarily due to infection). 8 patients were over 70 years of age, and one was a newborn infant whose mother was thought to be infected. The organism was identified as <i>S. zooepidemicus</i> .	45,59
1987	Australia	1	Group C	A 52-year-old man had septicemia complicated by glomerulonephritis caused by <i>S. zooepidemicus</i> , apparently from drinking unpasteurized milk from the family's house cow. Two other family members were asymptomatic throat carriers. rRNA gene restriction profile confirmed the identity of the "outbreak" strains.	54
2000	Brazil	253	Group C	An epidemic of acute glomerulonephritis associated with cheese made from unpasteurized milk. 7 patients required dialysis and 3 patients died.	67

<sup>a</sup> Griffith's typing system was based on agglutination of T antigens, whereas Lancefield's system used M protein types. T1, T2, T6, T9, T18, and T22 correspond to M types of the same numbers; both other T patterns include multiple M types.

**TABLE 4** Group A Streptococcal Epidemic Sore Throat Associated with Foods Prepared by Institutions, Hotels, or Commercial Caterers

	Location	Total cases	Organism identified	Comments	Ref.
1927	Massachusetts	138	$\beta$ -Hemolytic streptococci	Three simultaneous geographically distinct outbreaks of scarlet fever were traced to lobster meat used for salad prepared by a single caterer. It could not be determined if the lobster had been contaminated by the supplier or the caterer. 138/592 (23%) of persons attending the three banquets were affected, 98 with scarlet fever. There were 5 secondary cases.	36
1942	US	341	Group A T15	Outbreak at an army camp, thought to be food- but not milkborne, based on circumstantial and epidemiological features. Complications included 18 cases of peritonsillar abscess, 7 otitis media, and 14 with arthralgia.	26
1944	UK	89	Group A T9	An outbreak at an airforce station was traced to an infected cook who contaminated milk when diluting tinned milk and preparing it for use.	76
1945	US	104	Group A T5	This is the best studied outbreak of its kind, occurring at an Air Force infantry base and traced to creamed eggs served at breakfast. There were 86 primary cases of tonsillitis/pharyngitis (attack rate, 42%) and 9 primary carriers. The median incubation period was 38 hours. There were 18 secondary cases, with 22 secondary carriers. Treatment with sulfadiazine was modestly effective. Acute complications were limited to 1 peritonsillar abscess and a case of sinusitis; 1 patient had a symptomatic recurrence of tonsillitis. There were 3 cases of acute rheumatic fever, and 2 probable cases. Includes clinical detail, serological testing, excellent discussion and references.	39
1957	Cyprus	835	Group A T5/12/27	Several waves of infection struck an airforce station at biweekly intervals, following the pay parade. It seems that on these weekends the tinned milk was being diluted the night before use and allowed to sit, against regulations, and was contaminated by the night staff or by their convivial friends demanding late night snacks.	60
1958	Baltimore	600	Group A M25	At a catered charity luncheon, egg salad prepared by infected food handlers, 1 of whom later developed cellulitis of the hand.	79

**TABLE 4** Continued

	Location	Total cases	Organism identified	Comments	Ref.
1965	Buffalo, NY	64	$\beta$ -Hemolytic streptococci, <i>Shigella</i>	This unusual mixed epidemic probably originated from contaminated shrimp salad. Most patients had acute pharyngitis, and some also had diarrhea due to <i>Shigella flexneri</i> , and some had diarrhea alone.	77
1969	Colorado		Group A T12 M n.t. <sup>a</sup>	About 1200 (40%) of cadets at the U.S. Air Force Academy developed sore throat after eating tuna salad contaminated by the handling of boiled eggs. Antibiotic prophylaxis was credited in part with limiting the secondary spread to 5 cases. There were no nonsuppurative sequelae.	37
1974	Florida	290	Group A T9, M9	An epidemic at a jail in southern Florida was traced to egg salad. The attack rate was 49%; secondary spread was controlled by penicillin treatment of patients and by prophylaxis of asymptomatic inmates.	80
1980	Israel	41	Group A M3	An outbreak at an Israeli military base was attributed to egg salad prepared by a cook with inadequately treated tonsillitis and 5 other kitchen workers.	81
1981	Portland, OR	300	Group A T9, M n.t.	This outbreak originated at medical microbiology meeting banquet. No single food source could be positively identified, but 5/10 kitchen workers had sore throat and/or skin sores on their hands.	89
1984	Missouri	60	Group A	At a meeting of regional blood banking personnel held at a hotel, 60/102 (57%) eating the luncheon became ill, presumably from a contaminated chocolate mousse.	90,91
1984	Puerto Rico	23	Group A T12, M n.t.	Carrucho (conch) salad was identified as the likely source of this outbreak. The attack rate of 56%.	91
1985	Israel	162	Group A	In this outbreak a failure of antibiotic therapy seemed to be related to penicillin-tolerant strains. Susceptibility testing was reported in a follow-up study (94).	93

1986	Italy	179	Group A T28, M28	A prawn hors-d'oeuvre was implicated in people attending several wedding banquets held at the same restaurant. The restaurateur's family and kitchen staff were colonized by the same serotype.	95
1988	Israel	439	Group A M29	An epidemic of pharyngitis at a military base due to a new group A M29 variant.	96
1990	Israel	61	Group A T12	Outbreak at a military base attributed to boiled eggs prepared by a food handler with pharyngitis.	82
1991	Djibouti	304	Group A T11, M n.t.	A French military unit was immobilized for about 6 days. Gastrointestinal symptoms were seen in 24% of patients. 5 serological assays were evaluated.	97
1991	Israel	40	Group A T28, M56	Outbreak at a military training camp, attributed to a cook who contaminated the cabbage salad. 36 asymptomatic soldiers were also identified by throat cultures.	63
1991	Louisiana	71	Group A T5/9, M9	This outbreak at a school banquet was attributed to direct inoculation of a macaroni and cheese dish by a cook with an open wound on the hand, the result of a barroom fistfight 2 weeks before, treated with some antibiotic ointment but no systemic antibiotic.	88
1989, 1992	France	n.s. <sup>b</sup>	Group A T9 (1992) T4 (1989)	The use of rRNA gene restriction analysis was used to characterize strains from various sources. Little information was presented regarding the epidemiology of the outbreaks.	98
1996	Israel	197	Group A T8/25/ Imp19	An outbreak at an Israeli Airforce base in 1991 was attributed to processed white cheese contaminated by a food handler with pharyngitis.	41
1999	Greece	42	Group A M90	This outbreak was attributed to a vegetable-salad dressing made of mayonnaise, but none of the kitchen staff were infected. 30 asymptomatic infections were identified by culture.	64

<sup>a</sup> This strain was M "nontypable," i.e., it did not have one of the M antigens (M11, 27, or 44) usually associated with T12 strains (92).

<sup>b</sup> n.s., not stated.

caused by group L or by *S. iniae*. Topical murpiricin is effective for treatment of small superficial lesions and may also be used to prevent infection when applied to fresh cuts or abrasions. More extensive, multiple, or widely spread lesions are usually treated with oral antibiotics.

#### **D. Cellulitis**

Cellulitis (43) results from inflammation of skin and underlying tissues, causing local redness and pain, often accompanied by fever and chills. Lymph nodes draining the affected area are usually swollen and tender. Cellulitis often begins with minor trauma, such as a bruise, abrasion, or laceration, sometimes distant from the site of infection. Cellulitis is treated with oral or intravenous antibiotics, depending on the severity of infection.

#### **E. Erysipelas**

Erysipelas (43), recognized from ancient times, was known as St. Anthony's fire in the Middle Ages and derives its name from the Greek *erythros* (red) and *pelas* (skin). Erysipelas involves the subcutaneous lymphatics and is characterized by a red rash with sharply demarcated advancing margins. Classically, it occurs over the bridge of the nose and cheeks, but almost any skin surface may be affected. About 5% of adult cases are bacteremic.

#### **F. Severe Focal and Systemic Streptococcal Infections**

Foodborne streptococcal infections have historically been associated with occasional severe suppurative complications (see [Tables 2, 3, and 4](#)). The 16 deaths in the Baltimore epidemic of 1912 occurred in patients who developed sepsis, erysipelas, or peritonitis (4). The most frequent complications, such as adenitis, peritonsillar abscess, and otitis media, might be considered severe by today's standards but were rarely fatal even before penicillin. Few serious complications have been noted in foodborne outbreaks in recent decades, probably because most acute infections are recognized and treated promptly. The exceptions have been in small outbreaks of *S. zooepidemicus* (group C) disease. One in 1983–1984 was associated with consumption of raw milk from a single dairy (45). Twelve persons were hospitalized and 8 died. Most had meningitis or endocarditis, including 10 elderly patients (>70 years of age) and one newborn infant whose mother was thought to be infected. Another outbreak in 1983 was associated with homemade cheese (46). There were 2 deaths among 15 bacteremic illnesses that included patients with pneumonia, meningitis, endocarditis, and pericarditis.

#### **G. Streptococcal Toxic Shock Syndrome**

This severe manifestation of systemic streptococcal infection has not been reported as a complication of foodborne streptococcal disease (47).

#### **H. Nonsuppurative Sequelae**

There have been only a few reports of acute rheumatic fever (ARF), following infections transmitted by milk (48–50) or food (26,39). This may be because ARF was not generally recognized as a streptococcal sequella until the mid-1930s and 1940s, and in later years patients were usually treated with antibiotics during the acute primary illness. Diagnosis is made according to the revised Jones criteria (51). Similarly, few cases of acute glomerulonephritis (AGN) have been noted in foodborne group A streptococcal disease (48), although group C streptococci have been associated with AGN in at least three milkborne outbreaks (52–54).

## I. Gastrointestinal Disease

As noted above in the context of streptococcal pharyngitis, gastrointestinal symptoms are a variable accompaniment to foodborne infections. It is believed that the abdominal pain sometimes associated with group A disease is due to mesenteric adenitis. However, it is possible though unproven that streptococcal exotoxins or other bacterial products may play a direct role in stimulating intestinal secretion and motility. One of the few descriptions of a streptococcal “enteritis,” by Cary et al. in 1931 (32), involved “green” hemolytic streptococci apparently contaminating tins of Vienna sausage.

## IV. EPIDEMIOLOGY

Outbreaks of foodborne streptococcal disease have become relatively unusual in recent years. They are not routinely reported to the CDC, and the real number of cases is assumed to be 10-fold higher than reported. Mead et al., using CDC surveillance data from 1982 to 1992, estimated 50,000 cases per year, assuming 38 times the number of reported cases, by extrapolation from studies of salmonellosis (14). Streptococcal outbreaks recorded by the CDC in the three most recent 5-year surveillance periods include the following: 1982–1987, seven outbreaks totaling 1001 cases (55); 1988–1992, two outbreaks with 135 cases (56); and 1993–1997, one outbreak with 122 cases (57). During the same time frame, over two dozen reports of foodborne streptococcal disease appeared in the literature from various parts of the world (see tables). No doubt there is underreporting, but there have also been remarkably few deaths, which usually bring attention to an outbreak.

The presenting characteristics of foodborne streptococcal outbreaks have not changed appreciably over the years. Outbreaks are often described as “explosive” because the majority of affected persons become symptomatic in the first several days after exposure. With prompt identification and institution of control measures, epidemics are short-lived but may otherwise smolder for several weeks (58). A few reports describe cases appearing over a period of months all relating to the same source of infection, as was seen in a series of serious of group C infections eventually traced to unpasteurized milk from a local dairy (59). One epidemic at a military base had three fortnightly waves of illness that coincided with the soldiers’ pay period. The weekend kitchen crew diluted tinned milk on Friday night amidst pay-day reveling and allowed it to sit out unrefrigerated, where it was frequently sampled by their convivial but presumably infectious friends (60).

Exposed persons of any age may be affected, and the age distribution is generally dependent on the circumstances and demography of the outbreak. For example, in Boston (1911) and Christiana, Norway (1908), mostly adults were affected (5,6), whereas half the cases in the Baltimore epidemic (1912) occurred in children from 4 months to 15 years of age (4). The age range in outbreaks associated with institutional- or home-prepared foods generally reflects the ages of the persons partaking of the food served. One might suppose that the proportion of milk drinkers or the proclivity for certain kinds of food might be factors. Other factors that are similarly hard to assess may relate to bias in case reporting or identification, to prior immunity among exposed persons, or to dose phenomena, i.e., how much is eaten with what initial colony count (61).

Streptococcal outbreaks may occur any time of year, but there is a tendency for them to occur in the fall when there is a peak in pharyngitis. Skin sepsis in meat workers has been noted particularly in the fall, in association with the major slaughtering time of pigs and cattle (62).

Attack rates are typically about 50% of those presumed to be exposed to the contaminated food. In foodborne outbreaks it is important to consider three categories of infected individuals: (a) those primarily exposed who present with acute pharyngitis or other symptoms; (b) those who do not develop acute symptoms but who may be carriers or have subclinical infection; and (c) those with no initial exposure who develop pharyngitis as the result of respiratory spread, i.e., secondary cases. Primary infections are usually easy to identify, because the acutely ill persons generally, though not always, seek medical attention. Those with mild or subclinical infections have some potential risk for later developing rheumatic fever, although few such cases have been identified.

The rate of inapparent infections, that is, the case-to-carrier ratio, has been reported to be as low as 2–6% (37,39,58) and as high as 90% (63,64), as determined by performing throat cultures on potentially exposed persons. Secondary attack rates have been reported to be as high as 17%, mostly among family members of soldiers at a military base (37). Ideally, the secondary attack rate should approach zero when primary infections are identified and treated promptly (63). Penicillin prophylaxis has been used in a few outbreaks at military installations, with a modest or no apparent effect on the number of secondary cases (37,41).

It remains important to collect and preserve streptococcal isolates from suspected outbreaks. Accurate grouping and typing, and nowadays molecular identification methods, are essential to understanding a given outbreak, to distinguish between outbreak and unrelated strains, and to follow up on later suppurative and nonsuppurative complications.

## A. Milkborne Epidemics of Streptococcal Disease

The first clinical record of milkborne infection was probably that of Sagar, who in 1764 suggested that some throat infections and aphthous ulcers were due to drinking milk from a certain cow (see Ref. 4). Major outbreaks of milkborne scarlet fever were recognized in England beginning in 1867 (65), but controversy and uncertainty hindered epidemiological studies until better microbiological methods became available. Wilson (3) gives a thoughtful and detailed account of this interesting chapter of medical history. Although scarlet fever was a useful marker for streptococcal disease, early investigators regarded “septic sore throat” as a different disease. Investigators often had difficulty distinguishing it from cases of diphtheria by clinical characteristics until improved bacteriological methods came into use (2). Other important milkborne infections were common, especially typhoid, brucellosis, and tuberculosis (Bang’s disease in cattle). Pasteurization was initially employed to prevent these diseases, but it was the sore throat epidemics of 1911–1912 in Boston, Chicago, and Baltimore that probably did the most to bring pasteurization to the forefront of public health policy. It nevertheless took about two decades and slowly changing public opinion before 90% of milk was pasteurized in Massachusetts (3,10,66).

Hamburger (4) and other early investigators differentiated two epidemiological patterns of milkborne streptococcal infections: those associated with milk primarily infected from one or more dairy cows with mastitis, and those derived from milk contaminated secondarily during processing by individuals with sore throat. Examples of these patterns are found in [Tables 2](#) and [3](#).

The early investigators were unaware of the association of streptococcal sore throat and the later occurrence of nonsuppurative sequelae. Only a few reports mention patients with symptoms suggestive of acute rheumatic fever. Camps and Wood recorded 51 cases of “arthritis” and 2 cases of nephritis in their investigation of the 1935 epidemic scarlet fever affecting 1600 persons in Chelmsford, England (48). Watson reported 23 cases of “arthritis” among 1300 patients in the 1936 epidemic in Doncaster, England (49). Henningsen and Ernst noted 2 cases described as arthralgias, but no cases of acute glomerulonephritis, among 92 affected individuals in their account of the 1936 outbreak in Norway (50). Sequelae were looked for in later outbreaks but not observed.

Recent milkborne outbreaks have all been associated with unpasteurized milk and have been due to group C strains. Acute glomerulonephritis was seen in two outbreaks (52–54), and several deaths were reported in another (45). Homemade cheese made from unpasteurized milk was implicated in an outbreak of 16 cases of invasive disease (46). A recent epidemic in Brazil involved 253 cases of acute glomerulonephritis and 3 deaths, associated cheese made from unpasteurized milk (67). Although streptococcal mastitis continues to be common in dairy herds, few infections are now caused by group A or C strains (68). In England and Wales about half of unpasteurized goat’s and ewe’s milk on retail sale fails to meet the U.K. *Dairy Products* standards for indicator organisms (69). Curiously, there have never been any reports of streptococcal outbreaks associated with milk from mammals other than cows, although sporadic cases have been observed with unpasteurized goat’s milk in the United Kingdom (M. Barnham, personal communication).



## B. Foodborne Outbreaks

Foodborne streptococcal outbreaks can usually be traced to infected persons involved in preparing or handling the food. This includes outbreaks in which milk was the vehicle but was found to be contaminated by kitchen workers, rather than primarily infected with milk from cows with mastitis (60,76). The majority of reports and the larger outbreaks were associated with group A streptococci encountered at larger venues served by caterers or institutional food services (Table 4). There are fewer reports of outbreaks due to group C and G streptococci (Table 5). Outbreaks associated with home-prepared foods (Table 6) follow similar patterns but on a smaller scale.

In a few instances food components, such as lobster meat or shrimp, may have been already contaminated when received from the suppliers, but this pattern of transmission is infrequent and hard to prove (36,77). In most foodborne outbreaks it is the kitchen staff who are primarily infected and who contaminate the food directly. Growth of streptococci to infective concentrations is often facilitated by improper food handling and ignorance or breach of routine sanitary practices. Failure to refrigerate food components and complex prepared foods is commonly reported as a major factor in the spread and magnitude of the outbreak.

The most commonly implicated food component is boiled eggs served alone (78) or more often as in egg salad (38,79–82), potato salad (61,83), tuna salad (37), and other complex prepared dishes.

**TABLE 5** Epidemic Sore Throat Due to Non–Group A Streptococci in Association with Foods Prepared by Institutions

	Location	Total cases	Organism identified	Comment	Ref.
1931	US	75	“Green” hemolytic streptococci	This institutional outbreak involved 75/182 (41%) boys after eating Vienna sausages. Symptoms included nausea, vomiting, colicky pains, and diarrhea within 4–5 hours. A young adult laboratory worker developed similar symptoms after eating representative sausages, and 1 of 2 other volunteers became sick after drinking pure cultures of the organism.	32
1969	Eastern US	176	Group G	Epidemic at a small college; attack rate 31% after eating the egg salad, probably contaminated by food handlers with respiratory infections.	38
1982	Florida	72	Group G	Convention attendees at a hotel were sick after eating chicken salad served at a luncheon and banquet. The cook may have been the source.	40
1983	Israel	50	Group G	This outbreak was traced to a food handler who had developed pharyngitis 10 days before, but did not take penicillin as directed.	99
1990	Rochester, NY	69	Group G	Outbreak of pharyngitis among college students probably associated with imitation crab meat. The report is mainly on the use of restriction fragment analysis to characterize strains.	85

**TABLE 6** Epidemic Sore Throat Associated with Home-Prepared Foods Served at Parties and Picnics

Year	Location	Total cases	Organism identified	Comment	Ref.
1941	Massachusetts	182	Group A T2	Outbreak at a church reunion luncheon. The attack rate was 57%. The vehicle was ground ham salad sandwiches prepared by Mrs. A, who was in the preeruptive stage of scarlet fever.	86
1976	Arizona	121	Group A T3/13/ B3264 M n.t.	Attendees at a July 4 picnic developed pharyngitis after eating potato salad made with contaminated boiled eggs. The attack rate was 48%.	61
1982	New Hampshire	34	Group A T12	Several dips and a potato salad were implicated in this anniversary party. The one person preparing the food was culture-negative but had been medicating with a few doses of ampicillin after intimate contact with a family member with acute pharyngitis.	83
1984	Nashville, TN	20	Group A T8/25, M n.t.	After a potluck luncheon, more than half the staff of a hospital pediatric clinic became ill from eating a contaminated rice dressing. Gastrointestinal symptoms occurred in about a quarter of affected persons.	87
1992	Sweden	122	Group A T28	72% of people who attended a church birthday party or ate leftovers became ill. Sliced eggs on sandwiches were implicated. Complications included otitis media, peritonsillitis, arthritis, and pericarditis; a 45-year-old man died of septicemia.	78

Seafoods such as lobster, shrimp, conch, and imitation crab meat have been implicated (36,77,84,85). Meats, including sausage, ground ham, and chicken, have been reported (32,40,86). Vegetable salads made with mayonaise, macaroni and cheese, and dishes with prepared sauces are also common (41,63,87,88).

### C. Skin and Soft-Tissue Infections

“Skin sepsis” is an occupational hazard of meat and poultry handlers. Though probably unrecognized for many years, it was first reported in the 1970s (Table 7). Epidemics occur in slaughterhouses and meat packing factories, usually beginning in autumn, coinciding with the peak slaughtering

**TABLE 7** Streptococcal Skin Infections Associated with Meat Handling

Year	Location	Total cases	Organism identified	Comment	Ref.
1974–1975	UK	131	Group A T25/Imp 19	A series of outbreaks of skin sepsis in meat handlers was studied, revealing several new provisional M types, one of which appeared to be almost exclusively associated with meat packing.	112
		225	Various M types	A follow-up study of the first series included 166 isolates from 29 outbreaks and 55 from sporadic infections.	105
1977	Vermont	16	Group A M41	This study of the mode of transmission in an abattoir outbreak found that most infections occurred on the kill floor or cutting room, with the initial cluster of cases traced to one person, followed by multiple intermittent introductions by other workers.	100
1978	UK	82	Group A T3/13/B	An outbreak in a chicken factory had of 103 infections in 82 of 347 workers. The attack rate was 44% in the packing department. The epidemic subsided after identification and treatment of cases and carriers, along with specific control measures.	113
1978–1980	UK		several T, M types	A follow-up study added 30 patients from 1979 and 42 from 1980. Infections due group B, C, G, and other pathogens were also noted.	114
1980	Oxfordshire, UK	n.s.	Group A M49	Skin infections in abattoir workers and butchers in 3 districts were affected.	115
1980–1981	Oxfordshire, UK	16	Group A M81, M49	This study provides some epidemiological evidence, backed by serotyping data, to suggest spread of disease from slaughtermen to meat processors and retail butchers and on to housewives, relatives, a chef, and a restaurateur.	103
1981	Norway	7	Group A T8/Imp.19	2 butchers and 5 meat inspectors had skin infections (1 with a T12 strain). A total of 40 infections occurred in an 8-month period, most not cultured.	116

**TABLE 7** Continued

Year	Location	Total cases	Organism identified	Comment	Ref.
1981–1983	Yorkshire, UK	15	Group L, Groups A, B, C, E, G	Skin infections are described in meat handlers in chicken and pork slaughtering/processing plants. Almost all were wound infections; in 10 cases the same or a separate wound also grew <i>S. aureus</i> . Five additional patients from other areas of the UK were also noted. Surveys of streptococci from meat handlers in the UK, 1978–1986, found 287 group A, 9 group B, 5 group, 1 group E, and 13 group G.	107
1985	Germany	1	Group A	A butcher had repeated skin infections over a 4-month period.	117
1985	Oregon	32	Group A T14, M n.t.	There were 44 episodes of skin infection in 32/69 workers at a meat-packing plant. The men worked in boning or killing areas, and most had several skin lacerations. Epidemiological and typing evidence suggested spread of infection via the meat.	101
1996	North Devon, UK	46	Group A	Skin sepsis affected 46/194 workers in a meat plant over a 7-month period. Spread of infection from the plant to a retail butcher was documented.	104
1996	Canada	4	<i>S. iniae</i>	Bacteremic illnesses associated with preparing aquacultured fish (tilapia, species <i>Oreochromis</i> ). Three cases involved acute cellulitis from hand injuries; the fourth patient had septic arthritis, meningitis and endocarditis.	111
1999	UK	8	Group A M59	An indolent outbreak of skin infection occurred over a 3-month period among abattoir workers. This unusual strain had a T protein pattern (T5/27/44) not commonly associated with M59. DNA fingerprinting and <i>emm</i> gene type were done.	106

n.s., not stated.

time (62). Most affected workers have two or more hand lacerations, and most infections are seen in workers on the kill floor or cutting/boning rooms (100,101). Workers often shift job positions and share knives, whetstones, gloves, and sinks. Bone and bone fragments are the most common of many causes of initial minor injury (102). A variety of other skin infections have been reported, including paronychia in workers who bite their fingernails, infection in pierced ear lobes, and infected tattoos (62,103).

Although generally well tolerated by meat workers, who tend to regard skin infections as minor nuisances, secondary spread has been reported both from the infected workers and via the contaminated meat. Secondary cases have included the families of meat workers, retail butchers, restaurateurs, and housewives (62,103,104).

A few of the common group A streptococcal M types (M1, M49) and a host of new M types particularly associated with skin sepsis have been identified (101,105,106). Group L and occasional group B, C, E, and G streptococci have also been reported (107). In Hong Kong, Group C (*S. equi* subspecies *zoepidemicus*) infections have been associated with pigs and pork (108). Generally considered zoonoses, *Streptococcus suis* infections have been noted among pig farmers and pork handlers in Germany and the Netherlands (109,110).

*Streptococcus iniae* is a fish pathogen that has not previously been associated with human disease (111). The first cases occurred in Texas in 1991 and Ottawa in 1994, but potential sources were not identified. In 1996 four cases of bacteremic illness were reported in association with aquacultured tilapia fish. Three patients developed cellulitis within 2 days of injuring their hands while preparing fish purchased from a market. One patient presented with apparent septic arthritis 10 days after preparing a fresh tilapia; he developed meningitis and endocarditis, and recovered after treatment with antibiotics.

#### D. Prechewing of Food Fed to Infants

An unusual mode of foodborne infection is prechewing of food by adults before feeding to infants. Although this is not a recognized practice in North America or Europe, it may be the custom among some families or ethnic groups. Steinkuller et al. (118) reported three cases of group A streptococcal pharyngitis in infants  $\leq 1$  year of age whose parents routinely prechewed their food. Evidence for this mode of transmission was circumstantial, with one parent giving a history of recent sore throat, one with frequent "tonsillitis," and one mother whose throat culture was negative.

### V. ISOLATION AND IDENTIFICATION

Streptococci are spherical or ovoid facultatively anaerobic, gram-negative bacteria (1,119,120). They generally grow in chains and are nonmotile, non-spore-forming, and catalase-negative. Group A streptococci, as well as most of the group C and G species causing human disease form "large" ( $\geq 0.5$  mm)  $\beta$ -hemolytic colonies on blood agar. Colonies are 0.5–1.0 mm in diameter and quite firm, with a wide 1–3 mm diameter zone of clear hemolysis surrounding the colony. Colonies are opaque and off-white to grayish in color; they may be flat, dry, glossy, or matte; mucoid colonies have capsule material. Group C (*S. equi* subspecies *zoepidemicus*) are usually highly mucoid. The "small" colony streptococci, or "tiny betas," are usually members of the *S. anginosus-milleri* or *intermedius* group.

Bacteriological methods are now fairly standardized (1,119,120). An enriched medium, usually a trypticase-soy base with 5% sheep blood, is generally used in the United States, although some laboratories use horse blood, which is more sensitive to the oxygen-labile hemolysins. Plates are grown at 35–37° C in normal atmosphere. Cuts or "stabs" made in the agar at the time of inoculation enhance hemolysis, as does growth in anaerobic conditions. Cultures negative for typical colonies at 18–24 hours should be incubated and reexamined at 48 hours. Selective media, such as Columbia CNA (colistin-naladixic acid) with 5% sheep blood, are sometimes used in epidemiological applica-

tions but are not considered necessary for routine work. Swabs may be transported to the laboratory in Amies semi-solid or modified Stuart's transport media, which keep the organisms viable for at least 48 hours.

Presumptive identification of group A streptococci is usually determined by sensitivity to the "A" disc, containing 0.04 units of bacitracin. Some large-colony group C and G strains will also be sensitive and falsely identified as group A. Many laboratories now dispense with the bacitracin disc and instead do a 2-minute test for hydrolysis of pyrrolidonyl peptidase (PYRase), using paper discs or strips containing a chromogenic substrate (L-pyrrolidonyl- $\beta$ -naphthylamide). The test is positive for group A streptococci, enterococci, and some *Gamella* and aerococci (119). The streptococcal group may then be confirmed with a latex agglutination test. Grouping kits generally include reagents for detecting groups A–F and are available from various bacteriology supply companies. They compare well with the traditional immunoprecipitation tests using Lancefield's hot acid and other extraction procedures.

Serological typing is employed for research and epidemiological purposes on group A streptococci and on some group C and G isolates that have T or M antigens. The M proteins are the most important type determinants and are virulence factors that correlate with protection. T antigens are trypsin-sensitive surface proteins that have no relationship to virulence but are useful for classification. Certain T typing patterns are consistent markers for many of the important M types (121); many isolates can be assigned a T type even if the strain produces little M protein or if the strain cannot be typed with existing M antisera. Currently, there are 81 classically established M types (including 4 originally from group C and G strains) for which rabbit antisera have been made at reference laboratories. Several dozen strains isolated in recent years were unassigned because of lack of M antisera but are now being typed by molecular methods. Many strains can also be classified by opacity factor (OF), based on the observation that specific antibodies inhibit distinct streptococcal apoteinases from causing an increase in opacity of mammalian serum (122). OF types correlate well with associated M types and provide an adjunctive typing method.

A variety of molecular methods are now available for subspecies discrimination of streptococci (119). Restriction fragment length polymorphisms of the rRNA gene (ribotyping) has been applied to group A strains, including isolates from two foodborne epidemics in France (98). Chromosomal DNA fingerprinting was first applied to epidemiological studies of group C isolates by Skjold et al. (123). Similar methods have been used to study isolates from a milkborne outbreak of group C disease in Australia (54) and a foodborne outbreak at a college in the United States (85).

The molecular equivalent of M typing of group A streptococci is based on sequencing the 5'-variable region of the *emm* gene, which codes for the M protein (124). "emm typing" correlates exactly with the Lancefield's M types 1–51 and reference types 52–81, and it has enabled investigators to unequivocally assign *emm* types to 12 strains with provisional M types. Historical correlations with T types and OF types remain essentially unchanged, based on an analysis of over 3000 strains.

Several antibody tests are sometimes used to help document recent infection by group A streptococci. The most common of these are the antistreptolysin O (ASO) and the antideoxyribonuclease B (anti-DNase B), which become elevated 4–8 weeks after exposure (125). These are useful when it is necessary to confirm a clinical diagnosis but are not helpful in diagnosing acute suppurative streptococcal disease.

In addition to culture, rapid antigen-detection tests for onsite diagnosis group A streptococcal pharyngitis have been available for some years (34,119). Most tests are based on detection of cell wall antigens extracted from material on a throat swab. The specificity is usually excellent (>95%), and a positive result allows appropriate treatment to begin without having to wait 24–48 hours for conventional culture results. This permits patients to return to work or school a day or two earlier and helps avoid prescribing antibiotics unnecessarily. However, the sensitivity of the tests is usually <90%. Since a large portion of patients with false-negative tests are truly infected and not merely carriers, it is recommended that negative rapid tests be confirmed with conventional culture on blood agar. Some newer tests using an optical immunoassay (OIA) or a DNA probe have greatly improved

the sensitivity, but many experts remain unwilling to dispense with a back-up culture on negatives until more experience is gained under routine office conditions. Cultures are still recommended in any investigation of a foodborne outbreak, because strains of group C, G, or other species are not detected by the rapid antigen test and because intact live isolates may be required for further specific identification.

## VI. PATHOGENICITY

The pathogenicity of streptococci depends on a number of factors that interact in a complex fashion with the host (1,30,126,127). A list of the more important virulence factors, extracellular enzymes, and other products along with their functions is provided in [Table 8](#). Group C and G streptococci also elaborate their own versions of these products, with the exception of the pyrogenic exotoxins. The major virulence determinant for most (but not all) strains is the M protein, which protects the organism from being engulfed by the host's phagocytes. Also aiding the organisms is the C5a peptidase, which inhibits the chemotactic activity of complement. Several other components, including immunoglobulin-binding proteins, interfere with host defenses by interacting with antibodies or complement. The hyaluronic acid capsule produced by some strains is also antiphagocytic. M18 strains are almost entirely dependent on capsule expression for protection against phagocytosis.

Cell walls are necessary for the organism's integrity but also play a pathological role (30). The breakdown products of the peptidoglycan, especially the myramyl peptides, are powerful activators of the alternative complement pathway and of tumor necrosis factor (TNF) and cytokines, which cause much of the inflammation and detrimental effects in acute infection. The hemolysins streptolysin O and streptolysin S are cytotoxic, capable of killing phagocytes, and they may also be responsible for some secondary inflammation.

Fibronectin-binding protein is one of several factors that promote adherence, along with other proteins and lipoteichoic acids on the cell surface (1,30). The hyaluronidase is thought to facilitate the spread of infection within the tissues by breaking down basement membrane matrix. DNases may have a similar function and contribute to the formation of the thin watery pus often seen in streptococcal infections. Streptokinases are plasminogen converting factors, some of which may play a role in the renal damage leading to acute glomerulonephritis. A streptokinase from group C is also used therapeutically to dissolve clots in certain cases of acute thrombosis or empyema.

The pyrogenic exotoxins of group A streptococci, especially SPE A, B, and C, are responsible for the rash of scarlet fever. These "superantigens" induce lymphocyte blastogenesis, suppress antibody synthesis, potentiate endotoxic shock, and promote the febrile response (30,128). SPE A does not induce vomiting or gastrointestinal symptoms when fed to monkeys (129).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

The genetics of group A streptococcus are among the best studied of pathogenic organisms (141). Although its genome has been sequenced and many of its pathogenic features have been studied at the genetic level, investigators are only beginning to understand the complex interactions of the bacterium and its host environment and the factors that influence development of disease.

Extrachromosomal elements important in human disease are the lysogenic phages that infect certain streptococcus and code for the pyrogenic exotoxins (SPE) A and C (31,141). Strains expressing SPE A are generally more virulent and are more frequently associated with serious invasive disease. Other mobile elements include plasmids and transposons, mainly associated with resistance to erythromycin, aminoglycosides, tetracycline, and chloramphenicol. SPE B is encoded in the chromosomal DNA of a group A streptococcus, but it is unclear how the gene is regulated during virulent states.

The major virulence gene are located in a cluster called the *vir* regulon, which contains the *emm* genes coding for M and M-like proteins. There are several evolutionarily related families of

**TABLE 8** Antigens, Enzymes, and Virulence Factors of Group A Streptococci

	Comments	Ref.
M proteins	These cell surface proteins are major virulence factors, conferring resistance to phagocytosis and interfering with complement deposition. Type-specific antibody against a given M protein is associated with immunity. Over 100 different types of M proteins have been identified by serological and molecular ( <i>emm</i> gene) classification. Also produced by some group C and G strains.	31,130
T proteins	T agglutinins are surface proteins of unknown biological function that are associated with certain M types but are not themselves virulence factors. The T typing system of Griffith has nevertheless remained useful in classification, especially for M types for which antisera are unavailable.	121,131
Cell wall	The cell wall consists of multiple layers of cross-linked peptidoglycan to which are attached the group A carbohydrate, lipoteichoic acids, M and T proteins, etc. The peptidoglycan subunits (muramyl peptides) are potent activators of complement and cytokines.	132
Capsule	A large mucoid capsule of hyaluronic acid (structurally identical to mammalian hyaluronic acid) is elaborated by some strains, e.g., M18, for which it is a major virulence factor, protecting the bacteria from phagocytosis. Also elaborated by some group C strains.	133
Streptococcal pyrogenic exotoxins (SPE)	Antigenically distinct erythrogenic toxins responsible for the rash of scarlet fever, including SPE-A, B, and C. Antibodies to one type does not confer immunity to the others. They induce fever, act as superantigens, and play a role in streptococcal toxic shock syndrome by activating TNF and cytokines. SPE A does not induce vomiting or gastrointestinal symptoms; SPE B and C have not been evaluated in this regard.	30,128,129
Opacity factor (OF)	These antigenically distinct lipoprotein lipases of unknown biological significance are used as an adjunctive classification method that correlates with M typing mainly of skin strains. Also produced by some group C and G streptococci.	122,134
Streptolysin O and S	Streptolysin O is the oxygen-labile cytolysin responsible for the $\beta$ -hemolysis surrounding colonies on blood agar plates. It is antigenic and serves as the basis of the antistreptolysin O (ASO) test in serological diagnosis. Streptolysin S is a nonantigenic cell-bound enzyme that causes hemolysis under anaerobic conditions.	125–127
Deoxyribonucleases (DNases)	DNases are elaborated by group A, B, C, and G, serving uncertain pathogenic functions. Group A streptococci produce 4 enzymes, DNases A, B, C, D, of which DNase B is commonly used as an antigenic marker of infection.	125,127
Streptokinase	These antigenic proteins convert plasminogen to plasmin, which in turn lyses fibrin clots. A distinctive low molecular weight streptokinase has been associated with acute glomerulonephritis.	135



**TABLE 8** Continued

	Comments	Ref.
C5a peptidase	This enzyme inactivates C5a and reduces complement-derived chemoattractant activity. It also binds IgG. Intranasal immunization prevents colonization of mice.	136
Hyaluronidase	This extracellular enzyme is often referred to a “spreading factor” because of its assumed role in facilitating spread of infection in the tissues.	127
Nicotine-adenine dinucleotidase (NADase)	NADase, an antigenic extracellular protein of unknown function for the organism, used as a serological marker for previous throat infection. Also produced by group C and G streptococci.	127
Fibronectin-binding protein	This protein promotes adherence and interaction of bacteria with host components of skin and mucosal surfaces; not produced by some invasive types, e.g., M1, M3.	137,138
Immunoglobulin-binding proteins	Group A and other streptococci produce a family of surface proteins (M-like proteins) that bind IgG, IgM, and IgA and interfere with complement activation.	139,140

*emm* gene clusters. They are typically organized with a multiple gene activator (*mga*, a regulatory gene), one or more *emm* genes, sometimes additional elements, and *scpA*, the gene encoding C5a peptidase. In some strains, this cluster is followed by the gene for opacity factor (OF). The genes for streptolysin O, capsule production, immunoglobulin-binding proteins, and streptokinase are located away from the *vir* locus, each with its own promoter but still regulated according to environmental needs. In order to cause disease the organism has to protect itself while at the same time performing a number of different functions at different stages. Initially it must adhere to mucosal surfaces, and then may invade deeper tissue, produce toxins, and sometimes enter the bloodstream. Each of these, and no doubt other complex steps of the pathway, must be regulated in order for the organism to become a successful pathogen (141).

## VIII. CONTROL MEASURES

The developed world has been largely free of milkborne streptococcal infections for over half a century, and the important control measures have not changed. Proper dairy practices include the veterinary care of cows with mastitis and segregation of infected animals and avoiding contact with infected care takers. Careful attention to sanitary procedures and refrigeration during transportation and processing of milk is essential. Pasteurization has proved its usefulness over many years, although small local producers may avoid pasteurization because it is “unnatural” and does not appeal to some consumers in the “organic” or “health food” markets. Prompt identification and treatment of dairy employees with sore throat or skin infection is mandatory but must be coupled with benefit policies that do not discourage reporting of illnesses by employees. Cooperation of local physicians and public health authorities is also needed for prompt reporting and investigation of suspected outbreaks.

The U.S. Food and Drug Administration (FDA) publishes and regularly updates its *Milk Safety References*, consisting of recommended ordinances for state agencies and sanitation compliance and enforcement ratings of interstate milk shippers. In the U.K. milk standards are prescribed by the *Dairy Products (Hygiene) Regulations*. A wealth of information and practical guidelines are available from dairy organizations, such as the International Dairy Foods Association, the American

Dairy Science Association, the National Mastitis Council, the U.S. Department of Agriculture, and from state and local organizations.

Food handlers, cooks and caterers, and persons at home in their own kitchens, should all observe proper practices of hand washing, sanitation, and refrigeration, with special attention to complex foods such as those containing eggs, mayonnaise, sauces, and meat. The FDA publishes the *FDA Food Code*, a reference that guides retail outlets such as restaurants, grocery stores, and institutions, on how to prevent foodborne illness. Local, state, and federal regulators use the *FDA Food Code* to update their own food safety policies.

Appropriate diagnosis and treatment of food handlers with throat and skin infection is of obvious importance for the affected person's health as well as the health of others. For the most part this is done on a case-by-case basis. Antibiotic prophylaxis has been used during some outbreaks at military bases to prevent secondary infection (37,41,80), but there are presently no guidelines for this approach to prevention.

Skin sepsis can be largely controlled by safety and sanitary precautions (101). Meat packing plants should place special emphasis on worker safety, especially the use of protective devices, first aid and prompt treatment of injuries or infections, and association with a physician who is familiar with the plant's occupational hazards. Worker hygiene should include frequent hand washing, sterilization of shared equipment, disinfection of work surfaces in contact with meat, plastic or rubber covering of skin lacerations, and removal of workers with untreated skin lesions from meat handling activities. Also important are the maintenance of an injury and disease surveillance logs, sick leave benefits that do not discourage reporting of injuries and infections by workers, and reporting of infections to local health officials.

Experimental vaccines against group A streptococci are currently in the developmental stages (142). These are based on the N-terminal type-specific regions of M proteins, avoiding conserved epitopes of the protein that cross-react with human tissue components thought to play a role in development of acute rheumatic fever. Multivalent recombinant vaccines are envisioned that may protect against 12–16 of the most common M types. It is possible that protection against common infecting strains may also provide protection in the event of foodborne exposure.

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## ***Enterobacter sakazakii***

**M. Nazarowec-White**

*Canadian Food Inspection Agency, Ottawa, Ontario, Canada*

**J. M. Farber**

*Health Canada, Ottawa, Ontario, Canada*

**M. W. Reij**

*Wageningen University, Wageningen, The Netherlands*

**J.-L. Cordier**

*Nestlé Research Centre, Lausanne, Switzerland*

**M. van Schothorst**

*Wageningen University and Nestlé, Vevey, Switzerland*

### **I. BACKGROUND**

*Enterobacter sakazakii* is a member of the family Enterobacteriaceae, genus *Enterobacter*. In the past this organism was referred to as a “yellow-pigmented” *Enterobacter cloacae*. In 1980 *Enterobacter sakazakii* was designated as a unique species (1) based on differences from *E. cloacae* in DNA relatedness, pigment production, and biochemical reactions. This organism has been implicated in outbreaks causing either severe neonatal meningitis in premature babies or necrotizing enterocolitis. A mortality rate of 40–80% has been reported in the literature, and in many cases neonates die within days of birth. In surviving patients severe neurological sequelae such as hydrocephalus, quadriplegia, and retarded neural development can occur. Contaminated dry infant formulas have been identified as the source of *E. sakazakii* in some of the outbreaks and sporadic cases of meningitis. Data on its occurrence in the environment are still limited.

### **II. CHARACTERISTICS**

*E. sakazakii* has a biochemical profile very similar to that of *E. cloacae*, but it is always D-sorbitol negative, is positive for extracellular deoxyribonuclease, and forms yellow-pigmented colonies. The yellow pigment formation is stronger at 25 than at 36°C. All strains grow rapidly on tryptone soy agar and form colonies 1.5–3 mm in diameter after 1–2 days. Freshly isolated strains form typical smooth colonies. Dry mucoid colonies with scalloped edges and a rubbery consistency, which revert to typical smooth colonies on subculturing, have been described as well (2). In tryptone soy broth, strains of *E. sakazakii* produce a large amount of sediment, which appears to contain clumped cells and amorphous masses. Constitutive  $\alpha$ -glucosidase activity is demonstrated by all *E. sakazakii* strains, but not by any other *Enterobacter* strains (3). Postupa and Aldova (4) studied six *E. sakazakii* strains isolated from milk powder and dry infant formula and found that all produced Tween 80



esterase. The absence of the enzyme phosphoamidase is also very characteristic of *E. sakazakii* isolates (3). Strains of *E. sakazakii* isolated from dry infant formula on the Canadian market demonstrated minimum growth temperatures of 5.5–8.0°C, while at 4°C growth was inhibited and cells died off during storage at this temperature. Using these same isolates, average generation times in reconstituted formulas were 40 minutes at 23°C and 4.98 hours at 10°C. A study on the heat resistance of a pool of different *E. sakazakii* strains in reconstituted formulas indicated D-values of 54.8, 23.7, 10.3, 4.2, and 2.5 minute at 52, 54, 56, 58 and 60°C, respectively. The overall calculated z-value was 5.82°C (5).

Little is known about the presence of *E. sakazakii* in the environment. Farmer et al. (6) mention a variety of human and other sources, and the organism has been isolated from insects collected at a dairy farm. Muytjens and Kollee (7), however, could not isolate this organism from surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic animals, cattle, or raw cow's milk. Most of the *E. sakazakii* strains reported in the literature have been isolated from clinical sources, blood, or cerebrospinal fluid.

### III. DISEASES

*Enterobacter* spp. are frequently the cause of nosocomial diseases (8), with *E. sakazakii* among them (9). *E. sakazakii* is usually a rare opportunistic pathogen causing severe meningitis in neonates and premature babies. In some outbreaks it has been at the origin of neonatal necrotizing enterocolitis (NEC) (10), the most common gastrointestinal emergency in newborns. In a few cases the symptoms were limited to septicemia only.

The first case of meningitis was reported by Urmenyi and Frank (11). In most of the cases described, *E. sakazakii* overcomes the gastrointestinal barrier, gaining access to the blood stream and finally to the cerebrospinal fluid. Different severe symptoms developing at later stages of the disease such as ventriculitis, brain abscess, infarction, and cyst formation have been reported by several authors and summarized by Gallagher and Ball (12).

The mortality rate, as shown in Table 1, is usually high, and the severity of the symptoms can account for the remaining sequelae of babies surviving the illness. *E. sakazakii* seems to be more susceptible to commonly used antibacterial agents than other Enterobacteriaceae, and treatment with ampicillin/gentamicin is effective in most cases (28).

Several cases of bacteremia due to *E. sakazakii* have been described for older children or adults (29,30).

### IV. EPIDEMIOLOGY

Review of the literature related to *E. sakazakii* allows differentiation into two groups of cases: sporadic ones involving single patients or outbreaks affecting several patients. Available information has been compiled in Table 1. These data indicate an increase in the number of reported cases over the last 30 years. This tendency can probably be attributed to an increase in the number of sensitive consumers, in particular premature or ill babies in comparison to healthy neonates. Increased awareness of clinicians and medical laboratories is probably a further factor as well as that of consumers (parents).

The source of *E. sakazakii* has only been investigated in a few cases, and the origin of the opportunistic pathogen therefore remains unknown. Dry infant formulas have been implicated in some cases of meningitis and NEC (10,25,26). Quantitative studies on contaminated infant formulas have shown a prevalence of 1–12% and very low levels of 0.36–66 cfu/100 g in positive samples (2,31). Data on investigations in the bottled kitchen are scarce, but improper handling and the use of contaminated utensils such as mixers may have been contributing factors in some cases (25,26). When prepared bottles are stored overnight at room temperature, levels of  $>10^5$  cfu/mL are easily obtained (5).

**TABLE 1** Worldwide Reported Sporadic Cases or Outbreaks of Neonatal *E. sakazakii* Infection

Location	Number of cases (deaths)	Source implicated	Ref.
Sporadic cases			
Denmark	1(1)	Unknown	13
Georgia	1(0)	Unknown	14
Oklahoma	1(0)	Unknown	15
Indiana	1(0)	Unknown	16
Greece	1(0)	Unknown	17
Missouri	1(1)	Unknown	18
?	1(?) + 1(?)	Infant formula + blender	19
Maryland	1(0)	Unknown	20
North Carolina	1(?)	Unknown	21
Ohio	1(0)	Unknown	12
Outbreaks			
England	2(2)	Incubator	11
Netherlands	8(6)	Birth canal (not exclusively)	22
Greece	11(4)	Unknown	23
Massachusetts	2(1)	Unknown	24
Tennessee	4(0)	Dried infant formula	25
Iceland	3(1)	Dried infant formula	26
Canada	2(?)	Unknown	Gleeson, personal communication, 1991
Australia	3(1)	Unknown	27
Belgium	12(2)	Dried infant formula	10

## V. ISOLATION AND IDENTIFICATION

So far no standardized/validated or official method exists for the direct isolation of *E. sakazakii* from foods. *E. sakazakii* grows readily on enteric media, and most of the descriptions of detection are based on methods to isolate enteric pathogens from medical samples or coliforms/Enterobacteriaceae from foods, with subsequent identification of grown colonies. In studies performed to date, detection of the organism is performed either after an enrichment procedure or by direct plating on selective plates used to detect coliforms or enteric pathogens. Quantitative determinations are usually done using the most probable number (MPN) method. Presumptive colonies of coliforms or Enterobacteriaceae are purified and identified using biochemical tests such as the API 20E biochemical identification system (bioMérieux). Since constitutive  $\alpha$ -glucosidase activity is demonstrated for all *E. sakazakii* strains, but not for other *Enterobacter* strains (3), a quick screening method based on this characteristic is currently being developed and validated for milk powder and environmental samples (M. W. Reij and M. van Schothorst, personal communication).

A number of studies have been performed to implement more specific typing methods in addition to the biochemical identification of *E. sakazakii*. Nazarowec-White and Farber (32) found antibiograms to be of limited value in discriminating among strains of *E. sakazakii*. The 18 strains evaluated fell into only four patterns, thus confirming the results obtained by Clark et al (19). Furthermore, a high variability in antibiotic resistance was found among different colonies (clones) selected. A range of other biological and molecular techniques have been investigated and compared in different laboratories. Clark et al. (19) found ribotyping to be superior to restriction endonuclease analysis in the intraspecies identification of *E. sakazakii* strains. This was confirmed by Nazarowec-White and Farber (32), who classified 18 isolates into 10 ribotypes using the restriction endonuclease *EcoR*I. Comparison of the ribotypes of isolates from three factories manufacturing dry infant formu-

has enabled the identification of strains specific to each manufacturing site (33). Total DNA of strains isolated from a cluster of neonatal infections was digested with the restriction of enzyme XbaI and analyzed using pulsed-field gel electrophoresis (34). A relationship could be established between isolates from two patients and a strain isolated from a brush used for dishwashing in the milk preparation kitchen. Studies performed by Clark et al. (19) and Nazarowec-White and Farber (32) showed that the choice of the restriction enzyme was important when comparing isolates with molecular techniques such as ribotyping or pulse-field electrophoresis. The superior discriminatory power of genotypic typing schemes over phenotypic ones was further confirmed by Nazarowec-White and Farber (32) using random amplification of polymorphic DNA (RAPD).

## VI. PATHOGENICITY

There are no known published data describing any virulence factors or pathogenicity of *E. sakazakii*. However, 18 isolates of *E. sakazakii*, both from clinical samples and from dry infant formulas, were tested for enterotoxin production using the suckling mouse assay (M Nazarowec-White, unpublished data). In addition, suckling mice were challenged both orally and by intraperitoneal injection with these isolates. Out of the 18 strains evaluated, four clinical isolates and only one food isolate were positive for enterotoxin production and only two strains caused death after peroral administration. The clinical strains originated from three hospitals situated in very different regions in Canada. It is also interesting to note that out of three strains isolated from different lots of infant formulas, two did not form enterotoxins. This finding suggests that there are differences in virulence among the various isolates, which may in part be related to the organisms ability to survive the acidic conditions present in the stomach. If one extrapolates from the suckling mouse model, it would appear that the minimum infectious dose for infants would be fairly high, and therefore, some abuse allowing for growth to high levels in the reconstituted formulas would have to occur to cause infection in infants. Nazarowec-White and Farber (2) have shown that even very low initial counts of 1 CFU/mL in bottled infant formula would lead to levels of  $10^7$  per serving of 100 mL in bottles stored at room temperature for 10 hours. Potentially hazardous levels of *E. sakazakii* would be reached even sooner in formula held at 35–37°C. Further studies on the putative virulence factors of *E. sakazakii* are warranted.

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

The authors are not aware of any studies performed to elucidate the genetic determinants of virulence in *E. sakazakii*.

## VIII. CONTROL MEASURES—DRY INFANT FORMULA

Babies, and in particular prematures and neonates, belong to the most susceptible category of consumers, and all measures must be taken to avoid exposure and infection through reconstituted formulas. Dry infant formulas are not intended to be sterile, but levels of microorganisms must be kept as low as possible. While the target remains the absence of coliforms in 1 g of product, the current microbiological criterion according to the Codex Alimentarius considers the presence of low levels of coliforms (to which *E. sakazakii* belongs) acceptable, i.e., one out of five samples may contain up to 20 per gram. Stringent criteria have also been established for salmonellae (absence in 60 × 25 g) and for total viable counts which are used as a hygiene indicator (out of five samples, three must have  $<10^3$  cfu/g, the other two  $<10^4$  cfu/g).

The production of dry infant formulas therefore, requires particular attention and adherence to strict hygienic conditions. Heat treatments of the milk and other ingredients (as far as technically possible), such as pasteurization or even sterilization applied during processing, destroy all vegeta-

tive pathogens, including *E. sakazaki* (5,35). The potential for postprocess recontamination with low levels of *E. sakazaki* exists, and control over the processing environment is therefore of critical importance for the microbiological quality of the final packed product. Dust carried by air and aerosols produced through condensation or cleaning, etc., in the environment of drying and filling areas are the main sources of contamination for dried products (36). Investigations of the ecology of processing environments in factories have revealed that *E. sakazaki* can, with varying frequencies, be isolated from dust samples, indicating that it survives very well in warm and dry zones (Reij, Cordier, and van Schothorst, unpublished results).

In order to prevent pathogens from gaining access to the processing environment and finally to the manufactured product, many control measures are taken. For example, the air entering such areas should be filtered, and the rooms around the (spray-)dryer, transport, and filling should be kept at overpressure. In addition, the movement of personnel needs to be restricted, and cleaning procedures must be kept under control. Proper layout, including adequate separation of the various processing areas to protect the product as well as monitoring the efficacy of these control measures, are also of great importance (37). Despite the preventive measures available, the production of dry infant formulas without the sporadic presence of low levels of *E. sakazaki* seems difficult to achieve.

Control measures cannot only be restricted to the manufacturing process. They also have to be extended to the critical steps of preparation, handling, and use of the bottles with the reconstituted liquid formulas (38). The use of boiled water for reconstitution and of sterilized bottles and teats are part of the recommendations. Using hot (boiling) water for reconstitution assures the killing of low levels of Enterobacteriaceae, if present. This is important, because *E. sakazaki* is not the only Enterobacteriaceae species of clinical significance (6). *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Klebsiella pneumoniae*, and others have also been involved in outbreaks and have also been isolated from infant formulas (10,31,39).

Formulas containing living microorganisms added on purpose, such as probiotic bacteria, should of course not be prepared with hot water. Low levels of Enterobacteriaceae would, however, most probably not cause problems in this type of product. In any case, bottles should be used directly when lukewarm and not kept for prolonged periods in bottle warmers (26). The World Health Organization does not recommend storage of foods for infants and young children (40). However, when it is practiced, cooling should be rapid and bottles should be kept refrigerated (also for limited periods of time) in order to prevent multiplication of microorganisms such as spore formers that are still present. Although the use of in-pack sterilized liquid formulas is considered expensive and impractical for hospitals by some authors (10), this would represent, just as the reconstitution in boiling water, a safe manner to provide feeding for the most vulnerable group of neonates, in particular premature infants.

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## ***Mycobacterium avium* subsp. *paratuberculosis***

**Michael T. Collins**

*University of Wisconsin–Madison,  
Madison, Wisconsin, U.S.A.*

### **I. BACKGROUND**

*Mycobacterium avium* subsp. *paratuberculosis* (also known as *Mycobacterium paratuberculosis*) causes Johne's disease in ruminant animals. This agent may or may not belong in a book on food safety. Although mounting evidence suggests that this organism may be a human pathogen, medical science has not classified this bacterium as a zoonotic agent. Unless *M. paratuberculosis* infects and causes disease in humans, it is not a microbial risk in foods. Critical analysis of the zoonotic potential of *M. paratuberculosis* is outside the scope of this chapter, but interested readers are referred to several authoritative reviews on the subject (1–8). It should be noted, however, that the Food Standards Agency of the U.K., in its draft plan, has elected to exercise the precautionary principle by invoking controls on *M. paratuberculosis* at multiple levels of the food chain (<http://www.foodstandards.gov.uk/consultations/map.htm>).

### **II. CHARACTERISTICS**

*M. paratuberculosis* is a gram-positive, acid-fast staining, small rod-shaped bacterium (9–11). As with all members of the Mycobacteriaceae, it has a unique cell wall structure rich in complex lipids. The thick and chemically distinctive cell wall of mycobacteria are responsible in large measure for the tenacity of this bacteria, both inside host cells and in the environment.

The pathogenic potential of mycobacteria is closely correlated with their growth rate. Paradoxically, slow-growing mycobacteria such as *Mycobacterium tuberculosis* (the cause of tuberculosis in humans) and *Mycobacterium bovis* (the cause of tuberculosis in cattle) are more virulent than rapid-growing mycobacteria typically found in soil such as *Mycobacterium phlei*. With the possible exception of *Mycobacterium leprae* (the cause of leprosy in humans), which cannot be cultured in vitro, *M. paratuberculosis* has the slowest growth rate of the slow-growing, pathogenic mycobacteria. On primary isolation from infected animals, colonies of *M. paratuberculosis* are typically not visible for 3 months. The generation time of this organism under optimal growth conditions in vitro has been roughly estimated at 2 days as compared to 20 minutes for *E. coli* (12).

### **III. DISEASES**

Infection of ruminant animals with *M. paratuberculosis* leads to a chronic wasting (low body weight also known as cachexia) disease known as Johne's disease or paratuberculosis (13). In cattle, diarrhea is also a characteristic clinical sign of Johne's disease, while in other ruminants like sheep and goats it is not (9,14). The rapid weight loss seen in Johne's disease is due in part to thickening of the

intestinal tract caused by the host's immune response to *M. paratuberculosis* infection leading to a protein-losing enteropathy. It is likely also due to the systemic action of intercellular chemical communication molecules known as cytokines, such as tumor necrosis factor (TNF; formerly called cachectin), released by host lymphocytes and macrophages as they try to cope with invasion of *M. paratuberculosis*. Evidence for this is that cachexia is a clinical sign common of tuberculosis in humans even though *M. tuberculosis* infections largely involve the lungs, not the intestinal tract.

Infected animals excrete the largest numbers of *M. paratuberculosis* in feces. Excretion of the organism also occurs at much lower levels in colostrum and milk, and this occurs well before onset of clinical signs (15).

While the primary site of infection and pathology is the intestinal tract, specifically the terminal sections of the small intestine known as the ileum, in the latter stages the infection becomes systemic and *M. paratuberculosis* can be isolated from internal organs such as the liver and lymph nodes distant to the gastrointestinal tract. Infection dissemination has potentially important implications if *M. paratuberculosis* is a foodborne zoonotic agent.

#### IV. EPIDEMIOLOGY

Transmission of *M. paratuberculosis* infections is largely by the fecal-oral route. Consumption of contaminated milk, whether by natural nursing of an infected dam or by artificial feeding, is a major mode of infection transmission (16). However, any means by which feed or water can become contaminated with feces of infected adult animals is a potential mode of *M. paratuberculosis* transmission.

There appears to be an age-dependent increase in resistance to *M. paratuberculosis* infection in cattle (17). Cattle less than 6 months of age are considered most susceptible. However, while this is a commonly held belief with some support in the scientific literature, the actual infectious dose by age of animal has not been documented. Experimentally it has been shown that adult animals, if given a sufficient dose of *M. paratuberculosis*, can be infected. Therefore, resistance to infection is never complete at any age. Whether age-dependent resistance to *M. paratuberculosis* infection also occurs in animals other than cattle is purely speculative.

Following ingestion, the organism localizes in the terminal portion of the small intestine known as the ileum. During the protracted incubation period of 2–5 years in dairy cattle—shorter in other ruminants—an inflammatory response dominated by macrophages and T lymphocytes causes thickening of the intestinal wall, although the animals remain clinically normal. The clinical signs of Johne's disease, diarrhea and weight loss, start suddenly and seem to be precipitated by calving.

During the incubation, preclinical, phase of infection, *M. paratuberculosis* shedding in feces and milk steadily rises in frequency and quantity. By the onset of clinical Johne's disease, the organism has disseminated from the wall of the ileum to the regional gut-associated lymph nodes and then to the major internal organs such as liver and spleen and other lymph nodes in the body via the blood stream.

Surveys of most of the major dairy-producing countries indicate that 20–60% of herds are infected with *M. paratuberculosis* (18). Far fewer surveys have measured the prevalence of paratuberculosis in beef cattle, sheep, or goats, although the disease is commonly seen in these species.

#### V. ISOLATION AND IDENTIFICATION

*M. paratuberculosis* will grow on most of the culture media used for growth of mycobacteria provided such media are supplemented with an iron transport molecule produced by all other mycobacteria known as mycobactin. Mycobactin is available commercially: in the United States the sole supplier is Allied Monitor (Fayette, MO), and in Europe, BioMerieux sells mycobactin. Herrold's egg yolk agar is used in veterinary diagnostic laboratories in many countries, while modified Lo-



wenstein-Jensen medium is favored in The Netherlands and Denmark. Chemically defined media such as the various Middlebrook formulations are often not capable of supporting *M. paratuberculosis* growth unless enriched with egg yolk (19). Media used in automated liquid culture-based detection systems such as the BACTEC and Trek ESP II systems also require enrichment with egg yolk and supplementation with mycobactin in order to support *M. paratuberculosis* growth from clinical specimens (20,21).

After primary isolation, *M. paratuberculosis* will adapt to in vitro growth on less enriched media, and Middlebrook formulations and Watson-Reid are two of the more commonly used liquid media for growth of large numbers of the organism. Both require addition of mycobactin to support *M. paratuberculosis* growth.

Isolation of a slow-growing acid-fast bacterium from animals with clinical signs compatible with Johne's disease is presumptive evidence for detection of *M. paratuberculosis*. Definitive identification of *M. paratuberculosis* can be based on the phenotypic characteristic of mycobactin-dependency for in vitro growth or genetically by demonstration of an insertion sequence (IS) known as IS900 (22–24). Often, veterinary diagnostic laboratories will test for both mycobactin dependency and IS900.

Mycobactin dependency is evaluated simply by subculturing from primary isolation media to media with and without mycobactin. Growth on the former and not the latter indicates that the organism is dependent on mycobactin for in vitro growth and therefore is *M. paratuberculosis*. Clinical laboratories often expedite this step by simply inoculating media with and without mycobactin directly from clinical specimens. Caution must be exercised in performing this test by subculture to use only a small inoculum lest mycobactin carryover occur and growth be stimulated on the non-mycobactin-containing medium. Even with care, a small amount of *M. paratuberculosis* growth may be seen on the nonmycobactin medium, but if the organism is *M. paratuberculosis* the difference in quantity of growth between the two media should be substantial and obvious.

IS900 has heretofore only been found in *M. paratuberculosis*. Some related sequences have been shown to cause false-positive tests for IS900, but these are considered fairly rare events (25). Still, for maximum confidence in a PCR test for IS900 it is advisable to visualize and confirm the size the PCR product by electrophoresis. Multiplex reactions for the four or five most commonly isolated mycobacteria from animals shows promise as a fast, simple, genetically based method for identification of multiple mycobacterial species in a single assay.

There are no unique biochemical tests, nor is the mycolic acid HPLC chromatogram unique, making the more standard methods for mycobacterial identification useless (26). The commercial genetic probe kit for identification of *M. avium*, based on species-specific 16s rRNA sequence targets (GenProbe), reacts strongly with *M. paratuberculosis*, verifying the close genetic homology of these two organisms but negating use of this kit for distinguishing between these taxonomically closely related bacteria.

## VI. PATHOGENICITY

Improved diagnostics have led to expanded search for animals that are susceptible to *M. paratuberculosis*. The list of *M. paratuberculosis*-susceptible species continues to grow (27). In broad terms, all ruminants are likely susceptible, as are the pseudo-ruminants of the family Camelidae. Rabbits were recently shown to become naturally infected when resident on farms with infected cattle, and the predators of those rabbits too were shown to develop paratuberculosis (28,29). *M. paratuberculosis* has been isolated from nonhuman primates with pathology and clinical disease resembling Johne's disease (30,31). *M. paratuberculosis* has also been recovered from a small percentage of people with Crohn's disease and detected by IS900 PCR from an even larger subset of Crohn's disease patients (32–39).

Regardless of animal host, *M. paratuberculosis* has a strong propensity to infect the ileum. However, lesions and organism recovery from higher in the small intestine are common, particularly in animals other than cattle (40).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

There are no proven genetic markers of virulence in *M. paratuberculosis*. Because of the very high level of genetic homology of *M. paratuberculosis* with *M. avium*, the fact that *M. paratuberculosis* is much more virulent than *M. avium*, and the finding that IS900 is only found in *M. paratuberculosis*, it is tempting to label IS900 as a genetic marker of virulence. However, the only function ascribed to IS900 is that of a transposase enzyme, making a direct link to virulence unlikely (24,41,42). Some speculate that insertion of IS900 perturbs expression of *M. paratuberculosis* genes and that this then results in the virulent phenotype of an otherwise avirulent organism. The full genetic sequence of *M. avium* has been completed, and the full sequence of *M. paratuberculosis* is soon to be completed (<http://www.cbc.med.umn.edu/ResearchProjects/AGAC/Mptb/Mptbhome.html>). This information will significantly enhance our understanding of the genetic basis of virulence of these two similar organisms.

## VIII. CONTROL MEASURES

Control of paratuberculosis on farms is based on two fundamental strategies: (a) detect the infected adult animals and remove them from the herd, and (b) rear replacement animals under optimal hygiene conditions to limit the chances of becoming *M. paratuberculosis* infected. While diagnostic techniques for detection of subclinically infected animals are not 100%, they are sufficiently sensitive to aid significantly in on-farm paratuberculosis control programs. Because *M. paratuberculosis* cannot replicate outside the host animal, limiting exposure of susceptible neonates to the organism using common-sense hygiene is very practical and effective.

Control of paratuberculosis on a regional or national basis requires a program to identify and certify those herds that are not infected. Such herds represent a valuable resource for replacement animals to herds that are controlling paratuberculosis using a test-and-cull program. Formal programs are well developed in Australia, The Netherlands, and the United States (43–46).

## IX. FOOD SAFETY CONSIDERATIONS

The pathobiology of *M. paratuberculosis* in animals results in contamination of milk and meat. Milk can become contaminated both by direct excretion of the organism from the udder and by fecal contamination of milk at collection. For this reason dairy products have come under closest scrutiny for the potential of *M. paratuberculosis* contamination in finished products.

In the latter stages of infection the organism hematogenously disseminates from the primary site of infection, the ileum, to all other internal organs and lymph nodes. For this reason, although *M. paratuberculosis* has not been definitively classified as a zoonotic agent, investigators recently have been evaluating the ability of *M. paratuberculosis* to survive in ground beef.

Laboratory studies of the thermal resistance characteristics of *M. paratuberculosis* indicate that it is more heat-resistant than other bacterial pathogens, including other mycobacteria. Meta-analysis of four studies reporting D-value measurements for *M. paratuberculosis* suggest a  $D_{72C}$ -value of 7.9 seconds and a z-value of 7.43°C, which are substantially higher than those of *E. coli* O157:H7 (47), or *Mycobacterium bovis* (48), for example.

Studies of pasteurization using laboratory-scale pasteurizers are conflicting. Stabel and Pearce report that high-temperature/short-time (HTST) pasteurization is sufficient to kill *M. paratuberculosis* (49,51). Studies in the U.K. by Grant et al. and in Germany by Hammer et al. found survival

of *M. paratuberculosis* under the same conditions (52,53). Design of the pasteurizers used, treatment of *M. paratuberculosis* cultures used to spike milk, and methods of *M. paratuberculosis* recovery after pasteurization were different in all four studies, making direct comparison difficult. Klijn et al. provide an excellent discussion of the many factors affecting the results of pasteurization trials and emphasize the role of bacterial cell clumping, a notable characteristic of *M. paratuberculosis* (54). Only one study reported HTST pasteurization of naturally infected cow's milk using commercial-scale pasteurization equipment (55). That study found *M. paratuberculosis* survivors.

Survey of retail milk for *M. paratuberculosis* was first reported in 1996. The organism was detected by PCR, but cultures of the same milk samples could not definitively confirm recovery of *M. paratuberculosis* (56). A much larger and more comprehensive survey was conducted in 2000. This study found that 1.7% of retail milk samples in the U.K. contained viable *M. paratuberculosis* (57).

Two studies of *M. paratuberculosis* survival in cheese have been published with similar findings (58,59). Heat treatment of raw milk before making cheese is important in the reduction of viable *M. paratuberculosis*. Low pH, moisture content, and holding (ripening) time were all found to be significant in causing further reduction of viable *M. paratuberculosis* counts. Salt, by contrast, has no measurable effect on *M. paratuberculosis* viability. These findings suggest that high-moisture cheeses made from raw (not heat-treated) milk are more likely to harbor *M. paratuberculosis*.

The recently reported recovery of *M. paratuberculosis* from deep lymph nodes of thin cattle at slaughter has stimulated interest in the organism's response to cooking temperatures when suspended in ground beef (60). As of January 1, 2002, no such studies have been reported.

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## Major Fungal Toxins of Regulatory Concern

**Garnett E. Wood, Mary W. Trucksess, and Sara H. Henry**

*U.S. Food and Drug Administration, College Park, Maryland, U.S.A.*

### I. INTRODUCTION

Agricultural crops growing in the field are subject to invasion by many fungal species present in the soil. Under optimal conditions of temperature and moisture, some of these fungal species may infect growing plants and produce secondary toxic metabolites; these metabolites are referred to as mycotoxins. As plants mature and grain kernels develop, some fungal species may continue to proliferate and produce toxic metabolites that persist and are not eliminated or degraded during harvesting operations. If harvested grains (crops) are not stored properly, it is possible that some fungi brought in from the field on the crops may either continue to grow and produce mycotoxins, or may gradually be replaced by fungi that can proliferate under various storage conditions and produce additional mycotoxins (1). Mycotoxins can therefore enter the food supply during preharvest and postharvest periods. Small amounts of these substances may be legally permitted in foods and feeds, provided the amounts involved are not considered to be injurious to human and animal health.

The U.S. Food and Drug Administration (FDA) is the federal agency responsible for protecting the public health of consumers and ensuring the safety of food. The Agency is responsible for enforcing the Federal Food, Drug, and Cosmetic Act (FFD&C Act) (2). This statute and its amendments serve as the legal basis for regulating poisonous and deleterious substances in foods, which allows FDA to prohibit the entry of adulterated food into interstate commerce. The strategies used by FDA in enforcing the FFD&C Act include providing guidance to the food industry, establishing regulatory standards, monitoring the marketplace to ensure compliance with established guidance and food laws, taking precautionary steps to prevent situations that might expose the public to food hazards, cooperating with state and international governments, and interacting with other federal agencies in the area of food safety. The U.S. food supply is monitored by FDA through compliance programs in order to obtain information on the incidence and levels of selected mycotoxins in various commodities. The procedures used for implementing these programs have been published elsewhere (3,4).

FDA's involvement with mycotoxins began in the early 1960s after the discovery that aflatoxin, a potent hepatocarcinogen in rats, was present as an occasional contaminant in peanuts, peanut products, corn, and other commodities used for human consumption in the United States. The occurrence of these toxins on grains and other commodities is not entirely avoidable and is influenced by certain environmental factors. The extent of mycotoxin contamination of a particular commodity is unpredictable and may vary with geographic location, agricultural practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods. The worldwide occurrence of mycotoxins in foods and feeds has been recognized by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) for many years (5).

Analytical methods for mycotoxins in grains and grain products as well as animal tissues are needed for monitoring domestic and import surveillance programs, controlling quality of products, establishing regulatory standards and guidelines, validating decontamination procedures, and prepar-

ing standard materials for use in toxicological studies. These methods in general can be classified as chemical and immunochemical. Chemical methods for extracting mycotoxins from foods and feeds involves the use of various mixtures of water and organic solvents such as chloroform, methanol, acetonitrile, and acetone. Common approaches for clean-up procedures include solid phase extraction (SPE), liquid-liquid partition, dilution with water, and precipitation. Chromatographic techniques such as thin layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC), LC/mass spectrometry (MS), GC/MS, capillary electrophoresis, supercritical fluid chromatography, and other novel techniques are used for separation and quantitation. Chemical derivatization, LC/MS/MS, or GC/MS/MS is used to confirm the identity of the separated toxins.

Antibody-based immunochemical methods include the enzyme-linked immunosorbent assays (ELISA), immunoaffinity column assays, and immunosensors. These methods are rapid, simple, specific, sensitive, and portable; polyclonal and monoclonal antibodies are utilized as the binding agents for the toxins. All official samples of commodities collected by FDA for mycotoxin evaluation are analyzed by collaboratively studied methods described in Official Methods of Analysis of the Association of Official Analytical Chemists, International (AOAC Int.) (6).

The objective of this chapter is to give an overview of the major mycotoxins that are of significant public health concern at the national and international levels. The mycotoxins that are of current regulatory concern include the aflatoxins, ochratoxin A, deoxynivalenol, fumonisins, patulin, and zearalenone. The occurrence, toxicity, analytical methodology, and regulatory control measures associated with these toxins will be presented under the heading of the appropriate fungal genera associated with these toxins.

## II. TOXINS PRODUCED BY *ASPERGILLUS* SPECIES

Species of the genus *Aspergillus* are saprophytes and are widely distributed in nature. They are commonly found in soils in subtropical and tropical areas, also in plant material undergoing microbial decomposition, as well as in improperly stored oilseeds and grains, and on crops in the field under various climatic conditions. The best known of the *Aspergillus*-derived toxins are the aflatoxins. Ochratoxin A is also produced by certain species of *Aspergillus* in tropical and subtropical regions of the world. A comprehensive review of *Aspergillus* mycotoxins recently appeared in the literature (7).

### A. The Aflatoxins

The aflatoxins are a group of structurally related toxic compounds produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. Under favorable conditions of temperature and humidity, some strains of these fungi can grow on certain crops in the field and during storage, resulting in the production of aflatoxins. There are approximately 18 aflatoxins; the naturally occurring ones are aflatoxins (AF) B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, while the other 14 are metabolic products of animal systems (7). The naturally occurring aflatoxins are usually found together on various commodities in varying proportions; AFB<sub>1</sub> is normally predominant in amount and toxicity. *A. flavus* is the more common contaminant of corn in the temperate regions of the world, and it produces AFB<sub>1</sub> and AFB<sub>2</sub>. *A. parasiticus* is the most common contaminant of peanuts in tropical and subtropical regions, and it produces all 4 of the naturally occurring toxins (8). Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) is a toxic metabolite of AFB<sub>1</sub>, and it is produced in the liver of mammals. The aflatoxins are relatively stable compounds, therefore varying levels may be found in finished food products depending on the processing techniques or procedures used. The effects of various processing procedures on the reduction of aflatoxin levels in foods have been reviewed (8,9).

#### 1. Occurrence

The worldwide occurrence of the aflatoxins in foods and feeds is well documented in the literature, with the major contamination occurring in areas of high moisture and temperature (10). The



aflatoxins (except AFM<sub>1</sub>) have been found occurring naturally in corn and corn products, peanuts and peanut products, cottonseed, various tree nuts (e.g., Brazil, pistachio, almonds, pecans, walnuts), copra and its products, edible seeds (e.g., watermelon, sunflower), wheat, sorghum, spices, rice, unrefined vegetable oils, figs, and dried fruits. They have been found sporadically in other commodities at comparatively low or insignificant levels.

AFM<sub>1</sub> is produced in the liver of animals and humans that have ingested aflatoxin-contaminated commodities; it is usually excreted in the urine and milk of dairy cattle and other lactating mammals. The occurrence of AFM<sub>1</sub> in milk is transitory in nature, usually reaching a peak within 2 days after the ingestion of the contaminated commodity and disappearing within 4–5 days after the withdrawal of the contaminated source. Studies in the United States have shown that the ratio of AFB<sub>1</sub> in animal feed to AFM<sub>1</sub> in the milk averaged 75 to 1 (11).

## 2. Toxicology

A vast amount of literature has been published on the toxic effects of the aflatoxins in laboratory animals, livestock, and humans. Effects of aflatoxin exposure have been studied mostly in animal models (12). Aflatoxin B<sub>1</sub> is the most toxic and carcinogenic form of the naturally occurring aflatoxins; acute toxicity of the aflatoxins correlates generally to the susceptibility of species to liver cancer. LD<sub>50</sub> values within a given animal species vary by strain, sex, age, route of administration, nutritional status, and composition of the diet. In general, rats are more sensitive than mice, and males are more susceptible to both acute and carcinogenic effects than females. AFM<sub>1</sub>, when studied in laboratory animals, is about an order of magnitude less carcinogenic than AFB<sub>1</sub>.

Large doses of AFB<sub>1</sub> lead to liver failure and death in animals, while smaller doses lead to cell death and regeneration. Chronic exposure to small doses produces liver cancer. Long-term administration of AFB<sub>1</sub> to rodents induces metabolic enzymes that are involved in the biotransformation of aflatoxin, thus the metabolic fate of AFB<sub>1</sub> in an animal never exposed may be different from that in an animal that has been chronically exposed to the toxin.

In humans, the highest aflatoxin exposures are believed to occur in geographical areas where health statistics may not be well developed, and exposure to aflatoxin is not likely to be measured accurately. Immunosuppressive effects of aflatoxins have not been examined thoroughly in humans, but are known to occur in laboratory animals and livestock. These effects may help to explain the marked geographical variations seen in responses to some vaccines, as well as the high rates of infection seen in children in developing countries that have high levels of exposure to aflatoxin (13).

Aflatoxin has been implicated in several outbreaks of acute hepatitis in humans, e.g., in India and Kenya. Although causality was not clearly established, the known measurable high levels of aflatoxins in foods and the liver histology are highly persuasive (12).

Marasmus and kwashiorkor (protein-energy malnutrition disorders) affect millions of children worldwide. These diseases are found in tropical countries where corn, cassava, and peanut are staples and where aflatoxin contamination levels are likely to be high. Hence, some investigators have claimed an association between aflatoxins and marasmus and kwashiorkor. However, the epidemiological data are far from complete, relying mostly on anecdotal information, a small number of cases, and an unclear state of the relationship between levels of aflatoxin found in body tissues and the disease. More studies are needed before firm conclusions regarding these relationships may be drawn (12).

Aflatoxins have been associated with the development of human liver cancer. Liver cancer is most prevalent in some of the developing countries of the world, such as China, Southeast Asia, and sub-Saharan Africa, where levels of aflatoxins consumed may often be high. The complex etiology of liver cancer is now largely understood from the results of many laboratory and epidemiological studies. Viral infections of hepatitis B (HBV) or C virus (HCV) are associated with 65–100% of liver cancer cases studied. In countries with a low risk for liver cancer, HCV predominates; other relevant factors include the use of alcohol, tobacco, and oral contraceptives. In high-risk areas, HBV predominates and aflatoxin exposure plays a role, but quantification of this role has been

difficult. The evidence supports a synergistic interaction between HBV and/or HCV and aflatoxins in the etiology of liver cancer. Aflatoxin may not be a completely independent liver carcinogen in humans, despite its carcinogenic potency in sensitive animal species (12).

Strong support for the role of HBV in liver cancer in an area where aflatoxin exposure is relatively high and the rate of liver cancer is high comes from data on the immunization program against hepatitis B in Taiwan. The immunization program in infants and children reduced the rate of HBV carriage (as measured by hepatitis B surface antigen positivity) from about 10% to about 0.9% between 1981 and 1994. The reduction in the prevalence of hepatitis B surface antigen was accompanied by a decline in the average annual incidence of liver cancer in children 6–14 years of age, from about 0.7 per 100,000 (1981–86) to about 0.4 per 100,000 (1990–94). Thus, liver cancer may be virtually eliminated from a population simply by vaccinating for HBV without concurrent elimination of aflatoxin exposure (12,14).

### 3. Analytical Methodology

The aflatoxins have a strong ultraviolet (UV) absorbance with a maximum range of 350–365 nm when measured in methanol, acetonitrile, toluene-acetonitrile, or benzene-acetonitrile (15). The aflatoxins AFB<sub>1</sub>, AFB<sub>2</sub>, and AFM<sub>1</sub> exhibit a blue fluorescence, whereas AFG<sub>1</sub> and AFG<sub>2</sub> emit a blue-green fluorescence when exposed to long-wave UV light. In all TLC methods and most of the LC methods used for aflatoxin separation and quantitation, the toxin concentration is based on the intensity of the fluorescence observed. Silica gel G60 is the most common precoated TLC plate used for aflatoxin analysis. Two-dimensional TLC is a powerful separation technique that can be used for analyzing aflatoxins in many different matrices. The limit of detection is in the low picogram range using the modern instrumental high-performance TLC (HPTLC) technique (16). Trifluoroacetic acid (TFA) is the most common reagent used in preparing the chemical derivatives of the aflatoxins on a TLC plate. The difference between the resolution factors of the derivatives and the parent compounds confirm the identity of the toxins.

When LC methods are used, the fluorescence of AFB<sub>1</sub> and AFG<sub>1</sub> is quenched by the mobile phase used for reversed-phase LC, therefore it is necessary to prepare derivatives of the two toxins to overcome this quenching. Quenching problems can be eliminated to a large extent by precolumn derivatization with TFA/acetic acid/water (17), or postcolumn derivatization using iodine (18), bromine (19), or cyclodextrins (20). The difference in retention times of two chemical derivatives of the same toxin confirms the identity of the toxins. Other approaches such as thermospray MS, LC/MS, and MS/MS are also used for confirmation of identity of the aflatoxins.

Many commercial ELISA kits and immunoaffinity columns are available for the determination of aflatoxins. AOAC International and the International Union for Pure and Applied Chemistry (IUPAC) have sponsored many collaborative studies to evaluate some of the published methods. [Table 1](#) shows recently developed methods as well as collaboratively studied methods (21–23).

### 4. Regulatory Control

In 1965, FDA established an informal action level of 30 µg/kg total aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>) in peanuts and peanut products; this was a guideline for regulatory purposes (24). Action levels are used as a guide for FDA field staff to determine when it may be necessary to take enforcement action against a product or an establishment. In 1969, the action level was reduced to 20 µg/kg and was applied to all commodities (foods and feeds) susceptible to aflatoxin contamination. An action level of 0.5 µg/kg was established for aflatoxin M<sub>1</sub> in fluid milk products in 1977 based on studies on the metabolic conversion of B<sub>1</sub> in the feed of dairy cattle to aflatoxin M<sub>1</sub> in the milk (25). In recognition of the adverse economic consequences the 20 µg/kg action level may have during years when aflatoxin contamination is unusually high, FDA established higher limits (up to 300 ng/g) for certain contaminated products (corn, cottonseed meal, peanuts products) used as feed and/or feed ingredients for specific species of animals. The current action levels for aflatoxins in the United States are shown in [Table 2](#). The levels represent the best guidance available on levels that FDA considers to be of regulatory interest and will thus enhance the safety of the food supply

**TABLE 1** Analytical Methods for Total Aflatoxins

Method	Commodity	Extraction solvent	Clean-up	LOQ	Ref.
TLC	Peanut, peanut butter	Methanol/water	Partition	10 ng/g, total	6
TLC	Milk	Acetone/water	SPE C-18, silica gel	0.2 ng/mL, AFM <sub>1</sub>	6
TLC	Corn, peanut	Methanol/water	SPE silica gel	10 ng/g	6
LC	corn, peanut	Methanol/water	SPE silica gel	10 ng/g, total	6
LC	Corn, peanut, peanut butter, pistachio nut	Acetonitrile/water	Multifunctional column	10 ng/g, total	6
LC	Milk	None	Immunoaffinity column	0.02 ng/mL, AFM <sub>1</sub>	21
ELISA	Peanut, peanut butter	Methanol/water	Immunoaffinity column	9 ng/g	6
Fluorometric	Corn, peanut, peanut butter	Methanol/water	Immunoaffinity column	10 ng/g, total	6
Capillary electrophoresis	Corn	Methanol/water	Immunoaffinity column	5 ng/g	22
Biosensor	Peanut, oat	Methanol/water	Partition, or Spec C18	10 ng/g	23

LOQ, Limit of quantitation.

when implemented and adhered to by the food industry. Science-based risk assessments have supported these levels as being adequate to protect the public health of consumers. At least 80 countries have established regulatory limits for total aflatoxins or aflatoxin B<sub>1</sub> in foods and feeds (26,27). In 1998 the European Union (EU) established maximum levels for aflatoxin M<sub>1</sub> in milk, aflatoxin B<sub>1</sub> and total aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>) in certain foodstuffs that were more stringent than those in the United States (28). The regulation became effective in January 1999 in the EU member states.

**TABLE 2** FDA Action Levels for Total Aflatoxins

Commodity	Concentration (ng/g)
All products, except milk, designated for humans	20
Corn for immature animals and dairy cattle	20
Corn and peanut products for breeding beef cattle, swine, and mature poultry	100
Corn and peanut products for finishing swine	200
Corn and peanut products for finishing beef cattle	300
Cottonseed meal (as a feed ingredient)	300
All other feedstuffs	20
Milk	0.5 <sup>a</sup>

<sup>a</sup> Aflatoxin M<sub>1</sub>.

Source: Food and Drug Administration (FDA) Compliance Policy Guides, Sections 527.400, 555.400, and 683.100 (1996).

## B. The Ochratoxins

The ochratoxins (ochratoxin A and ochratoxin B) are produced by certain fungal species of the genera *Aspergillus* and *Penicillium*. These fungi are commonly associated with many food crops grown in various parts of the world. Infection of commodities with these fungi may occur in the field and/or during storage. There is some evidence suggesting that *Penicillium* infections in grains in temperate climates do not normally occur before harvest (29). Survey results from various countries suggest that *Penicillium verrocosum* is the main producer of ochratoxin A in cereal grains in temperate climates where high moisture and reduced temperature prevail during harvesting and storing of cereal grains (30,31). Some of the field conditions that may contribute to ochratoxin A production in harvested grains in temperate climates include frost damage, excessive rainfall, strong sunlight that dries out grains, and drought stress (29). Ochratoxin A is the major toxin of toxicological significance produced by *Aspergillus* and *Penicillium* genera on cereal grains. Ochratoxin A is a relatively stable molecule and is not degraded to a significant extent by milling and processing procedures, including baking, roasting, and brewing (30,32,33). *Aspergillus ochraceus* is the major producer of ochratoxin A in commodities such as coffee beans, spices, and vine fruit in warmer climates. There is very little information available on ochratoxin A production by *Aspergillus* species in cereals in warmer climates, e.g., whether the infection and toxin production starts in the field or during storage.

### 1. Occurrence

Ochratoxin A occurs naturally in a variety of cereal grains including corn, barley, wheat, rye, sorghum, and oats. It is also found in various beans (including soya, coffee, and cocoa), peanuts, dried vine fruits, spices, nuts, figs, wine, beer, various cheeses, smoked meat, sausages (30,34–38), and roasted, ground, and instant coffee. It has been detected in human milk and blood in some countries in the temperate zone (39,40). Survey data from around the world reflect variations in incidence and levels of ochratoxin A between countries from year to year and variations within countries from year to year depending on climatic conditions and/or agronomic practices.

### 2. Toxicology

The toxicology of ochratoxin A was recently reviewed in the literature (12). From studies involving rodent species, the levels of ochratoxin A associated with acute toxicity vary from 3.4 to 30 mg/kg of body weight (bw), and female rats are more sensitive than males to the oral administration of ochratoxin A. The kidney was the primary target organ, with kidney tubular function impaired and the ability to concentrate urine reduced. Embryotoxic effects seen in mice and rats treated with 3–5 mg/kg bw included increased prenatal mortality, decreased fetal weight, and various fetal malformations.

Immunological effects observed in rodents include reduced plasma fibrinogen, factors, II, VII, and X involved in blood clotting, as well as reduced megakaryocyte and thrombocyte counts. Myelotoxicity has been seen in mice. Lymphocytopenia has been shown to occur in chickens, and levels of serum immunoglobulins (IgA, IgC, and IgM) were reduced.

Ochratoxin A inhibits protein synthesis and tRNA synthetase in both microorganisms and mammalian cells. In mice and rats, ochratoxin A is carcinogenic, inducing kidney tumors, predominantly in male animals; liver tumors were observed in male and female mice. DNA single-strand breaks were observed in renal and hepatic tissues of mice and rats treated with single or chronic doses of ochratoxin A.

The toxicity of ochratoxin A was reviewed by the Joint Expert Committee on Food Additives of the World Health Organization/Food and Agriculture Organization (JECFA) at its 37th and 44th meetings (41). A provisional tolerable weekly intake (PTWI) of 112 ng/kg bw (later rounded to 100 ng/kg bw) was established using a safety factor of 500. Ochratoxin A was reevaluated by JECFA at its 56th meeting in 2001 (42); the PTWI of 100 ng/kg bw/week was retained pending the results of ongoing studies on the mechanisms of nephrotoxicity and carcinogenicity.

In humans, acute gastrointestinal poisoning from food contaminated with high levels of ochratoxin A has been described (43). In addition, in the 1950s in some geographically limited areas of Bulgaria, Yugoslavia, and Romania, a fatal chronic renal disease was identified as being associated with ochratoxin A. This disease, later called Balkan endemic nephropathy (BEN), is a bilateral noninflammatory, chronic nephropathy in which the kidneys are greatly reduced in size and weight and show diffuse cortical fibrosis extending into the corticomedullary junction, hyalinized glomeruli, and severely degenerated tubules.

Several theories have been advanced to explain how ochratoxin A (and possibly other mycotoxins) may be implicated in the etiology of BEN and the associated urinary tract tumors (UTT). Contamination of cereal grains and beans by ochratoxin A (and citrinin) has been found to be more frequent in endemic than in control areas and also more frequent in affected than in nonaffected families. Case-control epidemiological studies have revealed more frequent and higher blood and urine contamination of ochratoxin in patients with BEN and/or UTT in the Balkan area. Patients with BEN have impaired immunological functions, as do experimental animals exposed to ochratoxin A. BEN has a morphology and progressive course similar to the endemic porcine nephropathy observed in many countries that is caused by ingestion of ochratoxin A.

A recent study in the Balkan area (44) showed a correlation between ochratoxin A in food samples, serum, urine, and ochratoxin A–DNA adducts in tissues from patients with kidney tumors. The geographical correlation between BEN and UTT in that study strongly suggested a common etiology with ochratoxin A. However, other factors have been identified that argue against the implication of ochratoxin A as the sole risk factor for BEN and/or UTT (12). No direct epidemiological proof of a causal relationship between mycotoxins or ochratoxin A and BEN has been presented. Patients have generally been exposed to mixtures of mycotoxins in combination with other poorly characterized environmental agents. Differences in food storage and dietary practices may affect the unusual clustering of BEN and UTT. Villages affected in the past continue to be affected today, while nonaffected villages, located in close proximity to affected villages, have remained free of BEN. Long-term exposure to polycyclic aromatic hydrocarbons and other toxic organic compounds leaching into drinking water obtained from wells located near low-rank coal mines adjacent to endemic settlements has been proposed as a cause of BEN. Phenotypic and genotypic differences in activating and detoxifying enzymes may also play a role in BEN. The risk for developing BEN and/or UTT may be associated with the efficiency of the oxidative metabolism of debrisoquine.

### 3. Analytical Methodology

Ochratoxin A has a carboxylic acid functional group, therefore it can be extracted from grains or grain products using an acidic or basic solvent system. Ochratoxin A exhibits blue fluorescence on a silica gel plate or in a reversed-phase LC mobile phase. Analytical methods for ochratoxin A are mainly LC techniques with fluorescence detection. Both C-18 SPE and immunoaffinity columns are widely used for the cleanup step. The identity of OTA can be confirmed by forming the fluorescent methyl ester using boron trifluoride reagent. ELISA techniques for ochratoxin A are mostly in the microtiter well format. The analytical methods that can be used for the analysis of OTA are shown in [Table 3](#) (45,46).

### 4. Regulatory Control

No regulatory levels have been established for ochratoxin A in foods or feeds in the United States. Surveys of various cereal grains over a 20-year period revealed that less than 2% of the samples examined contained ochratoxin A, and these were at very low levels. A similar trend was noted in other commodities examined, therefore, the establishment of a level is not warranted in the United States at this time. Many other countries have established regulatory limits for ochratoxin A in foods and feeds. Generally, levels ranging from 1 to 50 µg/kg have been established for foods, and levels of 100–1000 µg/kg have been established for feeds (26,47). In some instances, the scientific basis for the established levels has not been clearly indicated.

**TABLE 3** Analytical Methods for Ochratoxin A

Method	Commodity	Extraction solvent	Clean-up	LOQ	Ref.
TLC	Barley	Chloroform/0.1 M phosphoric acid, then partition into sodium bicarbonate	C-18 SPE	10 ng/g	6
LC	Corn, barley, oats	Same as above	C-18 SPE	2 ng/g	6
LC	Wine	None	Immunoaffinity column	0.04 ng/mL	45
ELISA	Wheat, corn, barley	Dichloromethane/1 M hydrochloric acid, then partition into sodium bicarbonate	Immunoaffinity column	2.5 ng/g	46

LOQ, Limit of quantitation.

### III. TOXINS PRODUCED BY *FUSARIUM* SPECIES

Many *Fusarium* species are plant pathogens and are commonly found in the soil. The species of *Fusarium* that infect cereal grains are referred to as “field fungi” because they infect plants in the field and not during the storage of the grain (48). As of 1996, more than 60 *Fusarium* species had been reported in raw foods and feeds throughout the world and more than 100 toxigenic secondary metabolites had been identified (49). Variations in mycotoxin-producing ability have been observed between strains within the same species. Comprehensive reviews regarding the diverse nature of the various mycotoxins produced by *Fusarium* species have recently appeared in the literature (50,51). The most important *Fusarium* toxins, from the standpoint of human exposure and regulatory concern, are deoxynivalenol (vomitoxin), the fumonisins, and zearalenone.

#### A. Deoxynivalenol

Deoxynivalenol (DON), commonly referred to as vomitoxin, belongs to a class of mycotoxins that are produced by *Fusarium* fungi and referred to as trichothecenes. Deoxynivalenol is a natural toxin produced by several fungi of the genus *Fusarium*, especially *F. graminearum* and *F. culmorum*, which are pathogens of corn, wheat, and other cereal grains. Infection of susceptible cultivars by these fungi usually occurs when there is cool, wet weather at the silking or anthesis stage of grain development (52). The disease resulting from infection by these fungi is sporadic and causes some damage in wheat and other grains every year. The severity of the infection and the levels of DON produced depend on the weather conditions. The fungal strains that infect grains may produce mixtures of structurally related mycotoxins that can vary from one country or region to another (53). Methods to remove deoxynivalenol from various types of grains rely mainly on physical separation of the inner area of the kernel from the more heavily contaminated outer layers of the grain kernels. The extent to which deoxynivalenol is removed from contaminated grain during milling operations can vary, depending on the procedures used. Deoxynivalenol is relatively stable and shown to survive most processing and baking procedures, and it is not completely destroyed by ethanol fermentation processes (54,55).

##### 1. Occurrence

Deoxynivalenol is often found on cereal grains such as wheat, corn, barley, oats, rice, rye, and in their products, in many regions of the world (54,56,57). In limited surveys conducted in the United States, deoxynivalenol was found in milled and processed corn and wheat products, including bran, flour, meal, breakfast cereals, baby foods, bread, and snack foods (58–60). Deoxynivalenol has been found in kernels of sweet corn prior to harvest and is reported to be relatively stable during

the canning process (61,62). Trace amounts of deoxynivalenol may be transmitted from poultry feed into meat and eggs, but these residues are reduced to negligible levels once the feed source is removed (63). High concentrations of deoxynivalenol must be present in the feed of dairy cows to result in detectable levels being produced in the milk.

## 2. Toxicology

In laboratory animals and livestock, acute DON poisoning is characterized by skin irritation, feed refusal, vomiting, diarrhea, hemorrhage, neural disturbance, abortion, and death. LD<sub>50</sub> values range from 27 mg/kg bw in the Peking 10-day-old duckling to 43 mg/kg bw in the mouse to 140 mg/kg bw in the broiler hen. DON is an inhibitor of peptide chain elongation during protein synthesis.

DON has been shown to be noncarcinogenic in a long-term study in the mouse (64). In genotoxicity tests, DON was negative in in vitro gene mutation assays and positive in both in vitro as well as in vivo chromosomal aberration assays. DON has been demonstrated to be teratogenic in the mouse and has also shown reproductive effects such as retarded fetal ossification, embryotoxicity, and decreased fetal weight in various species (mouse, rat, pig) (42). Suppression of humoral and cell-mediated resistant immunity results from exposure to DON in mice, including decreased resistance to secondary infection by bacteria and viruses.

JECFA (42) established a provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg bw for DON on the basis of the NOEL of 100 µg/kg bw/day in mice in a 2-year feeding study and a safety factor of 100.

In humans, outbreaks of intoxication, including symptoms such as nausea, vomiting, diarrhea, and abdominal pain, followed by recovery within a few days, have been reported associated with consumption of *Fusarium* species or DON. Such outbreaks have occurred in Korea, Japan, India, Colombia, China, and South Africa (12).

A human disease known as alimentary toxic aleukia (ATA) occurred in the U.S.S.R. in 1931–47, reportedly related to the ingestion of overwintered grain invaded by toxic *Fusarium* species. The disease was fatal in a great number of cases. ATA may have been related to contamination with DON and/or a related trichothecene toxin, T-2. In Japan and Korea from 1946 to 1963, cases of scabby grain toxicosis were reported in humans as well as farm animals, and the grains were found to be contaminated with *Fusarium* species. Features of these human diseases were similar to trichothecene-type toxicoses, as observed in experimental animals, particularly symptoms caused by DON, nivalenol, and T-2 toxin. Recent outbreaks of trichothecene-related disease have been reported in Japan, India, and China. None of these outbreaks have been studied using rigorous epidemiological criteria. Although relatively high levels of DON were detected in some samples of moldy corn and scabby wheat and *Fusarium* species were identified, a rigorous correlation could not be demonstrated between DON and the human symptoms (12). The epidemiological data available, however, point toward trichothecene- or specifically DON-contaminated grain products as the causative factor of the acute human toxicoses.

## 3. Analytical Methodology

DON contains three hydroxyl groups and does not fluoresce but absorbs UV light at 220 nm, therefore determination by TLC requires derivatization after separation. The most common reagent used is aluminum chloride, which can be coated on silica gel TLC plates before use or can be applied as a spray after development. The plate is heated at 120°C for 7–8 minutes and is then examined under long-wave UV light for the blue fluorescence. Most LC methods for DON employ UV detection. The application of postcolumn derivatization to form the fluorescent derivative has been investigated recently. To increase the volatility in a GC column and the detectability with the GC detectors, DON is usually derivatized with trimethylsilyl ethers or fluorinated propionyl or butyryl esters prior to the determinative step with a flame ionization detector (FID) or an electron capture detector (ECD). GC/MS has also been used. The analytical methods that can be used for the analysis of DON are shown in Table 4 (59,65,66).

**TABLE 4** Analytical Methods for Deoxynivalenol

Method	Commodity	Extraction solvent	Clean-up	LOQ	Ref.
TLC	Wheat	Acetonitrile/water	Charcoal-alumina-celite column	300 ng/g	6
LC	Wheat flour, bran	Acetonitrile/water	Multifunctional SPE	500 ng/g	59
GC	Wheat, barley, corn	Acetonitrile/water	Florisil SPE	10 ng/g	65
ELISA	Corn, wheat, barley	Water	Immunoaffinity column	500 ng/g	66

LOQ, Limit of quantitation.

#### 4. Regulatory Control

No U.S. action level has been established for deoxynivalenol. FDA has established guidance levels for deoxynivalenol in finished wheat products that may be consumed by humans and for grains designated for animal feed. Guidance levels are issued to provide guidance to state and federal officials, as well as industry, about levels of a particular contaminant that FDA believes will provide an adequate margin of safety to protect human and animal health. The current guidance level for deoxynivalenol in finished wheat products and other grains is shown in Table 5 (67). No level was set for raw wheat intended for milling because normal manufacturing practices and additional technology available to millers can substantially reduce deoxynivalenol levels in finished wheat products. Regulatory levels for deoxynivalenol have been established by several countries. The levels range from zero to 2 µg/g for grains designated for human food and up to 10 µg/g for feedstuffs (26).

## B. Fumonisin

*Fusarium verticillioides* (*F. moniliforme*) is one of the most prevalent fungal species associated with corn throughout the world (68,69). It is among the most common fungi colonizing symptomless corn plants (70). Symptomless infections can exist throughout the plant, and seed-transmitted strains of the fungus can develop systemically to infect the kernels. Fumonisin are fungal toxins produced by the molds *Fusarium verticillioides*, *F. proliferatum*, and other *Fusarium* species that can grow on agricultural commodities in the field and during storage. Approximately 20 fumonisin analogs have been isolated and characterized (51,71–73). Of these, fumonisin B<sub>1</sub> (FB<sub>1</sub>), fumonisin B<sub>2</sub> (FB<sub>2</sub>),

**TABLE 5** FDA Guidance Levels for Deoxynivalenol in Wheat-Derived Products, Other Grains

Product	Concentration (µg/g)
All finished wheat products, e.g., flour, bran, and germ, that may be consumed by humans	1
All grains and grain by-products destined for ruminating beef and feedlot cattle older than 4 months and for chickens; these ingredients should not exceed 50% of the diet of cattle or chickens	10
All grains and grain by-products destined for swine; these ingredients should not exceed 20% of the diet	5
All grains and grain by-products for all other animals; these ingredients should not exceed 40% of the diet	5

Source: Letter to State Agricultural Directors, State Feed Control Officials and Food, Feed and Grain Trade Organizations from R. G. Chesemore, Associate Commissioner for Regulatory Affairs, FDA, dated Sept. 16, 1993.



and fumonisin B<sub>3</sub> (FB<sub>3</sub>) are the major naturally occurring fumonisins. The most prevalent of these mycotoxins in contaminated corn is FB<sub>1</sub>, which is also believed to be the most toxic (72,74,75). Fumonisin B<sub>1</sub> has been found to be relatively stable during most processing procedures for corn and corn products (76).

### 1. Occurrence

The extent of fumonisin contamination of raw corn varies with geographic location, agronomic and storage practices, and the vulnerability of the plants to fungal invasion during all phases of growth, storage, and processing. The levels of fumonisin in raw corn are also influenced by environmental factors such as temperature, humidity, and rainfall during preharvest and harvest periods (77). High levels of fumonisins are associated with hot and dry weather, followed by periods of high humidity. High levels may also occur in raw corn that has been damaged by insects (78,79). Agricultural practices and the genotype of corn are factors to be considered in determining the susceptibility of corn plants to fungal invasion and fumonisin production (80). Moisture levels between 18 and 20% have been found to support the growth of the fungus in stored corn (70). The worldwide occurrence of fumonisins in corn and corn-based products has been noted (81–83). A comprehensive study of the incidence and levels of fumonisin B<sub>1</sub>, fumonisin B<sub>2</sub>, and *Fusarium* molds in food-grade corn and corn-based food products in the United States was recently published (84). In addition to corn, fumonisins have occurred naturally in sorghum, rice, navy beans, and other commodities (85–89). FB<sub>1</sub> has been isolated from cultures of *Alternaria alternata*, which is a host-specific pathogen of tomato plants, and from cultures of *F. proliferatum* obtained from date palms in Saudi Arabia (90,91). Recent studies suggest that there is very little accumulation of fumonisins in edible tissues of food animals, and no detectable levels have been found in milk from cows dosed with high levels of fumonisins (92,93).

### 2. Toxicology

Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is the most toxic of the fumonisins. The severity and effects of the fumonisins vary widely among species. The most sensitive species is the horse, which succumbs to equine leukoencephalomalacia when exposed to relatively low doses of fumonisin. Swine exhibit pulmonary edema, which is secondary to cardiovascular depression, apparently induced by elevated levels of sphingosine and inhibition of cardiac L-calcium channels. Rats exhibit renal lesions and liver lesions are the most sensitive endpoint in mice (94). In two long-term studies in rats, FB<sub>1</sub> caused liver cancer in one study and in another caused kidney cancer in male rats and liver cancer in female mice (95).

Fumonisin has not been shown to be unequivocally genotoxic. No published data support the theory that fumonisin causes developmental or reproductive toxicity in farm animals. JECFA (42) established a group provisional maximum tolerable daily intake (PMTDI) for fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub>, alone or in combination, of 2 µg/kg bw/day on the basis of the NOEL of 0.2 mg/kg bw/day (renal toxicity in the rat) and a safety factor of 100.

Epidemiological studies in the Transkei, South Africa, China, and Italy have attempted to clarify the relationship between fumonisin exposure in maize and liver, oral, pharyngeal, and esophageal cancer. In studies in the Transkei region of South Africa, occurrence of fumonisin [or DON and nivalenol (NIV)] in corn and the *Fusarium* species producing the toxin(s) does not always correlate with incidence of esophageal cancer over a number of seasons. Residents of the high-rate esophageal cancer incidence area in Transkei compared to the low-rate incidence area have been shown to possess nutritional deficiencies such as vitamins A, E, and B<sub>12</sub>, and selenium, and folate. This population is also exposed to aflatoxin B<sub>1</sub>, fusarin C, and other compounds produced by *Fusarium* species (42).

It has been suggested that fumonisin is involved in the development of neural tube defects in the Transkei, Hebei Province, China, and in the lower Rio Grande Valley in southern Texas, all areas of high corn consumption in certain populations. Adequate data to confirm this suggested relationship are lacking (42). Indeed, the Centers for Disease Control and Prevention (CDC) has

reported that neural tube defects in the United States decreased by 19% following the FDA-mandated folic acid fortification of enriched grain products in the U.S. food supply (96). A role for fumonisin in neural tube defects remains unconfirmed.

One can only conclude that fumonisins are toxic to humans, but their relationship to human esophageal or liver cancer has not been definitively demonstrated. Present-day estimates of exposure to a carcinogen do not necessarily reflect long-term exposures, especially when human cancers may be expected to have a long latency period. In addition, analyzed samples of a food crop which give a statistically valid representation of the levels of fumonisins are rarely available (12).

### 3. Analytical Methodology

Fumonisin do not absorb UV light or exhibit a fluorescence. Most chromatographic methods for the determination of total fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> or fumonisin B<sub>1</sub> (FB<sub>1</sub>) alone require derivatization, but LC/MS methods do not require derivatization. The most commonly used reagents are *ortho*-phthalaldehyde/mercaptoethanol and naphthalene-2,3-dicarboxyaldehyde for LC pre column derivatization and fluorescamine for pre- or post-C18 TLC plate development derivatization to form the fluorescent derivatives. GC methods center on the separation and detection of the hydrolysis products after the formation of the trimethylsilyl derivatives of the hydrolysates. Both ELISA and immunoaffinity methods have been commonly used for fumonisins in grains and grain products, including beer and wine. Analytical methods that can be used for the analysis of fumonisins are shown in Table 6 (97–101).

### 4. Regulatory Control

In the United States, FDA has established guidance levels for fumonisins that are considered adequate to protect human and animal health and that are achievable in human foods and animal feeds with the use of good agricultural and good manufacturing practices. The levels for total fumonisins (FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub>) in human food products and animal feeds are shown in Table 7. A regulatory level of 1 µg/g has been established for fumonisins (FB<sub>1</sub> + FB<sub>2</sub>) in corn products in Switzerland. No other country has published a regulatory limit (102).

## C. Zearalenone

Zearalenone is a mycotoxin produced by several *Fusarium* species including *F. graminearum* and *F. culmorum*. Infection of grains by these fungi usually occur when there is cool, wet weather at the silking or anthesis stage of grain development (52). Zearalenone occurs naturally on cereal grains in the field, but it is believed that the toxin is produced to a larger extent during the storage of grains that have not been properly dried before storage (103). Zearalenone is relatively stable during storage, milling, and processing of food. A recent study of the effects of extrusion cooking on the stability of zearalenone in corn grits revealed that this process was more effective for reducing zearalenone concentrations than other thermal processes (104). Wet-milling of corn concentrates

**TABLE 6** Analytical Methods for Fumonisin

Method	Commodity	Extraction solvent	Clean-up	LOQ	Ref.
TLC	Corn	Methanol/water	Strong anion exchange (SAX) SPE	1000 ng/g, total	97
LC	Corn	Acetonitrile/water	SAX SPE	800 ng/g, total	98
LC	Beer, wine	None	Immunoaffinity column	1 ng/g FB <sub>1</sub>	99
Fluorometric	Corn, grits	Methanol/water	Immunoaffinity column	1000 ng/g total	100
ELISA	Corn	Methanol/water	Immunoaffinity column	500 ng/g, total	101

LOQ, Limit of quantitation.

**TABLE 7** Guidance Levels for Fumonisin in Human Foods and Animal Feed

	Total fumonisins (FB <sub>1</sub> + FB <sub>2</sub> + FB <sub>3</sub> )
<i>Human food, product</i>	
Degermed dry-milled corn products	2 µg/g
Whole/partially degermed dry-milled corn product	4 µg/g
Dry-milled corn bran	4 µg/g
Cleaned corn intended for masa production	4 µg/g
Cleaned corn intended for popcorn	3 µg/g
<i>Animal feed, corn and corn by-product for:</i>	
Equids and rabbits	5 µg/g <20% of diet
Swine and catfish	20 µg/g <50% of diet
Breeding ruminants, poultry and mink; lactating dairy cattle, and laying hens	30 µg/g <50% of diet
Ruminants > 3 mos, raised for slaughter and mink for pelt production	60 µg/g <50% of diet
Poultry raised for slaughter	100 µg/g <50% of diet
All other species or classes of livestock and pet animals	10 µg/g <50% of diet

Source: Food and Drug Administration. Mycotoxins in Domestic Foods. Compliance Program Guidance Manual, 7307.001. Feed Contaminants Compliance Program, 7371.003.

the toxin in the gluten fraction while the starch fraction remains relatively free of the toxin (55). Dry-milling of corn results in the toxin being concentrated in the bran fractions with lower levels being present in grits, meal, and flour fractions (55,105). Ethanol fermentation processes used on corn resulted in zearalenone being concentrated by a factor of 2 in the recovered solids, compared with the starting concentration in the corn. There was no carry-over of the toxin to the distilled ethanol (55).

### 1. Occurrence

Zearalenone is associated primarily with corn and corn products, but the toxin has been reported in oats, barley, rye, wheat, soybeans, rice, sorghum, walnuts, pecans, and beer (54,56,105). Surveys of the occurrence of zearalenone in various commodities throughout the world were recently published (106,107). From a global standpoint, the incidence and levels of zearalenone vary from year to year and are dependent on the weather conditions. There is limited evidence of carry-over of zearalenone from feed to milk and other tissues in ruminants at levels typical of a natural exposure (63).

### 2. Toxicology

Zearalenone has been demonstrated to possess estrogenic and anabolic activity in various species, such as rodents, rabbits, pigs, and monkeys. The major toxic effects are on reproduction, including reproductive organs and their function, leading to hyperestrogenism. The pubertal pig is the most sensitive species tested, with a no-adverse-effect-level (NOAEL) of 0.06 mg/kg bw.

The most estrogenic metabolite of zearalenone is  $\alpha$ -zearalenol, which is formed in greater amounts in humans and the pig than in rodents. The half-life of this substance was longer in humans than in other species tested. In a number of assays, binding of zearalenone to estrogen receptors was approximately 20-fold lower than that seen with 17  $\beta$ -estradiol, a standard compound for estrogen receptor binding (64).

Bioassays in mice and rats are considered "positive evidence of carcinogenicity" by the National Toxicology Program. The International Agency for Research on Cancer (IARC) considered the evidence for zearalenone's carcinogenicity as "limited." Mutagenicity data are mixed. Zearalenone induced sister chromatid exchange, chromosomal aberrations, and polyploidy in Chinese hamster cells, but did not induce mutations in *Salmonella typhimurium* (12,105).

The relationship between exposure to *Fusarium* toxins, such as zearalenone, and esophageal cancer has been examined in several ecological studies. However, most of the studies considered mixtures of many toxins from many species of fungi on corn. There are no analytical epidemiological studies on the carcinogenicity of any individual *Fusarium* toxin or zearalenone alone (12).

### 3. Analytical Methodology

Zearalenone has a greenish-blue fluorescence under short-wave UV (256 nm) on a silica gel TLC plate and is not visible under long-wave UV light except at high concentration. Zearalenone fluoresces blue under long-wave UV light after spraying with aluminum chloride solution (6). The limit of quantitation is about 0.1 µg/g. An LC method with fluorescence detection for the determination of  $\alpha$ -zearalenol and zearalenone in corn was collaboratively studied recently (108). A GC/tandem mass spectrometry method has been used for the confirmation of identity (109). An ELISA method, employing a monoclonal antibody, was evaluated and found to be applicable for the detection of zearalenone in corn, wheat, and pig feed at >800 ng/g (6). A specific recombinant antibody for zearalenone was produced to serve as an alternative to monoclonal and polyclonal ELISA approaches (110). The limitations have been low sensitivity and low affinity.

### 4. Regulatory Control

No regulatory level has been established for zearalenone in the United States. There do not appear to be any human health problems or major international trade problems associated with this fungal toxin at this time. There is a need, however, to obtain monitoring data on zearalenone levels in various foods over a number of years before consideration is given to setting an action level or international standard. Nine countries have established guidelines or maximum tolerable levels for zearalenone in foods, mainly cereals. The levels range from 0 to 1000 µg/kg (107).

## IV. TOXINS PRODUCED BY *PENICILLIUM* SPECIES

Species belonging to the genus *Penicillium* are prolific producers of toxic metabolites, some of which have been found in foods. It has been estimated that 70–80% of the *Penicillium* species have the potential for production of mycotoxins. A comprehensive review of the literature pertaining to *Penicillium* was recently published (111). Patulin, a mycotoxin produced by fungi of several genera including *Penicillium*, has received much attention in recent years because of its suspected carcinogenic properties, although no concrete evidence has been presented to date that support that suspicion. (The production of ochratoxin A by *Penicillium verrocosum* was in Sec. II.)

### A. Patulin

Patulin is a toxic metabolite of several species of fungi including *Penicillium*, *Aspergillus*, and *Byssochyلامys*. These fungi grow on a variety of foods including fruits, grains, and cheese. *Penicillium expansum* is the most commonly encountered species of *Penicillium* that produces patulin in various fruits and fruit juices (112–114). Patulin in apple juice is almost completely destroyed by alcoholic fermentation processes. It is not detectable in wine prepared from contaminated grapes (115,116). Thermal processing causes only moderate reductions in patulin levels, therefore patulin present in apple juice will survive pasteurization processes (113).

#### 1. Occurrence

Patulin is most frequently found as a natural contaminant of apples and apple products. It has also been found in various other fruits (e.g., pears and grapes), fruit juices, barley, wheat, and corn (113,117). Under natural conditions, patulin has been isolated almost exclusively from apples and apple products contaminated with *P. expansum*, therefore these are probably the main dietary sources of patulin in the human diet (113). The presence of certain chemicals in foods, particularly compounds containing sulfhydryl groups, can reduce the level of patulin present in some foods (112).

The reaction of patulin with sulfur-containing compounds in foods, such as grains, meat, and cheese, may account for the lack of high patulin levels in those foods (113). The occurrence of patulin in apple juice/products can be greatly reduced if apple processors follow good manufacturing practices by not using rot-damaged apples for further processing (118).

## 2. Toxicology

The World Health Organization summarized the toxicological characteristics of patulin in a 1990 publication (119). In mice, the LD<sub>50</sub> of patulin ranges from 15 to 35 mg/kg, depending on the mode of administration. Patulin has cytotoxic effects, including antibiotic, antifungal, and anti-protozoal properties. Patulin appears to act by affecting membrane permeability and disorganizing cytoplasmic microfilaments (119,120). In vitro, patulin inhibits several enzymes including RNA and DNA polymerase and affects DNA directly by its effects on transcription and translation (119).

In classical mutagenicity tests, such as the Ames test, patulin is generally negative. Patulin is positive in sister-chromatid exchange and chromatid break tests and in chromatid translocation tests. It has been shown to have aneuploidogenic potential, i.e., the ability to induce numerical chromosomal aberrations in cultured Chinese hamster V79 cells (121).

Patulin has been shown to be negative for carcinogenicity in three long-term exposure rodent studies. In whole rat embryo culture, patulin is toxic (122). Patulin is fetotoxic and embryotoxic in rodents only at doses overtly toxic to the mother (119). It is not toxic to the immune system in mice at doses based on human exposure levels, despite earlier reports of its immunotoxicity (123).

When the 35th JECFA evaluated patulin in 1990, a maximum provisional tolerable weekly intake (PTWI) of 7 µg/kg bw/week was proposed (116). A second evaluation of patulin was done at the 44th JECFA meeting in 1995, which took into account the fact that most of the patulin ingested in rats is eliminated within 48 hours and 98% within 7 days. JECFA therefore established a maximum provisional tolerable daily intake (PTDI) of 0.43 µg/kg bw/day (124). This PTDI has a 100-fold safety factor built in.

Patulin was at one time evaluated as an antibiotic for treatment of the common cold in humans. When WHO (119) evaluated two reports available in the literature on test of patulin for its antibiotic activity in humans, it was not possible to determine from the information given which clinical tests were performed to support the authors' assertion that no ill effects were observed. Humans have been exposed to patulin from apples and apple products for centuries, and there have been no reported cases of human illness associated with patulin (119,123).

## 3. Analytical Methodology

Methods of analysis for patulin are mainly developed for the determination of the toxin in apple juice. Patulin shows UV absorption at 275 nm and does not fluoresce under long-wave UV light on a silica gel TLC plate. A recently developed method utilizes a semi-permeable membrane for the diphasic dialysis extraction of patulin from apple juice (125). The test extract was applied to a normal phase TLC plate. After development, an alcoholic solution of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) is used to spray the plate to prepare the yellow-brown fluorescent patulin-MBTH derivative necessary for quantitation.

A method based on liquid-liquid partition, reversed-phase LC coupled with UV detection at 276 nm was evaluated by an international collaborative study (126). Patulin was well resolved from another compound, 5-hydroxymethyl-2-furaldehyde (HMF), with similar chromatographic properties, commonly found in processed apple juice. The limit of determination of this method is 10 ng/mL. The negative ion chemical ionization technique has been used with a variety of capillary columns in quadrupole, ion trap, and magnetic sector GC/MS instruments to confirm the identity of patulin (127). There has not been any development of high-affinity antibody for patulin; therefore, there are no suitable ELISA methods for patulin.

## 4. Regulatory Control

In the United States, FDA believes that patulin levels in apple juice can be controlled by processors, principally by removing spoiled and visually damaged apples from the product stream used

for the production of apple juice. The results from a safety assessment conducted by FDA suggested that adequate public health protection will be achieved if processors control patulin levels in apple juice to a level not exceeding 50 µg/kg. FDA has established an action level of 50 µg/kg for patulin in apple juice, apple juice concentrates, and apple juice products based on the level found, or calculated to be found, in single strength apple juice or in the single strength apple juice component of a juice product. In arriving at the action level of 50 µg/kg, FDA considered consumption of apple juice by drinkers of all ages and by small children in two age categories: children less than one year old and children 1–2 years old. The two age categories for small children were considered because small children consume higher amounts of apple juice relative to their body weight than other age groups. At least 11 countries regulate patulin in apple juice at levels of 30–50 µg/kg, with lower levels in infant foods (26).

## V. CONCLUSIONS

The maintenance of a wholesome food supply is a major responsibility that must be shared by the food industry (producers and processors) and the regulatory agencies involved. It is incumbent upon the regulatory agencies, as well as the food industry, to keep abreast of technological advances in processing techniques and agronomic practices that are available. Current technology cannot completely prevent fungal contamination of field crops before harvest. Research is underway in many laboratories with the objective of controlling preharvest contamination through genetic manipulations, use of irrigated plots, the application of various chemicals, and other innovative measures. Some progress has been made in this research, but no commercial, large-scale controls have been adopted.

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# 25

## Foodborne Marine Biotoxins

**Mark A. Poli**

*U.S. Army Medical Research Institute of Infectious Diseases,  
Fort Detrick, Maryland, U.S.A.*

### I. BACKGROUND

Marine biotoxins, which are globally distributed, are responsible for numerous human intoxication syndromes due to the ingestion of cooked or uncooked seafood. Over 2500 cases of foodborne diseases caused by fish and shellfish toxicity were reported to the Centers for Disease Control and Prevention (CDC) between 1993 and 1997 in the United States (1). Marine biotoxins are estimated to cause over 60,000 foodborne intoxications worldwide each year (2). In addition to human intoxications, they cause massive fish kills, negatively impact coastal tourism and fishery industries, and have been implicated in mass mortalities of birds and marine mammals. The long-term environmental and public health effects of chronic exposure to these toxins are poorly understood; research needs are only beginning to be addressed (2,3).

Ingestion of seafood containing marine biotoxins causes six identifiable syndromes: paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), ciguatera fish poisoning (CFP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), and azaspiracid poisoning (AZP). With the exception of CFP, which, as the name implies, is caused by eating contaminated finfish, all are caused by the ingestion of shellfish. And, with the exception of ASP, the causative toxins are all isolated from marine dinoflagellates. ASP is notable as a syndrome caused by the only known toxin produced by a diatom. These toxin-producing species are a small minority of the thousands of known species of phytoplankton. However, under the correct environmental conditions, they can proliferate to high cell densities known as blooms. During these harmful algal blooms (HABs), they may be ingested in large quantities by zooplankton, filter-feeding shellfish, or grazing or filter-feeding fishes. Through these intermediates, toxins can then be vectored to higher trophic levels, including humans.

In recent decades, there has been a perceived increase in both geographic distribution and occurrence of HABs (2,4,5). While a portion of this increase is undoubtedly a result of increased monitoring and reporting programs as well as improved detection technologies, a global expansion in geographic range of several syndromes has been well documented (2,4,5). It seems likely that anthropogenic effects have contributed to this expansion, but to what extent is currently a subject of debate. What seems certain, however, is that foodborne marine biotoxins will continue to be an important issue to the seafood industry, recreational harvesters, and consumers for the foreseeable future.

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The views, opinions, and/or findings contained herein are those of the author and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

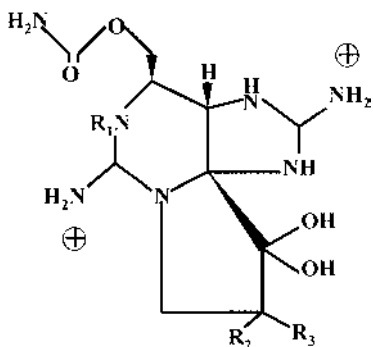
This chapter will briefly describe the six major toxic syndromes mentioned above and the implicated toxins. Space limitations do not allow a discussion of cyanobacterial toxins obtained through contaminated drinking water. Readers interested in this topic should refer to Ref. 6 and references therein.

## II. HUMAN INTOXICATION BY MARINE BIOTOXINS

### A. Paralytic Shellfish Poisoning

PSP results from ingesting shellfish contaminated with a family of heterocyclic guanidines called saxitoxins (Fig. 1). The name saxitoxin (STX) is derived from the giant butter clam *Saxidoma giganteus*, from which the toxin was first isolated (7). While STX was the original toxin isolated, the family of PSP toxins is now known to consist of over 20 derivatives of varying potency. They are associated with species of marine dinoflagellates belonging to the genera *Alexandrium*, *Pyrodinium*, and *Gymnodinium*, as well as several species of freshwater cyanophytes. More recently, the isolation of STX from species of *Moraxella* associated with dinoflagellate cultures suggests a bacterial origin (8). In filter-feeding shellfish such as clams, oysters, mussels, and scallops, toxins accumulate after ingestion of dinoflagellate cells during bloom conditions or resting cysts from the sediment. The toxin profile in the originating dinoflagellates can be modified by metabolic biotransformation reactions in the shellfish (9). Ingestion of toxic shellfish by humans results in the characteristic signs and symptoms of intoxication. These toxic dinoflagellates occur in both tropical and temperate oceans. Approximately 2000 cases of PSP are estimated to occur annually across regions of North America, South America, Europe, Japan, Australia, Southeast Asia, and India (2,5). The overall mortality rate has been estimated at 15% (2). In addition to human intoxications, PSP toxins have also been suspected in the deaths of birds (10) and humpback whales (11).

STX and its derivatives elicit their physiological effects by interacting with the voltage-dependent sodium channels in excitable cells of heart, muscle, and neuronal tissues. High-affinity binding to neurotoxin receptor site 1 on the sodium channel blocks ion conductance across the cellular membranes, thereby inhibiting depolarization. While all voltage-dependent sodium channels are susceptible to saxitoxins, pharmacokinetic considerations make the peripheral nervous system the primary target in seafood intoxications.



**FIGURE 1** The basic toxin structure of the paralytic shellfish poisons. In the parent carbamate compound (saxitoxin), R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> = H. A number of sulfated and hydroxylated derivatives at these locations make up the carbamate class of PSPs. In addition, the same sulfated/hydroxylated derivatives occur as carbamoyl or decarbamoyl classes of PSP toxins where the carbamate group is replaced by OCONHSO<sub>3</sub><sup>-</sup> or OH, respectively.

Ingestion of PSP toxins results in a rapid-onset (minutes to hours) complex of paresthesias including a prickling, burning, or tingling sensation in the lips and mouth that rapidly progresses to the extremities. At low doses these sensations can disappear in a matter of hours with no sequelae. At higher doses numbness can spread from the extremities to the trunk, followed by weakness, ataxia, hypertension, loss of coordination, and impaired speech. At lethal doses respiratory failure results from paralysis of respiratory musculature (8,12). Children appear to be more susceptible than adults. In an outbreak in Guatemala in 1987 involving 187 cases, the highest attack rate occurred in patients 13–17 years old, and children <6 years of age had a much higher mortality rate (50%) than that of victims 18 years and older (5%) (12,13). The lethal dose for young children may be as low as 25 µg STX equivalents\* (13), while that for adults is probably about 1–4 mg STX equivalents (2). In adults, clinical symptoms can occur upon ingestion of approximately 0.7 mg equivalents (14). Shellfish can contain up to 10–20 mg equivalents per 100 g of meat, so ingestion of only a few shellfish can cause illness (2,13,15). Fortunately, clearance from the blood via the urine is rapid. After a series of outbreaks of PSP on Kodiak Island, Alaska, in 1994, serum half-life was estimated to be less than 10 hours. Respiratory failure and hypertension resolved in 4–10 hours in these victims, and toxin was not detectable in urine 20 hours after ingestion (15).

Treatment of PSP centers upon removing unabsorbed material from the gastrointestinal tract and supportive care. In severe cases, mechanical ventilation may be necessary. Neither antidote nor vaccine is currently available.

Toxins in clinical samples can be detected by several methods. High-performance liquid chromatography (HPLC) can detect individual toxins, but typically requires derivatization reactions (16,17). While this allows elucidation of toxin profiles that can provide valuable comparative data, it provides no information on toxicity. Receptor-binding assays based upon either rat brain membranes (18,19) or purified STX binding proteins from frogs or snakes (saxiphilins) (20) measure total biological activity without regard to toxin profile. All of these assays have been used to detect PSPs in the urine and serum of intoxicated victims (15). Immunologically based assays can detect major toxins such as STX or neosaxitoxin, but cross reactivity with other PSPs is highly variable. At present, the “gold standard” assay for PSP toxins in shellfish is still the mouse bioassay (21), although a rapid-throughput microtiter plate–based version of the receptor-binding assay (19) shows great promise as a regulatory screening tool.

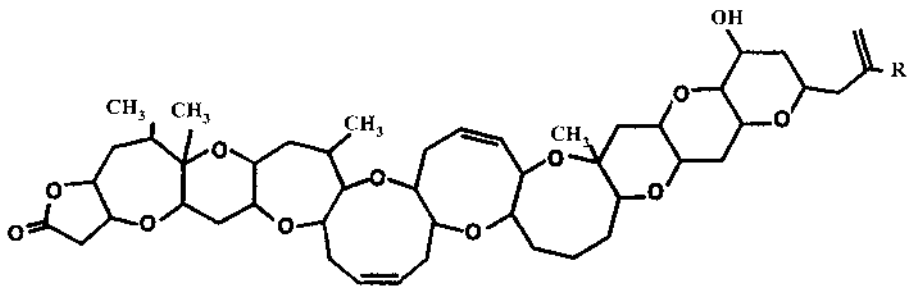
Many countries, including the United States, have established regulatory limits for PSP and closely monitor the commercial shellfish banks. In the United States the guidance level is 80 µg/100g of shellfish meat (22), and individual states monitor the toxin levels by mouse bioassay. Thus, commercially purchased shellfish is typically of low risk to the consumer. Recreationally harvested shellfish, however, are not monitored, and private individuals may not be cognizant of commercial regulatory restrictions. The greatest risk to the consumer is from shellfish harvested outside the regulatory sphere. For the recreational fisherman, the greatest safety will come from being aware of any harvesting prohibitions in the area and restricting recreational harvesting accordingly. While visible signs of toxicity, such as discoloration of the water or dead or dying fish, may be present, they are not reliable indicators of the shellfish safety.

## B. Neurotoxic Shellfish Poisoning

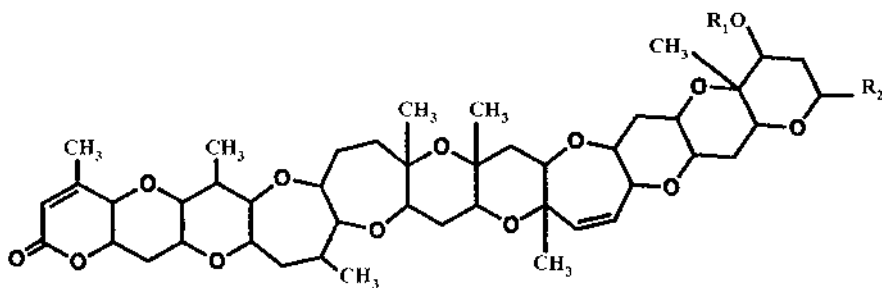
NSP results from the consumption of shellfish contaminated with brevetoxins (Fig. 2), a group of cyclic polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis* (formerly

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\* Toxicity in shellfish is routinely measured using a mouse bioassay. This assay measures the composite toxicity of the solution, which consists of a mixture of PSP components of varying toxicity. The assay results are compared to a standard curve derived from reference standard saxitoxin, and thus the total activity of the extract is expressed as “saxitoxin equivalents.” For pure saxitoxin, 1 mouse unit (MU) = approximately 0.2 µg.



A



B

**FIGURE 2** Polyether backbone structure of the PbTx-2-type (A) and PbTx-1-type (B) brevetoxins. Derivatization at R, R<sub>1</sub>, and R<sub>2</sub> account for the nine known members of this class of toxins, which are the causative agents in neurotoxic shellfish poisoning.

*Ptychodiscus brevis*). Like PSPs, brevetoxins accumulate in filter-feeding molluscs that are then consumed by humans (23). Unlike PSP, however, it appears that the causative agents in NSP are actually molluscan metabolites of the parent brevetoxins (24). This syndrome has historically been limited to the American states bordering the Gulf of Mexico, although in 1993 an outbreak of shellfish poisoning in New Zealand was identified as NSP. The causative organism was a novel *Gymnodinium* species (*Gymnodinium* cf. *breve*), which produced brevetoxin-like neurotoxins (2). In 1987, an outbreak of NSP was reported in North Carolina. In this case, a mass of Florida Gulf Stream water containing *K. brevis* was trapped in a warm-core eddy and was carried into coastal waters, where it remained intact for several weeks (25). During this time, 48 cases of NSP were reported. However, this rare concurrence of a sea surface temperature anomaly and favorable local conditions has not been repeated. Recently, a bloom of *Chattonella verruculosa* appeared in Rehoboth Bay, Delaware, coincident with mass mortalities of menhaden. Cell samples were found to contain significant levels of brevetoxins (Dr. Carmelo Tomas, University of North Carolina at Wilmington, personal communication). While no cases of human illness were reported, this event opens the possibility of a range extension of NSP into the Delaware and Chesapeake Bays.

Because *K. brevis* is an unarmored dinoflagellate and therefore relatively fragile, it is easily lysed by wind or wave action. Consequently, blooms are frequently associated with massive fish kills when lysed cells release toxins into the water column. These lipophilic compounds easily diffuse across gill membranes in fish, where they rapidly exert their toxicity. In an analogous manner,

brevetoxins can be aerosolized by wind, wave, and surf action and cause irritation, coughing, and burning of the throat and upper respiratory tract in beachgoers during coastal red tide blooms. In 1996, a mortality event occurred when at least 149 manatees were unable to escape a Florida red tide (26).

Brevetoxins and their metabolites bind to neurotoxin receptor site 5 on voltage-sensitive sodium channels where they alter the voltage-dependence of activation and inhibit channel inactivation (27,28). This results in inappropriate and prolonged channel opening.

Symptoms of NSP can manifest within an hour of consumption of contaminated shellfish. These typically include nausea, oral paresthesias, ataxia, myalgia, and fatigue. In severe cases, tachycardia, seizures, and loss of consciousness can occur, but a fatal case of NSP has never been reported. Treatment consists of removing unabsorbed material from the gastrointestinal tract and supportive care. Patients typically improve dramatically in 24–48 hours.

Brevetoxins are eliminated primarily in the bile, although urinary excretion plays a significant role. Animal models suggest that most of the toxin is eliminated within 48–72 hours, although some residue remains in lipophilic tissues for extended periods (29,30). Human pharmacokinetic data are very limited. However, a severe NSP outbreak occurred in Florida in 1996 when a family ingested whelks collected in Sarasota Bay. Two children were hospitalized with severe symptoms, including seizures. Brevetoxin metabolites were detected in urine samples collected 3 hours postingestion but were undetectable 4 days later (24). With supportive medical care, symptoms resolved in 48–72 hours.

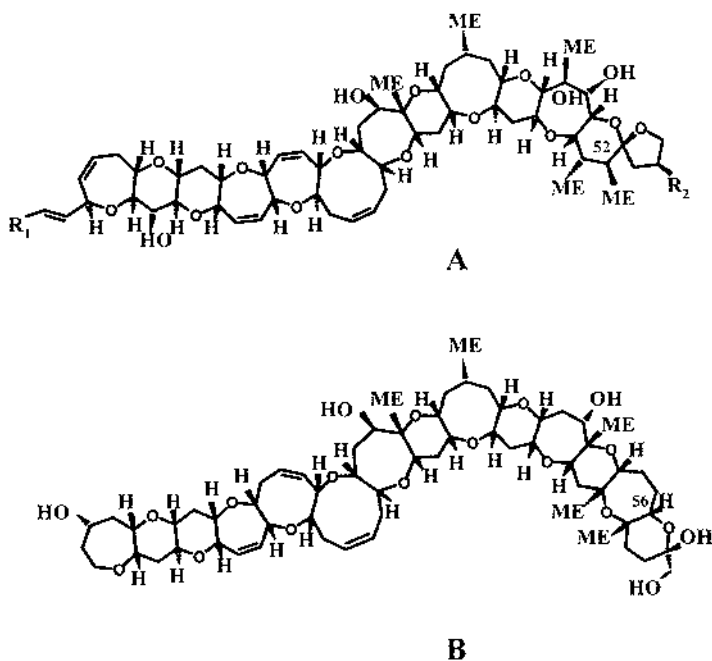
The toxic dose of brevetoxins in humans has not been established, although it is clear that eating only a few shellfish can result in severe intoxication. Toxins in clinical samples can be detected either by HPLC coupled to mass spectrometry, receptor-binding assays, or immunoassays (24). Because metabolic conversion of the parent toxins occurs in shellfish, and metabolites are less pharmacologically active than the parent toxins, it appears at this time that immunological assays are preferable as screening tools. However, the question of secondary metabolism in humans may impact this issue, and it awaits further study.

The FDA guidance level for brevetoxins in shellfish is 80 µg/100 g of shellfish tissue (22), and Gulf Coast shellfish are closely monitored. State laboratories monitor both toxin activity in the shellfish and *K. brevis* cell counts in the water column. Fishing grounds are closed when cell counts are significant and reopened when toxin activity in shellfish reach safe levels. For this reason, commercially harvested shellfish are very safe. Once again, the greatest risk occurs from recreational harvesters. As with PSP, avoiding intoxication is best accomplished by limiting consumption to commercially caught shellfish and avoiding privately caught shellfish of unknown origin. Recreational harvesters should keep abreast of closures and harvesting limitations set forth by state agencies.

### C. Ciguatera Fish Poisoning

CFP is a syndrome caused by exposure to ciguatoxins through the consumption of fresh fish. Like brevetoxins and PSP toxins, ciguatoxins originate with dinoflagellates, in this case the benthic species *Gambierdiscus toxicus*. This organism is an epiphyte, growing in association with filamentous algae on coral reefs and reef lagoons. Specific strains of *G. toxicus* produce precursors of ciguatoxins, which are ingested by grazing herbivorous fish and invertebrates (31). As these precursors move up the food chain to higher trophic levels through predation, they are metabolically modified to form a family of very potent neurotoxins (Fig. 3) (32,33). At present, over 20 members of this family have been identified. The Pacific form of the toxin varies slightly from the Caribbean form, although both are long cyclic polyether compounds reminiscent of the brevetoxins. Large, predatory reef-dwelling carnivores such as grouper, snapper, barracuda, and jacks are especially recognized as frequent carriers of ciguatoxins. However, small reef-dwelling herbivores can also cause ciguatera, especially when consumed whole. This is especially true in the tropical Pacific, where these small herbivores are more widely eaten.





**FIGURE 3** Polyether backbone structure of the Pacific (A) and Caribbean (B) forms of ciguatoxin, the causative agent in ciguatera fish poisoning. Derivatization at R<sub>1</sub> and R<sub>2</sub> and less energetically favorable epimers make up the more than 20 members of this class.

Although CFP occurs globally in tropical and subtropical latitudes approximately paralleling the distribution of reef-building corals, it occurs most frequently in the Pacific Ocean, western Indian Ocean, and the Caribbean. However, modern advances in the shipping of fresh fish has expanded the range of CFP to virtually anywhere in the world. Even within endemic regions, however, it is highly variable and spotty in distribution. In most areas only a small percentage of the large fish are toxic. Difficulties in predicting toxic areas and detecting toxicity in fish has always been a major impediment to the implementation of control measures.

CFP is estimated to affect more than 25,000 people annually (33), although substantial underreporting undoubtedly occurs. The symptomatology is complex. In severe cases, symptoms can develop in as little as 30 minutes; in milder cases onset can be delayed 24 hours or more. The early symptoms are typically gastrointestinal, including nausea, vomiting, diarrhea, and abdominal pain. These generally last only 24–48 hours and may co-occur with neurological symptoms such as tingling of the lips and extremities, reversal or abnormalities in hot/cold temperature sensations, and severe localized itching. These neurological symptoms occur in nearly all cases and are often accompanied by a wide range of other signs and symptoms (34). Fatigue, muscle and joint pain, and mood disorders such as anxiety or depression occur in 50% or more of cases (33). Severe cases may also manifest cardiac symptoms such as bradycardia and hypotension. There are also regional differences in symptomatology, probably resulting from regional differences in toxins (33). Although the gastrointestinal symptoms resolve early, neurological symptoms often persist for weeks or even months. Late in the course of recovery, symptoms may become episodic, recurring during periods of stress or after consumption of certain foods or alcohol.

Like brevetoxins, ciguatoxins bind to neurotoxin receptor site 5 on the voltage-dependent sodium channel and cause a hyperpolarizing shift in the voltage dependence of channel activation (33). For mammals they are the most potent sodium channel toxins known.

Treatment of CFP consists primarily of symptomatic care and preservation of electrolyte and fluid balances. While not beneficial in all cases, patients diagnosed early may respond to intravenous mannitol treatment (35,36). During the recovery period, avoiding the consumption of fish and alcohol are recommended.

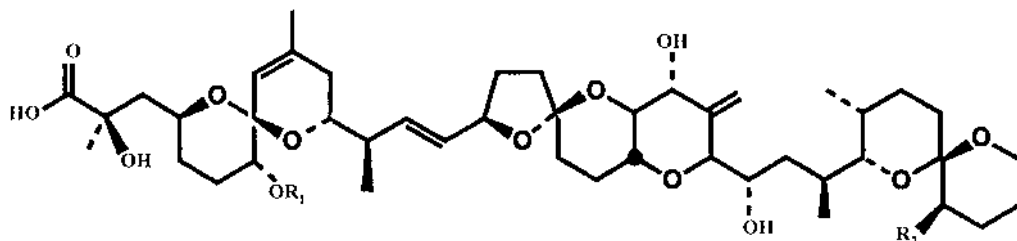
Ciguatoxins in fish are best detected with analytical methods such as liquid chromatography coupled to mass spectrometry. However, such analytical techniques are not useful for analyzing large numbers of samples. Because levels in fish tissues are typically in the parts-per-billion range or below, most techniques lack the required sensitivity. Development of immunological assays has been hindered by the lack of purified toxins with which to vaccinate animals for specific antibody production. Synthesis of the backbone structures is extremely difficult, but progress is being made for some of the Pacific forms (37,38). At present, the most useful *in vitro* assay available is still the competitive receptor-binding assay with rat brain membranes (18,31). This assay takes advantage of the fact that ciguatoxins and brevetoxins bind to a common receptor site and therefore radiolabeled brevetoxin can be used as the probe. It is sufficiently sensitive to detect ciguatoxins at levels that are believed to cause human intoxication but not to provide the desired safety margin for regulatory testing. Improved assays for these toxins are desperately needed in many parts of the world.

At present, there is no regulatory guidance level for ciguatoxins and the commercial fish harvests are not monitored. Some local jurisdictions have implemented bans on the sale of certain toxic species. The primary control measure for CFP is avoidance of toxic fish. For consumers this means being aware of the local situation with regard to prevalence and implicated species. It is prudent to avoid eating locally implicated species in particular and large coral reef-dwelling predators in general. If in doubt, eating small portions reduces the risk. Although screening individual high-risk fish is not yet fully feasible for either the seafood industry or the individual consumer, research continues for new assay technologies to achieve this goal.

#### D. Diarrhetic Shellfish Poisoning

Diarrhetic shellfish poisoning occurs after consumption of shellfish containing okadaic acid or its derivatives (Fig. 4). Okadaic acid was named for the black sponge *Halichondria okadai*, from which it was first isolated. However, it was later determined that the origin of the toxin was actually dinoflagellates of the genera *Prorocentrum* and *Dinophysis*. These organisms also produce at least seven okadaic acid derivatives denoted dinophysistoxins (DTXs). Other toxins, such as the pectenotoxins and yessotoxins, often co-occur with the DTXs in shellfish tissues. However, because these toxins differ from the DTXs in their mechanism of action and are much less potent orally, there is some question as to whether they should be considered as part of the DSP complex (8).

Toxic species of *Dinophysis* and *Prorocentrum* are distributed worldwide. Consequently, DSP is also widespread. It occurs seasonally and is a major problem to the shellfish industry in Europe and Japan, but has also been documented in South America, South Africa, New Zealand, Australia, Thailand, Mexico, Scandinavia, and Canada (2,39). The primary vector to humans is cultured or



**FIGURE 4** Diarrhetic shellfish poisons. In okadaic acid, R<sub>1</sub> and R<sub>2</sub> = H. Methylated or acylated derivatives make up dinophysistoxins 1–3.

wild mussels, which accumulate toxins in the digestive glands after filter-feeding. Although the toxin-producing species *Proocentrum lima* has been found associated with cultured mussels in Maine and low levels of okadaic acid have been measured in shellfish from the Gulf of Mexico (40), no outbreaks of DSP have yet been reported in the United States.

Symptoms of DSP intoxication in humans can occur within 30 minutes of ingestion and consist entirely of gastroenteritis, including nausea, vomiting, diarrhea, and abdominal pain. Symptoms typically resolve in 2–4 days. Treatment is symptomatic, including maintenance of fluid and electrolyte balances, and no deaths have been reported.

DSPs are potent inhibitors of serine/threonine protein phosphatases. Activity is highest for the PP2A class, lower for the PP1 class, and minimal or absent for the PP2B and PP2C classes of phosphatases (2,8,12). Diarrhea is thought to be a result of hyperphosphorylation of proteins controlling sodium secretion by intestinal cells (39), causing impaired water balance and fluid loss. In addition, through their effects on diverse protein phosphorylation and dephosphorylation reactions, DTXs can impact such diverse cellular processes as signal transduction, memory, cell division, and apoptosis (8). Whether these effects are important in human intoxications is not fully understood.

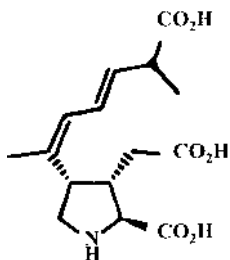
Both okadaic acid and at least one of the DTX derivatives are potent tumor promoters (41,42). The mechanism of this activity is thought to be increased phosphorylation of critical cellular proteins and/or intermediate filaments and changes in DNA gene expression resulting from phosphorylation of suppressor elements (12). Again, it is not known whether this activity poses a significant public health threat to the seafood consumer. However, the wide distribution of toxic *Proocentrum* and *Dinophysis* species and detection of low levels of DTXs in shellfish in diverse regions raises the question of whether chronic ingestion of subsymptomatic doses of these compounds could pose a health risk. One study, although only tentative in its conclusions, has suggested an association between long-term regular shellfish consumption and digestive cancers in France (43). More study of this issue is urgently needed.

Currently, the U.S. regulatory action level for DSP toxins is 20 µg/100 g (22). The minimum dose of the major DSP toxins necessary to produce symptoms in humans is estimated to be 35–40 µg (12). Internationally, guidance levels vary. Canada has informally adopted the Japanese limit of 5 MU/100 g of meat (about 20 µg/100 g) (44); the European community has no current standard but is in the process of adopting a limit of 16 µg/100 g of shellfish (Douglas McLeod, President, European Mollusc Producers Association, personal communication; Dr. Kevin James, Cork Institute of Technology, personal communication).

Detection of DSP toxins at the regulatory level is still dependent primarily upon the mouse bioassay (45). Other techniques, such as an in vitro phosphatase inhibition assay, immunoassays, and a variety of in vitro bioassays have been proposed and are currently in various stages of validation (17). In most areas of the world where DSP is a problem, shellfish stocks are closely monitored for the presence of toxins and the waters monitored for toxic dinoflagellates. Thus, as with other shellfish intoxication syndromes, the best recourse for avoiding DSP is to restrict consumption to commercially regulated shellfish products. Recreational harvesters, as always, should closely monitor the local restrictions on harvesting and remember that there will probably be no visible indicators of shellfish toxicity.

## E. Amnesic Shellfish Poisoning

ASP first came to the attention of public health authorities during an outbreak in Prince Edward Island, Canada, during the winter of 1987. In this event, over 100 people became ill after eating contaminated mussels, and 3 people died. The causative agent was soon identified as domoic acid (Fig. 5) (46). Domoic acid was not an unknown compound; it had been isolated from red macroalgae in 1958 (47) and was the active ingredient in an algal extract used as an anthelmintic in fishing villages in rural Japan. It had been evaluated and subsequently rejected as a potential insecticide. Consequently, it was quite surprising to discover link between domoic acid and an outbreak of human seafood intoxication. Even more surprising was the identification of the diatom *Nitzschia*



**FIGURE 5** Domoic acid, the causative agent in amnesic shellfish poisoning.

*pungens* f. *multiseriis* (now known as *Pseudo-nitzschia multiseriis*) as the causative organism. This remains the first and only known seafood toxin produced by a diatom. Since that time, several other species of *Pseudo-nitzschia* around the world have been demonstrated to produce domoic acid (48).

Since the first outbreak in Canada in 1987, the wide distribution of domoic acid-producing species of *Pseudo-nitzschia* has become clear. In 1991 a die-off of numerous cormorants and pelicans occurred in Monterey Bay, California. These birds had been feeding on anchovies containing high levels of domoic acid in their guts after filter-feeding during a bloom of *P. australis*. This bloom later moved up the coast and caused the toxification of razor clams and Dungeness crabs in Washington and Oregon. Several cases of human intoxication are thought to have resulted from the ingestion of these razor clams, although a definite connection was not made (49). In 1998, over 400 California sea lions died and numerous others displayed signs of neurological impairment in the Monterey Bay area during another bloom of *P. australis*. Again, high levels of domoic acid were detected in anchovies and in the feces of the sea lions (50). Domoic acid has since been found to be seasonally widespread along the Pacific coast of the United States (51) as well as the Gulf of Mexico. Around the world, domoic acid has been reported in such diverse locales as New Zealand, Mexico, Denmark, Spain, Portugal, Scotland, Japan, and Korea. Occasionally, levels in shellfish become sufficient to stimulate bans on harvesting. Fortunately, since the initial 1987 Canadian outbreak and the suspected cases in Washington in 1991, no further human cases have been reported. This is no doubt attributable to effective survey and monitoring programs.

Domoic acid is a neuroexcitatory amino acid, structurally related to kainic acid. It binds with high affinity to the kainate and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtypes of the glutamate receptor throughout the central nervous system and elicits nonsensitizing or very slowly sensitizing currents. The nonsensitizing nature of these currents causes a protracted influx of cations into the neurons through the receptor channels, stimulating a variety of intracellular biochemical events that lead to cell death in susceptible cells (52). Kainate and AMPA glutamate receptor subtypes are present in high concentrations in the hippocampus, a portion of the brain associated with learning and memory processing.

Symptoms of intoxication occur within hours of ingestion and include vomiting, diarrhea, or abdominal cramps within 24 hours and potentially confusion, disorientation, memory loss, and, in serious cases, seizures, coma, and death. The memory loss involves primarily short-term memory, and in the Canadian outbreak was more prevalent in elderly patients (53). Diverse neurological deficits may occur and can persist for months. Potential treatment regimens are discussed in Ref. 53.

Based upon levels measured in Canadian shellfish, it was estimated that mild symptoms can occur after ingesting about 1 mg/kg domoic acid; severe symptoms occur when 2–4 mg/kg are ingested. The current regulatory limit for domoic acid in shellfish in Canada, the European Community, and the United States is 20  $\mu\text{g/g}$  (22). Assuming a serving size of 250 g, this approximates an oral dose of 0.1 mg/kg or less, 10-fold less than the lowest reported toxic dose (3). Even so, the European Community is in the process of reducing their regulatory limit to 4.6  $\mu\text{g/g}$  in harvested

shellfish. The 20 µg/g limit will still apply to sales (Kevin James, Cork Institute of Technology, personal communication).

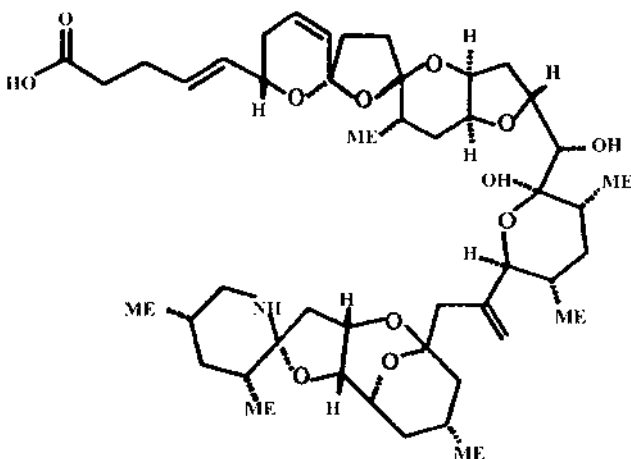
The official regulatory testing method for domoic acid in the United States and the European Community utilizes analytical HPLC. However, both immunological methods and a very simple and inexpensive thin-layer chromatographic method have been reported to work very well (17).

As with other recognized shellfish intoxications, avoiding toxic shellfish is paramount. Effective monitoring programs are in place and commercial products are typically safe.

## F. Azaspiracid Poisoning

In November 1995, at least eight people in the Netherlands became ill after consuming cultured mussels harvested in Killary Harbor, Ireland. The symptomatology included nausea, vomiting, diarrhea, and stomach cramps, and thus was reminiscent of DSP. However, analysis of the offending shellfish demonstrated negligible levels of either DSP or PSP toxins. After further investigation, a new class of cyclic polyether shellfish toxins, known as the azaspiracids (Fig. 6), was isolated from these toxic mussels (54). After the initial outbreak in the Netherlands, further outbreaks in 1997 and 1998 occurred in Ireland, France, and Italy, all of which traced back to mussels harvested in Ireland. During 1998–2000, monitoring efforts in Ireland showed that most of the major shellfish-producing areas experienced periods of contamination by azaspiracids (55). The causative organism in AZP is not yet known. However, the cyclic polyether nature of the molecules and their seasonal occurrence suggests a dinoflagellate origin (54).

Although the human symptoms of AZP are quite similar to those of DSP, animal studies have demonstrated major differences. As with okadaic acid, azaspiracid, when administered orally to mice at 500–700 µg/kg, caused necrosis, erosion of epithelial cells, and fluid accumulation in the small intestine. Unlike okadaic acid, however, azaspiracid also caused dilation of the stomach, hepatitis and fatty accumulation in the liver, and decreased lymphocyte counts in the thymus and spleen (56). While the effects of okadaic acid were transient, the deleterious effects of azaspiracids lasted for many days. These results were later confirmed in chronic exposure studies, which revealed that the stomach and intestinal damage took several months to heal (57). Even more importantly, this latter study revealed a tumorigenic property of the azaspiracids. At much lower doses (20–50 µg/kg) than that required for GI damage, azaspiracids caused lung tumors and hyperplasia in the stomach. And unlike the DSP toxins, these tumors occurred in the absence of added initiators.



**FIGURE 6** Azaspiracid. Methylated and acylated derivatives have also been isolated and associated with azaspiracid poisoning.

To date, only azaspiracids from mussels cultured in Ireland have caused human intoxication. ASP is a serious threat to the local industry, not only because of the seriousness of the intoxication, but because it occurs in the winter when shellfish are free of contamination by DSP toxins. In addition, toxicity can extend for many months. However, azaspiracids are not restricted to Irish waters. Levels below the current regulatory limits have recently been demonstrated in mussels from northeastern England and southwestern Norway (58). Once the causative organism is identified, a wider distribution of these toxins in shellfish will likely come to light. The European community is expected to soon endorse a regulatory limit of 16 µg/100 g shellfish tissue for all member nations (draft Commission Decision SANCO/2227/2001 Rev-4). An immediate need is increased worldwide surveillance.

Azaspiracids are inefficiently extracted by the procedures used to extract DSP or PSP toxins. However, efficient extraction techniques and a sensitive HPLC-MS assay procedure have been developed (59). Many regions may detect low levels of azaspiracids in shellfish, well below those required for acute intoxication. However, the tumorigenic properties identified by (56) will no doubt make this a sensitive issue. Much more research is urgently needed to better delineate appropriate safety levels for these compounds.

### III. SUMMARY

Marine foodborne biotoxins are a potential threat to human health through the consumption of various seafood products. Because these toxins occur naturally in fresh and otherwise wholesome foods, possess no visual or olfactory clues to their presence, and are impervious to typical cooking temperatures, they can be a difficult problem for both the industry and the consumer. To combat this problem most nations have employed vigorous monitoring programs to ensure the quality and wholesomeness of their seafood. For the most part, these programs are extremely effective. However, occasional outbreaks of poisoning occur, primarily from recreationally harvested seafood. The exception to this is CFP, where present technology is insufficient to adequately test the product. Luckily, CFP is relatively rare; only 205 cases were reported to the CDC from 1993 to 1997 (1). Because this number undoubtedly reflects significant underreporting, and because many areas of the world have a much higher incidence of CFP than the United States, there is an urgent need for the development of new testing technologies for these toxins.

For the recreational shellfish harvester, the best defense against biotoxins is to keep abreast of information from the state monitoring labs and apply the same standards as the industry. Consumers are best served by consuming only commercially harvested shellfish or that harvested personally or by a known source.

In the event of outbreak of seafood poisoning from any source, victims should seek medical care immediately. Urine and serum samples should be collected and frozen, and any remaining implicated seafood frozen and retained for investigators. Call the U.S. Food and Drug Administration's Center for Food Safety and Applied Nutrition for further instructions. These steps can aid in timely diagnosis and treatment of victims as well as supply important information on human pharmacokinetics and elimination of toxins. And, as was the case in Canada in 1987 and Ireland in 1995, new types of marine biotoxins are often discovered in this manner.

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## ***Entamoeba histolytica***

**Terry F. H. G. Jackson and Selvan G. Reddy**

*South African Medical Research Council, Durban, South Africa*

### **I. BACKGROUND**

*Entamoeba histolytica* is a protozoan parasite responsible for the disease amebiasis. Although the organism generally asymptotically parasitizes the human gastrointestinal tract, it can invade the intestinal mucosa, giving rise to amebic dysentery, and in some cases it spreads to other organs, primarily the liver, where it causes amebic liver abscess. It is estimated that amebiasis is responsible globally for approximately 70,000 deaths per annum and is the fourth leading cause of death due to a protozoan infection after malaria, Chagas' disease, and leishmaniasis (1).

The first definite description of *Entamoeba histolytica* can be attributed to Fedor Lösch in 1875 (2). Lösch described the clinical and autopsy findings of a case of fatal dysentery in a Russian migrant laborer. He recorded detailed descriptions of the amebae found in the stool and intestinal ulcers. He described their size, varying shape, their biphasic cytoplasm, the presence of ingested foreign bodies, among which he recognized red blood corpuscles, the characteristics of the nucleus, and the peculiar movements of the organisms. He concluded that these amebae were different from those known at that time and decided that they were a new species for which he proposed the name *Amoeba coli*. Although he was able to successfully infect dogs using stools from the autopsy and observed ulcerative lesions similar to that found in the migrant, Lösch did not attribute any etiological significance to the presence of these amebae in stools and lesions. Over the next 30 years, several authors added to these observations:

In 1891 Councilman and Lafleur (3) reported on detailed studies of lesions found in intestines and hepatic abscesses. They confirmed the pathogenic role of *Amoeba coli* by isolating them from bacteriologically sterile liver abscess and introduced the terms "amebic dysentery" and "amebic abscess of the liver."

Quinke and Roos, in 1893 (4), described the cyst stage of dysenteric ameba and successfully inoculated kittens with these cysts.

In 1903, Schaudinn (5) differentiated *E. histolytica* from *Entamoeba coli*.

In 1913, Walker and Sellards (6) fed cysts to human volunteers and conclusively proved that *E. coli* and free-living amebae were nonpathogenic to humans. They provided definitive proof of the pathogenic potential of *E. histolytica*.

In 1928 Emile Brumpt (7) introduced the concept that *E. histolytica* comprised two morphologically identical species. In his epidemiological surveys of temperate and tropical zones, Brumpt noted high infection rates with the parasite in certain regions where invasive disease was almost unknown. He concluded that most infections in temperate countries were due to a distinct species that was morphologically identical to the *Entamoeba* that caused invasive amebiasis. He proposed the names *E. dispar* for the nonpathogenic species and *E. dysenteriae* for the pathogen that was more prevalent in the tropical zones.

Although Brumpt's proposal best described the epidemiology of the parasite and has proved to agree closely with current opinion based on modern scientific analysis of the organism, it was never widely accepted during the following 50 years.

During the late 1970s and early 1980s, Sargeant and coworkers demonstrated that *E. histolytica* could be divided into two groups using isoenzyme electrophoresis (8). The first group was termed pathogenic *E. histolytica* and included all isolates from individuals with invasive disease and a few from asymptomatic carriers. The second group, termed nonpathogenic *E. histolytica*, were all isolated from asymptomatic carriers.

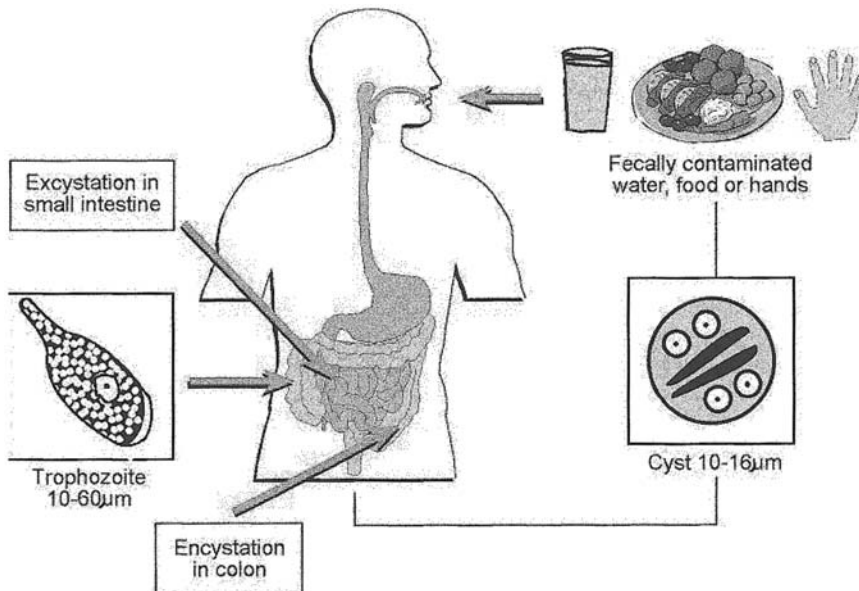
More recently, compelling genetic evidence (9,10) reinforced the "two-species theory" leading to a formal redescription of *E. histolytica* by Diamond and Clark in 1993 (11). They proposed that these two species be named *E. dispar* and *E. histolytica*. The latter is the causative organism of invasive amebiasis; the former is widely believed to be a gut commensal. This nomenclature has been incorporated into the latest WHO recommendations (12) and is now in use worldwide.

## II. CHARACTERISTICS

*E. histolytica* belongs to the protozoan genus *Entamoeba* of the rhizopodian order Amoebida. This genus is found within the family Entamoebidae, order Amoebida, and class Lobosea (13). Other species within the *Entamoeba* genus include *E. dispar*, *E. hartmanni*, *E. moshkovii*, *E. coli*, *E. gingivalis*, *E. polecki*, and *E. chattoni*. *E. histolytica* is the only species known to cause invasive disease.

### A. Trophozoite

*E. histolytica* trophozoites vary in size from 10 to 60  $\mu\text{m}$  (Fig. 1). Living trophozoites exhibit progressive, sometimes explosive, motility by means of a single well-defined finger-like pseudopodium.



**FIGURE 1** Diagrammatic representation of the life cycle of *Entamoeba histolytica*. Following ingestion of the infectious cyst stage, excystation occurs in the lower small bowel. Trophozoites mature in the cecum, migrate toward the rectum, and encyst. Infectious cysts are evacuated in the stool.

The single nucleus is not visible in unstained preparations. With stain, the spherical nucleus (4–7  $\mu\text{m}$  in diameter) has a small compact karyosome (0.5  $\mu\text{m}$  in diameter) that is usually centrally located but may be placed eccentrically. The nucleus is surrounded by a double membrane with pores and is lined by a single layer of small chromatin granules.

The cytoplasm is finely granular and rich in glycogen. Ingested bacteria and leukocytes are often visible in trophozoites from symptomatic individuals. The presence of ingested red blood cells was classically used to confirm a diagnosis of invasive infection with *E. histolytica*. Cytoplasmic organelles such as mitochondria, Golgi apparatus, rough endoplasmic reticulum, centrioles, and microtubules are lacking. The ribosomes are arranged in helical arrays that condense to form chromatoidal bars that are visible in the cyst stage.

## B. Cyst

Mature cysts are spherical in shape and contain four nuclei (Fig. 1). They range from 10 to 16  $\mu\text{m}$  in diameter. Although smaller, nuclei are similar in structure to those found in the trophozoite. In immature cysts glycogen may be concentrated into a discrete mass, but it becomes diffuse as the cyst matures. Chromatoid bodies made up of ribosomes, when present, are elongated cigar-shaped bars with bluntly rounded ends.

## C. Life Cycle

*E. histolytica* has a simple life cycle (Fig. 1) characterized by three distinct stages: trophozoite, precyst, and cyst. The cyst is the infectious form, which can be acquired by ingestion of contaminated food or water or direct fecal-hand-mouth transmission. The cyst is resistant to gastric acid, and on ingestion it passes into the small intestine. In the lower small bowel, a metacystic ameba containing the four cystic nuclei emerges from each cyst. Cytoplasmic division occurs and eight small metacystic trophozoites are formed. The trophozoites are carried to the cecum, where they complete their maturation. As trophozoites are carried towards the rectum, they become dehydrated, lose their cytoplasmic inclusions, and assume a spherical shape known as a precyst. The precyst forms a cyst wall and following two mitotic divisions matures into a quadrinucleate cyst. Cysts are evacuated in the stool and discharged into the environment.

## III. DISEASES

Infection with *E. histolytica* can result in a range of clinical presentations. Although most individuals remain asymptomatic, depending on various parasite and host factors, trophozoites may invade the gut mucosa, giving rise to amebic colitis, from here the amebae metastasize to the liver.

### A. Intestinal Infection

In most endemic areas the majority of intestinal infections are asymptomatic. However, in approximately 10% of infected individuals invasive amebiasis develops (14). Invasive amebiasis includes acute amebic colitis, fulminant colitis, and ameboma.

#### 1. Amebic Colitis

Onset of disease is usually gradual with abdominal discomfort or pain and frequent loose watery stools containing blood and mucus (dysentery). In individuals that have been ill for many weeks, profuse diarrhea has been observed (15). Stools may number 20 or more a day, leading to dehydration. Weight loss may be recorded.

During the early stages of invasion, ulceration of the colon and rectum may be superficial. The lesions are 1–5 mm in diameter and are rounded with slightly elevated areas of the mucosa. They have irregular necrotic centers surrounded by a rim of edematous tissue. Larger irregular ulcers

(30–50 mm) may also be present. There is usually normal mucosa between the sites of invasion, which becomes reduced as ulceration becomes more extensive and confluent.

### 2. *Fulminant Amebic Colitis*

Fulminant colitis is the result of confluent ulceration and necrosis of the colon. The bowel is usually dilated, particularly in the transverse position. Patients are severely ill with fever, leukocytosis, profuse bloody mucoid diarrhea, and widespread abdominal pain.

### 3. *Ameboma*

An ameboma may result from the repeated invasion of the colon by *E. histolytica* complicated by pyogenic infection. They are inflammatory thickenings of the bowel wall that are firm, hard, well-defined lesions resembling a carcinoma. They occur in 1% of patients with colonic amebiasis.

## B. Extraintestinal Disease

Amoebic liver abscess is the most common manifestation of extraintestinal disease and may occur in the presence or absence of intestinal symptoms. Onset is rapid (<2 weeks) with abdominal pain and fever (16,17). Pain is usually localized to the right upper quadrant and can be referred to the shoulder accompanied by a nonproductive cough. Anorexia, weight loss, nausea, vomiting, and fatigue may be present (16). In the case of a left lobe abscess, pain may be localized to the epigastrium.

Abscesses are usually single with the right lobe of the liver more often involved (5:1 ratio) than the left lobe. An abscess has a thin capsular wall with a necrotic center composed of a thick fluid, an intermediate zone of coarse stroma, and an outer zone of normal tissue. The “amebic pus”/abscess fluid is usually odorless, typically opaque, and variable in color from reddish to dirty brown or pink (16). The amebic pus is usually sterile except when secondary infection occurs. Liver abscesses may heal, rupture, or disseminate.

Pleuropulmonary amebiasis is the most common complication of amebic liver abscess. Invasion of the pleural cavity or lung parenchyma may occur through rupture or extension of the abscess. Symptoms include pain, cough, hemoptysis, or dyspnea (16). Pain may be pleuritic, occurring in the right lower chest or the tip of the shoulder. Cough may be productive if abscess material drains into the bronchus, and large amounts of reddish-brown, dirty brown, purulent, or frank pus may be expelled.

Peritoneal amebiasis results from the rupture of liver abscesses causing a sudden increase in abdominal pain. About 2–7% of cases result in peritoneal amebiasis (16).

Pericardial amebiasis is the most serious complication of an amebic liver abscess. Rupture into the pericardium may present as progressive tamponade or shock.

Other complications are rare and include cerebral, genitourinary, and cutaneous amebiasis.

## IV. EPIDEMIOLOGY

The true epidemiology of *E. histolytica* remains equivocal. Although there is a large body of literature based on the prevalence of *E. histolytica*, both worldwide and in selected populations, most of these data fail to distinguish between *E. histolytica* and *E. dispar*. Although the definition of amebiasis is “infection with the protozoan parasite *E. histolytica*,” the distribution of both this parasite and *E. dispar* will be considered in this section as they have been considered together in past epidemiological texts.

In 1986, Walsh (18) assessed the existing global prevalence data and concluded that in 1981, 480 million people harbored *E. histolytica* worldwide. She went on to extrapolate that about 36 million develop clinically overt disease, with 40,000–100,000 deaths each year. As these data do not distinguish between *E. dispar* and *E. histolytica*, revision of the prevalence and incidence estimations are necessary while that of mortality would remain the same. Unfortunately, reliable epidemiological

logical data on populations that do not have selection bias and from whom confirmed *E. histolytica* and *E. dispar* isolations have been made are limited. Studies over many years in Durban, South Africa (19), have indicated the overall prevalence of both *E. histolytica* and *E. dispar* together to be 10%, with *E. histolytica* accounting proportionately for 1% and *E. dispar* for 9%. Interestingly, the situation in Durban may be a reflection of the overall estimates of 10% prevalence worldwide (18,20). Consequently, the global prevalence of *E. histolytica* could be approximately 50 million and that of *E. dispar* approximately 450 million.

*E. histolytica* and *E. dispar* are ubiquitous organisms but are more prevalent in the tropics and subtropics. The highest incidence is usually found in communities with poor socioeconomic conditions and poor sanitation. This is believed to be related to poorer sanitation and nutrition and decreased resistance in those living in these environments. Recognized high-risk areas for acquiring amebiasis include Mexico, the western portion of South America, West Africa, South Africa (particularly in the black population), parts of the Middle East, and South, and Southeast Asia. Invasive disease seems to be more common in these areas. Many of the cases identified in North America and in Europe are imported, but a level of endemicity is present and occasional waterborne epidemics have occurred (18,21).

*E. histolytica*/*E. dispar* prevalence may vary with changes in seasons. High prevalence is usually associated with the onset of rainy seasons with lower levels as the rainy season progresses (22). However, high prevalence of infection has been reported in Nigeria during the dry months of the year, associated with the use of contaminated water resulting from limited supplies (23). In addition to temperature and rainfall, humidity has also been implicated in parasite transmission with a higher prevalence at the coast (52.3%) when compared to the highlands (13.7%) (22).

Accurate determination of the true prevalence of amebiasis is difficult. Surveyed populations may differ, and the prevalence in certain groups is higher than in the general population, e.g., in families of infected patients, in male homosexuals, and in persons in mental hospitals, prisons, and institutions for children. Differences in laboratory techniques, competence of laboratory personnel, stool-collection methods, intermittent excretion of cysts in the stool, and number of specimens examined from each person may lead to either under- or overdiagnosis. Most studies rely on examination of one stool specimen. This approach will detect only 40–50% of those infected, while three consecutive examinations on separate days can be expected to yield 60–80%; >90% can be identified if more than five examinations are performed (18). A study in the Gambia using single stool examinations indicated that an average of 20–30% of the population excreted cysts at any one time, but longitudinal follow-up over a year proved that almost all the study subjects (>98%) excreted cysts at some time (22).

In a study of 1381 asymptomatic individuals living in a semi rural endemic area South of Durban, South Africa, *E. dispar* occurred more frequently in females of all age groups whereas asymptomatic *E. histolytica* infection was equally common in males and females (19). In a subsequent longitudinal study of asymptomatic carriers of *E. histolytica*, infections were equally common in males and females (14). This is at variance with the higher prevalence of invasive amebiasis in males than females, which has been reported to be in a ratio of 2:1 (males:females) in patients with amebic dysentery and 7:1 in patients with amebic liver abscess (24). It seems that while males and females have an equal chance of being asymptotically infected with *E. histolytica*, females are protected by some mechanism from acquiring invasive amebiasis.

Sexual transmission of *Entamoeba* has been widely documented, especially among homosexual men (25–27). In 1978 the New York City Department of Health recorded 1875 infections with *E. histolytica*/*E. dispar* in the male homosexual population; this exceeded city totals for most of the other major infectious diseases of public health importance, such as tuberculosis (1307) and hepatitis (1260), and approached that of primary and secondary syphilis (2060) (28). In Toronto, Canada, *E. histolytica*/*E. dispar* infections were observed in 27% of homosexual men compared to only 1% of heterosexual men (25) and notably no cases of amebic colitis or liver abscess were documented in any of these cases, implying that they were all *E. dispar* infections. In London, isoenzyme electrophoretic studies revealed that 11.1% of male homosexuals harbored *E. dispar* (26). The above-

mentioned three reports are a few of the substantial number in which it appears that homosexuals in western countries have a high prevalence of infection with nonpathogenic *E. dispar* based most commonly on the absence of clinical symptoms and negative serology as well as a few reports employing isoenzyme characterization. It is, however, highly unlikely that male homosexuals in western countries are refractory to infection with pathogenic *E. histolytica*. This is borne out by a case of amebic liver abscess in a male homosexual described by Thompson et al. (29) and by reports from Japan where amebic infection was first observed in male homosexuals with invasive amebiasis and where more than 50 cases have been detected (27). The anomaly observed in western countries could be explained in part by once-off *E. dispar* in these regions and in part as an outcome of the studies relying on recruitment of study subjects from sexually transmitted disease clinics in preference to gastroenterology clinics. Considering the comments above regarding sampling procedures and the prevalence levels, it is highly likely that a much higher proportion of subjects are infected with *E. histolytica*/*E. dispar* in homosexual populations than reported in the supporting literature.

In a semi-rural area south of Durban, South Africa, 9% of asymptomatic individuals were found to be infected with nonpathogenic *E. dispar*, while 1% infected with *E. histolytica* (19). The 1% with *E. histolytica* represent a public health hazard as they are reservoirs of infection with a pathogenic organism. Asymptomatic carriers are more likely to spread the disease than symptomatic patients with invasive amebiasis because the latter individuals will seek medical attention. The necessity to identify and treat asymptomatic carriers of *E. histolytica* is emphasized by the observation that 10% of them develop invasive amebiasis in due course (14). In those who do not develop invasive amebiasis, "self-cure" apparently occurs within 12 months (14); this observation concurs with that of Nanda et al. (30), who followed subjects in India with both *E. histolytica* and *E. dispar* and found that all had apparently undergone "self-cure" within 19 months. It is important to note that the serological response in asymptomatic carriers of *E. histolytica* is as marked as that seen in patients with invasive amebiasis, implying subclinical tissue invasion (31).

*E. histolytica* occurs more frequently in family groups and closely associated individuals (14). This phenomenon has been reported by others (32,33) in studies in which differentiation between *E. histolytica* and *E. dispar* was not attempted and highlights the importance of direct person-to-person transmission. Overcrowding can therefore be expected to increase incidence rates. Furthermore, the persistence of infection with *E. histolytica* for 6–12 months without developing invasive amebiasis provides good evidence that a carrier state does exist. Good public health practice would require follow-up and treatment, where necessary, of all contacts of patients with invasive amebiasis as well as asymptomatic carriers of *E. histolytica*.

## V. ISOLATION AND IDENTIFICATION

Although food and water are sources of contamination by *E. histolytica* cysts, isolation from these sources is generally not attempted. In epidemic situations, water sources may be examined and stool from food handlers may be investigated for the presence of cysts. Generally stool and serum specimens from infected individuals are assessed using available laboratory tests/kits.

### A. Microscopy

Microscopic observation of cysts is not considered useful for differentiating *E. histolytica* from *E. dispar* as they are morphologically similar. When quadrinucleate cysts are observed, they should be reported as *E. histolytica*/*E. dispar* (12). Furthermore, microscopy is relatively insensitive when performed on a single specimen (40–50%); however >90% of cyst passers can be identified if more than five consecutive specimens are examined (18). The observation of hematophagous trophozoites in direct wet mount preparations of stool confirms the diagnosis of *E. histolytica* infection.

## B. Serology

Serological methods can be used to differentiate between *E. histolytica* and *E. dispar* infection. *E. histolytica* and not *E. dispar* is responsible for eliciting a serological response in humans whether they are symptomatic or not (31,34). In an endemic area a positive serological response indicates current or past infection with *E. histolytica*, and in nonendemic regions seropositivity has a high diagnostic specificity for current *E. histolytica* infection (35,36).

In endemic regions a combination of serology and stool microscopy can be useful in differentiating between *E. histolytica* and *E. dispar*. Regardless of symptoms, the presence of quadrinucleate cysts in the stool and a negative serological response indicates that the cysts are those of *E. dispar*.

## C. Isoenzyme Analysis

The culture of stool samples (37) followed by isoenzyme analysis (38) can accurately distinguish between *E. histolytica* and *E. dispar*, and this has been considered to be the gold standard for differential diagnosis. However, this is a time-consuming and fastidious process and as a result has been limited to a few laboratories in various parts of the world. Typically, lysates generated from cultured amebae are electrophoresed on starch gels. The migratory patterns of the isoenzymes malic enzyme (ME), phosphoglucomutase (PGM), glucose phosphate isomerase (GPI), and hexokinase (HK) are then assessed. Based on the migration of the bands, the amebae may be characterized into one of more than 20 zymodemes (strains allocated on the basis of the particular isoenzyme patterns observed). Initially, all these zymodemes were attributed to *E. histolytica* before its separation from *E. dispar* was widely accepted, but most importantly they were categorized as pathogenic zymodemes (PZ) and nonpathogenic zymodemes (NPZ), according to their observed association with invasive amoebiasis. Consequently in older texts PZ is a synonym for *E. histolytica* and NPZ for *E. dispar*.

## D. Antigen Capture Tests

The development of assays for detection of amebic antigen in feces has been designated a priority in amoebiasis research by the WHO (12). Antigen capture assays offer many advantages over existing tests such as determination of the currency of infection, indication of extent of infection, increased objectivity, and the potential for automation. Several enzyme-linked immunosorbent assays (ELISAs) have been developed to detect amebic antigens in fresh stool specimens (39–44). Although all ELISAs detect amebic antigens, only two are known to differentiate between *E. histolytica* and *E. dispar* (39,42). Both these tests utilize monoclonal antibodies to the galactose/*N*-acetylgalactosamine–inhibitable lectin, a molecule that is conserved and highly immunogenic. In addition to detection of antigen in stool samples, Abd-Alla et al. (39) reported the successful detection of antigen in serum. When compared to stool culture–detection methods, these ELISAs are relatively rapid, sensitive, and specific.

## E. PCR-Based Methods

Several methods that amplify and detect *E. histolytica* DNA in stool samples have been described (45–49). They offer high specificity and sensitivity, detect minute amounts of parasite material, and are fast. When used to detect the presence of trophozoites in culture, PCR amplification and detection of small ribosomal RNA (rRNA) genes is 100 times more sensitive than the best available ELISA kit for the detection of *E. histolytica* antigens (50). Clark and Diamond (10) described a technique known as “riboprinting,” which permits differentiation by examination of restriction enzyme site polymorphisms in PCR-amplified small subunit ribosomal RNA genes. Apparently riboprinting can be used for both intra- and interspecific differentiation.



## VI. PATHOGENICITY

Following the ingestion of cysts and excystation in the small bowel, trophozoites colonize the colon. If trophozoites are abundant, there is an increased chance that some will make contact with the mucosa long enough to grow, multiply, and eventually invade the tissues. Several factors contribute to the pathogenicity of *E. histolytica*. Three such factors have been extensively studied and characterized at the molecular level:

Adherence of trophozoites to host cells predominantly mediated by a galactose/*N*-acetylgalactosamine–inhibitible surface lectin

Killing of host cells by pore-forming peptides known as amebapores

Proteolysis of the host's extracellular matrix mediated by cysteine proteinases

Galactose/*N*-acetylgalactosamine–inhibitible lectin (Gal/GalNAc lectin) is a multigene family of 260 kDa heterodimers consisting of heavy (170 kDa) and light (35/31 kDa) subunits linked by disulfide bonds (51). The heavy subunit has been implicated as the molecule that effects adherence as it is recognized by adherence-inhibitory monoclonal antibodies (52). This surface lectin mediates attachment of the *E. histolytica* trophozoites to rat and human colonic mucosa and submucosa (53), human leukocytes (54), Chinese hamster ovary and Chang liver cells (55), and certain bacteria. Monoclonal antibodies to the lectin inhibits the adherence and cytotoxicity of *E. histolytica* to human cells in vitro (56). In addition to its role in adherence, the lectin has been implicated in the cytolytic process. In in vitro experiments, contact-dependent lysis of target cells is diminished in the presence of galactose. Further, monoclonal antibodies to the heavy subunit partially inhibited cytolysis without affecting adherence (57).

Another interesting feature of the lectin is that it binds to purified C8 and C9 complement components, blocking the formation of the complement membrane attack complex on the amebic plasma membrane. This suggests a possible role in mediating amebic resistance to complement lysis through components C5b through C9 (58).

Amebapores are a small group of pore-forming proteins contained in the cytoplasmic granules of the trophozoite. They are able to bind and insert into lipid membranes forming ion channels in the target cells (59). Ions and other molecules are able to pass through these channels, changing the contents of the target and ultimately resulting in its lysis. In addition to their ability to kill nucleated cells, amebapores are able to disrupt the activity of bacterial membranes (60,61). This antibacterial activity has been proposed to be the primary physiological role of amebapores as in the colon large numbers of bacteria are usually phagocytosed by the parasite.

Three isoforms of amebapores, namely, A, B, and C, have been identified (60). They are made up of 77 amino acid residues, have a 35–57% sequence identity, and are found in a ratio of 35:10:1, respectively. Although amebapore C is least abundant, it has substantially higher cytolytic efficacy. While amebapore genes have been demonstrated in nonpathogenic *E. dispar* (62), only amebapore A and B proteins are detected in their lysates and in reduced concentrations compared to that found in *E. histolytica*. Amebapore C is virtually absent from *E. dispar*, possibly explaining its inability to destroy nucleated cells.

Cysteine proteinases are considered to be a significant virulence factor in the pathogenesis of amebiasis and have been suggested to play vital roles in tissue invasion, disruption of host tissues, and modulation of the cell-mediated immune response (63–65). Cysteine proteinases, ranging in size from 16 to 116 kDa, are the most abundant proteolytic enzymes found in amebic extracts (66–69). During their passage to deeper layers of the intestine, trophozoites must lyse surrounding cells and degrade the extracellular matrix (ECM) components of the colonic mucosa. The potential role of the cysteine proteinases in the latter activities is supported by its ability to degrade purified fibronectin, laminin, and collagen (70–72). Furthermore, cultured fibroblasts are disrupted by purified *E. histolytica* cysteine proteinases (73). Although cysteine proteinases are found in both *E. histolytica* and *E. dispar*, much lower activities have been recorded in the latter (74,75).

Cysteine proteinases from *E. histolytica* can interfere with the host immune system. The proteinases are able to specifically cleave complement component C3, which enables *E. histolytica* to activate complement in the fluid phase (76). The proteinase also degrades immunoglobulin A (IgA) and the anaphylatoxins C3a and C5a, which may explain the relative paucity of neutrophils noted in amebic liver abscesses (77). By inhibiting cysteine proteinases, using specific inhibitors, amebic liver abscess formation can be diminished (78,79).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

Evidence based on zymodemes (80), restriction fragment length polymorphism (49), and nucleotide sequences of highly conserved genes (11) is highly suggestive that the two species, *E. histolytica* and *E. dispar*, are genetically different. However, there is not yet an adequate explanation of the lack of the invasive property of *E. dispar*.

Although the estimated genetic distance between *E. histolytica* and *E. dispar* is as great as that between human and mouse (10), the three well-characterized groups of molecules implicated in pathogenesis and virulence, namely, galactose/*N*-acetylgalactosamine–inhibitable lectin (81), amebapores (62), and cysteine proteinases (74), are found in both species. At a glance it appears that the differences between the two species are quantitative rather than qualitative, but there is emerging evidence that differences may exist at the gene level. The most striking difference reported thus far was found in the expression of cysteine proteinases. Compared to *E. histolytica*, *E. dispar* contains much less cysteine proteinase activity, apparently as a result of a lower number of cysteine proteinase genes (74,75). Six genes (*ehcp1*–*ehcp6*) encoding cysteine proteinases in *E. histolytica* have been identified, four of which (*ehcp1*, *ehcp2*, *ehcp3*, *ehcp5*) are expressed in cultured trophozoites. At least 90% of the total cysteine proteinase activity can be attributed to *ehcp1*, *ehcp2*, and *ehcp5* (74). Functional genes homologous to 2 (*ehcp1* and *ehcp5*) of the *E. histolytica* genes are absent in *E. dispar*. Recently, analysis of respective genomic regions from *E. histolytica* and *E. dispar* show that a sequence corresponding to *ehcp5* is present and positionally conserved in *E. dispar* (82). However, the gene is highly degenerated and does not contain any overt open reading frame, suggesting that the gene has been nonfunctional for a considerable period of time during the evolution of the nonpathogenic ameba species. There is about 95% shared similarity between *ehcp2*, *ehcp3*, *ehcp4*, and *ehcp6* in *E. histolytica* and *E. dispar*.

Although the genetic make-up of the organisms is slowly being unraveled, there is limited information on genes vs. virulence. Currently, an *Entamoeba histolytica* Genome Project is underway at The Institute for Genomic Research in Washington, D.C. The project aims to determine 99% of the genomic sequence of *E. histolytica* strain HM1:IMSS, analyze and annotate the data, and provide ready equal access to the sequence information and analysis.

## VIII. CONTROL MEASURES

Strategies for prevention of spread of amebiasis in endemic areas must be multifaceted. Although provision of clean water supplies and adequate sanitation facilities is crucial, health education of individuals is also important as good hygiene principles are important in parasite transmission. Furthermore, early detection and treatment of cases of infection will assist in curbing the spread.

Amebic infection may be prevented by eradicating fecal contamination of food and water. The most commonly contaminated foods are fresh, ground-grown vegetables such as lettuce. Freshening of vegetables and fruits with contaminated water and using human feces as fertilizer may result in serious contamination of fruits and vegetables that are usually eaten raw. Fruits should ideally be peeled, whereas vegetables should be thoroughly washed in weak detergent and rinsed in treated potable water. Soaking the fruits or vegetable in vinegar for 10–15 minutes will ensure eradication of cysts.

Water is always a prime source of infection. Fecal contamination of springs, unprotected shallow wells, and streams may occur as a result of surface runoff from areas where there is indiscriminate defecation. Contamination may also result from discharge into rivers. In endemic areas, water should be boiled before drinking (temperatures of  $>60^{\circ}\text{C}$  will kill cysts). Water may also be effectively treated with iodine water purification tablets, tincture of iodine, or liquid chlorine laundry bleach.

Travelers to endemic areas should avoid eating uncooked vegetables and fruits. As normal levels of chlorination in drinking water do not kill *E. histolytica* cysts and neither does freezing, the drinking of tap water in such areas, including the use of ice, is not recommended.

Asymptomatic carriers of *E. histolytica* are important sources of infection as they can pass large numbers of cysts in their stools. Where it is possible, carriers should be identified and prompt chemotherapy administered. In the male homosexual population, avoiding sexual practices that allow fecal-oral contact can prevent infection. In institutionalized individuals, especially the mentally retarded, improved supervision and hygiene may be beneficial.

The future hope in control and prevention lies in vaccine development. Three different *E. histolytica* proteins have formed the basis for recombinant antigen vaccines: the serine-rich *E. histolytica* protein (SREHP), the Gal/GalNAc-binding lectin, and the 29 kDa antigen. All three have proven extremely effective in prevention of amebic liver abscess in gerbils (83–86). However, no data are available on the efficacy of any of these candidate vaccines in preventing intestinal amebiasis. There is currently no reliable animal model for intestinal amebiasis. As a result, it may be difficult to appropriately assess the protective efficacy of any candidate vaccine in preventing intestinal amebiasis before human trials can be initiated.

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# 27

## *Giardia lamblia*

Rodney D. Adam

*University of Arizona College of Medicine, Tucson, Arizona, U.S.A.*

### I. BACKGROUND

*Giardia* is a flagellated protist that belongs to the order Diplomonadida (two karyomastigonts, eight flagella, two nuclei, and no mitochondria or Golgi) and the family Hexamitidae (along with the mole parasite *Spironucleus muris* and the free-living organism *Hexamita inflata*). The assignment to the family Hexamitidae was based on morphological criteria, but is also supported by molecular phylogenies (1,2).

*Giardia lamblia* was initially described by Leeuwenhoek in 1681 as he was examining his own diarrheal stool under the microscope. His description, as translated by Dobell (3), is as follows:

I have ordinarily of a morning a well-formed stool; but now and then hitherto I have had a looseness . . . when I went to stool some 2, 3, or 4 times a day. . . . My excrement being so thin, I was at divers times persuaded to examine it. . . . All the particles aforesaid lay in a clear transparent medium, wherein I have sometimes also seen animalcules a-moving very prettily; some of 'em a big bigger, others a bit less, than a blood-globule, but all of one and the same make. Their bodies were somewhat longer than broad, and their belly which was flatlike, furnished with sundry little paws, wherewith they made such a stir in the clear medium and among the globules, that you might e'en fancy you saw a pissabed (woodlouse) running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but slow progress.

Lambl independently discovered *Giardia* in 1859 and described it in greater detail (4), and the organism was subsequently named after him. The name *Giardia* was first used in 1882 for organisms from a tadpole. A proliferation of species names ensued based on host of origin. However, only three morphological types were distinguishable by light microscopy: *G. agilis* from amphibians, *G. muris* from rodents, and *G. lamblia* (also *G. duodenalis* or *G. intestinalis*) from a variety of mammals and birds (5). Subsequently, several additional species have been described that appear similar to *G. lamblia* by light microscopy but can be distinguished from *G. lamblia* by electron microscopy: *G. ardeae* (herons), *G. psittaci* (psittacine birds), and *G. microti* (voles and muskrats) (6). Even within the organisms called *G. lamblia*, there are a number of distinct genotypes, only some of which have been identified in humans. The bird isolates previously classified within *G. lamblia* (*G. ardeae* and *G. psittaci*) have not been identified in mammalian specimens. Likewise, *G. lamblia* sensu strictu have not been found in birds.

*G. lamblia* sensu strictu consists of a species complex with multiple genotypes that can be distinguished from each other on the basis of enzyme electrophoresis (zymodeme analysis), restriction fragment length polymorphism (RFLP), and related forms of DNA typing, and by sequence analysis. Two major genotypes, A-1 and 2 (Nash Groups 1 and 2, Mayrhofer Assemblage A, Polish isolates) and B (Nash Group 3, Mayrhofer Assemblage B, Belgian isolates), have been associated

with infections in humans and a variety of other mammals, including beavers and cats (6). Although some dog isolates have belonged to genotypes known to infect humans, the majority of dog isolates have belonged to genotypes not associated with human infections (7,8). Likewise, most livestock isolates have belonged to genotypes not yet documented as causes of human infections (9). Thus, the zoonotic potential for *Giardia* isolates from dogs or livestock is uncertain at this time.

Despite its early recognition, *G. lamblia* was not generally accepted as a human pathogen until the 1960s after its association with a definable clinical syndrome and outbreaks of diarrheal disease. The evidence for the pathogenicity of *G. lamblia* came from two approaches. First, a number of investigators described the presence of trophozoites along with abnormalities of the small intestinal mucosa in patients with diarrhea. Second, a number of epidemiological studies showed a link between diarrhea and the presence of *Giardia* trophozoites or cysts in fecal specimens (10). Subsequently, *G. lamblia* became quickly recognized as the most common cause of waterborne outbreaks of diarrhea (11).

## II. CHARACTERISTICS

The *Giardia* species have a two-stage life cycle. Infection is initiated when the environmentally stable cyst is ingested, followed by excystation to form two trophozoites. Excystation occurs when the cyst passes through the acidic environment of the stomach followed by entry into the duodenum. However, excystation has been induced in bicarbonate at a neutral pH, and achlorhydria does not prevent human infections; these observations indicate that acidic pH is not required for excystation. Pancreatic and endogenous proteases probably play an important role in excystation. The trophozoite is the vegetative form, colonizing and replicating in the proximal small intestine, where it replicates and causes disease. Trophozoites then encyst in the distal small intestine, and the cysts are passed in the feces to continue the cycle of infection. Encystation is induced by exposure to a mildly alkalotic pH and conjugated bile salts plus fatty acids (12,13), or possibly by cholesterol starvation (14). Cytokinesis is nearly complete before encystation, so each cyst in effect contains two trophozoites with a total of four nuclei. The cyst is covered by a hard filamentous layer composed primarily of *N*-acetylgalactosamine. It has a metabolic rate substantially less than that of the trophozoite, allowing it to persist for a long period of time in the environment. The cyst is infectious as soon as it is passed in the feces and thus represents an immediate risk for transmission of infection.

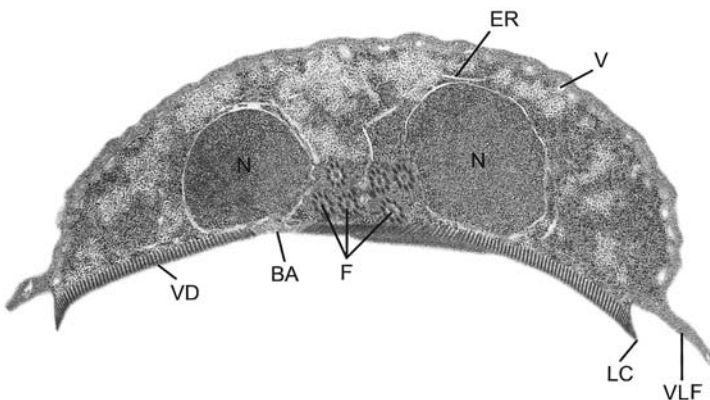
The trophozoites are pear-shaped and are approximately 12–15  $\mu\text{m}$  in length and 5–9  $\mu\text{m}$  in width (Fig. 1). They have a convex dorsal surface and a concave ventral surface that attaches to the intestinal epithelium (or glass with *in vitro* cultivation) by mechanical means. They have four pairs of flagella and two symmetrically placed nuclei that are similar in all ways that have been studied (Fig. 2). Each has a complete genome (15), is transcriptionally active (16), and divides at approximately the same time (17). It is not known why the trophozoites have two nuclei. A number of organelles typical of eukaryotes are absent from *Giardia*, including nucleoli, peroxisomes, and mitochondria. The mitochondrial genes, *cpn60* (18) and *valyl-tRNA synthetase* (19), have been identified in *Giardia*, suggesting the possibility that they may have had mitochondria at one time (or a premitochondrial symbiont). Endoplasmic reticulum is present, and even though Golgi are not readily visible in vegetative trophozoites, they can be seen in encysting organisms (20), and the typical Golgi functions can be found in vegetative trophozoites (21). Multiple vacuoles with lysosomal and endosomal functions can be found in the periphery of the cytoplasm. The median body is an organelle characteristic of *Giardia* and is a part of the cytoskeleton, but its function is not known. Its claim to fame is that it allows distinction of *Giardia* trophozoites into the three major morphological types.

The metabolism of the trophozoites is strictly anaerobic, in keeping with the lack of mitochondria (6). The pathways of oxidative phosphorylation are absent, and ATP is generated by substrate level metabolism. The trophozoites have relatively little capacity for *de novo* synthesis of the major building blocks. Purines and pyrimidines are obtained from the host, as are most amino acids and





**FIGURE 1** Fluorescence in situ hybridization (FISH) showing the location of the nuclei with Yo-Yo 1 and showing the trophozoite outline by overexposure after hybridization with the  $\beta$ -actin gene (15). (Photograph courtesy of LiZhi Yu.)



**FIGURE 2** Trophozoite coronal section—a coronal view of a trophozoite demonstrates the nuclei (N), endoplasmic reticulum (ER), flagella (F), and vacuoles (V). A mechanical suction is formed when the ventral disk (VD) attaches to an intestinal or glass surface. Components of the ventral disk include the bare area (BA), lateral crest (LC), and ventrolateral flange (VLF). A magnified view of the ventral disk is shown in [Figure 10](#). (From Ref. 6.)

fatty acids. Glucose is the only carbohydrate known to be an energy source, while the amino acids arginine and aspartate may also be important sources of energy.

Because of its rudimentary metabolism and the lack of certain organelles that are characteristic of eukaryotes, *Giardia* was suspected of being an early diverging eukaryote. This was supported by small subunit rRNA gene sequencing (22) and subsequently by phylogenetic trees of other genes (6). The basal phylogeny has been questioned (23), but the bulk of evidence continues to support its early divergence (6).

### III. DISEASES

The spectrum of disease from *G. lamblia* infection varies from severe weight loss and malnutrition to an asymptomatic state. The percentage of patients with symptoms varies greatly in different studies from greater than 90% in some studies of travelers and water- or foodborne outbreaks to no greater than the background level of diarrhea in some studies in daycare centers and in developing nations. The reasons for these differences are not well understood but probably include different levels of host immunity as well as difference in virulence of the infecting organisms. The incubation period is approximately one week but can be as short as 1–2 days and as long as 2 weeks. In two groups of infected human volunteers, the average incubation period was 7 days, with a range of 6–9 days (24). Perhaps the narrow time range of the incubation period was due to the uniformity of the method and inoculum size of the infecting dose.

An understanding of the illness associated with *Giardia* infections has been facilitated by epidemiological investigations of point source outbreaks of disease, as well as human volunteer studies. The initial human volunteer studies were conducted by Rendtorf in prisoners in 1954 (25). The volunteers were administered *Giardia* cysts and developed patent infections. Four of six men given 10 or 100 cysts developed infection; two from 10 cysts and two from 100 cysts. When given larger numbers of cysts (>10,000), all developed infection and excreted cysts for 5–41 days. However, none of the subjects developed symptoms. These results demonstrated that human infection could be readily induced with as few as 10–100 cysts but cast doubt on the pathogenicity of *Giardia*. In the 1960s, a number of reports associated waterborne *Giardia* infections with outbreaks of diarrheal illness. In a large outbreak among skiers at a single ski resort, 59 persons were found to be excreting *Giardia* cysts (10). Fifty-six had symptoms that included loose stools (93%), increased numbers of stools (88%), and malaise, abdominal cramps, foul-smelling stools, weight loss, abdominal bloating, decreased appetite, nausea, and greasy, frothy, or bulky stools in 48–80% of infected persons. Among the 73% of patients with weight loss, the mean weight loss was 4.3 kg. The mean duration of illness was 44 days (median 38 days), and only two had fewer than 10 days of illness. Frequently, spontaneous improvement was followed by a return of symptoms.

Studies of American and Finnish travelers to the Soviet Union, especially Leningrad (St. Petersburg), have also given valuable information regarding the spectrum of illness associated with *Giardia* infection. These studies, as well as those of epidemics of giardiasis in the United States, have confirmed the spectrum of illness documented in the ski resort–associated outbreak (Table 1). Diarrhea is present in most patients and is frequently accompanied by nausea or vomiting, abdominal bloating, and flatulence, although uncommonly patients may present with constipation. The stools are typically described as greasy, foul-smelling, or frothy. Bloody stools are rare and should suggest an alternative diagnosis or dual infection. Fatigue and weight loss are remarkably common, occurring in a many as 70–90% of patients with giardiasis. Fever is not typical but has been reported in nearly 20% of patients.

The mean duration of symptoms in the various studies has been as low as 11 days (28), but an average duration of 6 weeks (10,26) to 10 weeks (27) in the absence of treatment has been documented in other studies. A waxing and waning course of illness is not uncommon, and patients with illness lasting from months to years have been reported (31).

**TABLE 1** Signs and Symptoms of Giardiasis

Symptom	Frequency (%)		
	Average <sup>a</sup>	Individual studies	Ref.
Diarrhea or loose stools	95	96, 93, 72, 98, 92, 100	10,26–30
Abdominal cramps	71	61, 77, 59, 85, 70, 83	10,26–30
Foul-smelling stools	67	75, 52	10,27
Malaise or weakness	67	72, 80, 88, 86, 20, 97	10,26–30
Nausea	64	59, 59, 60, 74, 58	10,26–29
Greasy stools	57	57, 55	10,26
Weight loss	54	62, 73, 69, 13, 59	10,26,28–30
Decreased appetite	50	60, 56, 82, 2	10,27–29
Bloating or distension	47	42, 62, 75, 9, 79	10,26,28–30
Flatulence	47	35, 56, 89, 6, 76	26–30
Vomiting	30	29, 34, 36, 23, 17	26–30
Belching	26	26, 30	26,27
Fever	19	17, 17, 15, 28, 21	26–30
Constipation	3	9, 1	10,29

<sup>a</sup> Weighted average.

#### IV. EPIDEMIOLOGY

*Giardia* is the most commonly identified intestinal protozoan in the world. In the United States the incidence rate is estimated to be between 50 and 1000 cases per 100,000 population annually (32). The rate of hospitalization for severe giardiasis is 2 per 100,000 population annually, a rate that is comparable to that of hospitalization for shigellosis (33). In most developing nations, the prevalence is substantially higher. For example, the infection rate in Peruvian children living in a shantytown near Lima was 95% by age 2, and 98% became reinfected within 6 months after treatment (34). From the tropics to northern Russia or Alaska, infection rates with *Giardia* are extremely high when inadequately purified drinking water is used.

There is a marked seasonality for infections acquired in the United States, with a peak in the late summer (35). Waterborne infections are the most commonly reported, but infections are also quite common in daycare centers (35–37). Cases have also been associated with homosexual activity (38,39), camping (40), or foodborne acquisition. In most studies *G. lamblia* has been the most commonly identified cause of waterborne outbreaks of gastrointestinal disease associated with drinking water (41). Acquisition of giardiasis has been associated with shallow well water (42), untreated surface water, surface water that is chlorinated only, and filtered water that is contaminated after filtration (43,44). Giardiasis has also been reported after recreational water exposure (45), but this is less common (44).

Although foodborne transmission is less commonly documented than waterborne transmission, there have been a number of well-documented reports of foodborne transmission. Many cases of foodborne transmission of pathogens are sporadic rather than epidemic and are even less adequately recognized than those occurring as parts of epidemics. Since the cyst is infectious immediately after being passed, foodborne transmission is a risk whenever contaminated water is used to wash fruits or vegetables that are subsequently consumed without cooking and is likely to be a common source of infection in areas with contaminated water supplies, as is found in many of the developing countries. In one outbreak, half of 60 employees of a school in rural Minnesota developed giardiasis, but none of the students were infected (30). The outbreak was traced to home-canned salmon. The salmon had been opened by a woman caring for a grandson subsequently found to have asymptom-

atic giardiasis, probably acquired at a daycare center. Other outbreaks of giardiasis have also been tentatively linked to food handlers who had contact with *Giardia*-infected children (46–49).

The relative importance of human versus animal sources of contamination of drinking or recreational water has remained controversial. The potential for zoonotic transmission of *G. lamblia* has remained controversial, in part because of conflicting results with attempts at cross-species transmission (50,51). For example, human-derived *Giardia* cysts were used to infect beavers, but a high inoculum was required to establish infection (52). Dogs and cats are commonly infected with *Giardia*, but well-documented human infections resulting from exposure to these or other domestic or livestock animals have not been reported. In addition, infection of these animals with *Giardia lamblia* from a human source has been difficult to achieve. In contrast, beavers have been well documented as sources of water contamination and subsequent human cases of giardiasis (53–56).

In areas where human infection is hyperendemic and where water purification is nonexistent or inadequate, it is reasonable to believe that humans are the major source for water contamination. However, human infections acquired by camping in the wilderness are more likely the result of zoonotic contamination of water by beavers or other animals.

## V. ISOLATION AND IDENTIFICATION

Clinical cases of giardiasis are usually documented by examination of fecal specimens (ova and parasite examination) for cysts or trophozoites. When freshly obtained diarrheal stools are examined immediately, motile trophozoites can sometimes be identified. The cyst is the more usual form to be identified and can be found in fresh or preserved specimens. The yield of cysts is improved by concentration using formalin-ether (57) and by the examination of up to three specimens submitted over a several-day period. The cysts and trophozoites are identified by their typical morphology under light microscopy. Immunofluorescence and enzyme immunoassays are also available for diagnosis and generally comparable to standard microscopic techniques in sensitivity and specificity, but they may be less time consuming (58). PCR-based diagnostic testing is limited by PCR inhibitors in feces but may be useful for molecular typing of cysts that are present (59).

Sometimes fecal examinations in patients with symptomatic giardiasis are negative, but the trophozoites can be identified in small intestinal contents. Specimens can be obtained by endoscopic aspiration or biopsy. The biopsy is often useful in ruling in or out other potential etiologies of diarrhea with malabsorption. Alternatively, patients can swallow a string with a capsule on the end (Entero-Test), followed by microscopic identification of trophozoites that adhere to the capsule (57). Human or animal specimens can also be isolated by animal inoculation (e.g., gerbils) or by axenization into a cell-free medium, but these approaches are laborious and in the case of axenization are frequently unsuccessful; therefore, they are used only in research settings.

*Giardia* cysts are detected in environmental water samples by filtration of large quantities of water (e.g., 10 L) using 2  $\mu$ m pore size filters, followed by immunofluorescent detection of the cysts (60) (<http://www.epa.gov/nerlcwww/1623.pdf>).

## VI. PATHOGENICITY

A number of human factors may result in increased or decreased risk of infection or development of symptomatic disease with *G. lamblia*. Data from epidemiological studies suggest that people develop partial immunity after infection with *G. lamblia* (10,61). In a large outbreak of giardiasis at a ski resort in Colorado, a much higher rate of symptomatic giardiasis in visitors to the area suggested partial protection from giardiasis for residents of the area, presumably due to prior exposure (10). Giardiasis may be increased in frequency in certain HLA types (62) as well as cystic fibrosis (63). Numerous reports have shown an association with hypogammaglobulinemia and with X-linked agammaglobulinemia (64). Therefore, humoral immunity has generally been considered as the major arm of protective immunity. Isolated IgA deficiency may or may not be associated

with increased risk of giardiasis. More recently, studies using animal models of giardiasis have suggested that cellular immunity may be important in the resolution of *Giardia* infection (65), although other studies have stressed the importance of intestinal IgA (66). Although reports of refractory giardiasis in AIDS have been published (31), AIDS does not appear to be an important risk factor for giardiasis. The relative importance of cellular and humoral immunity remains to be determined (64).

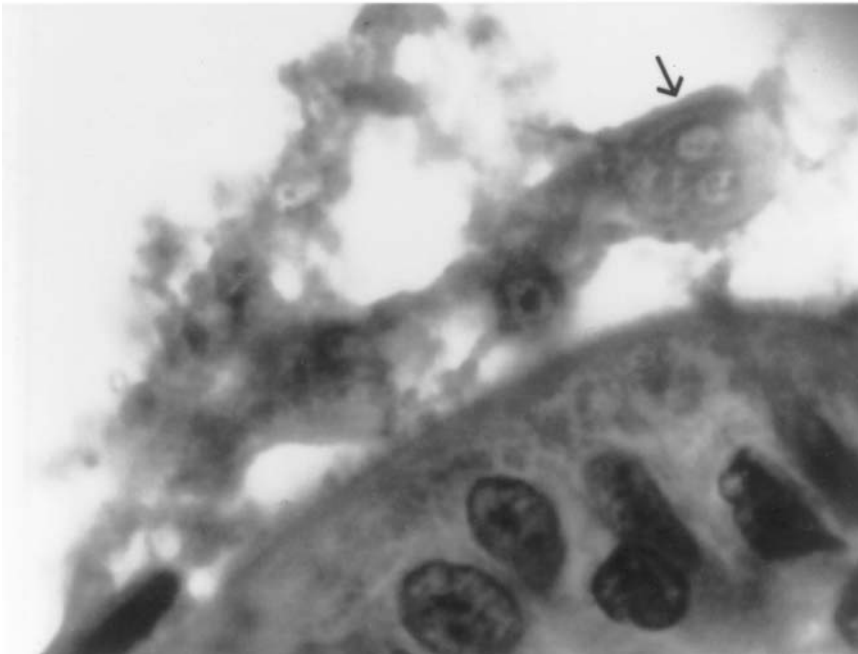
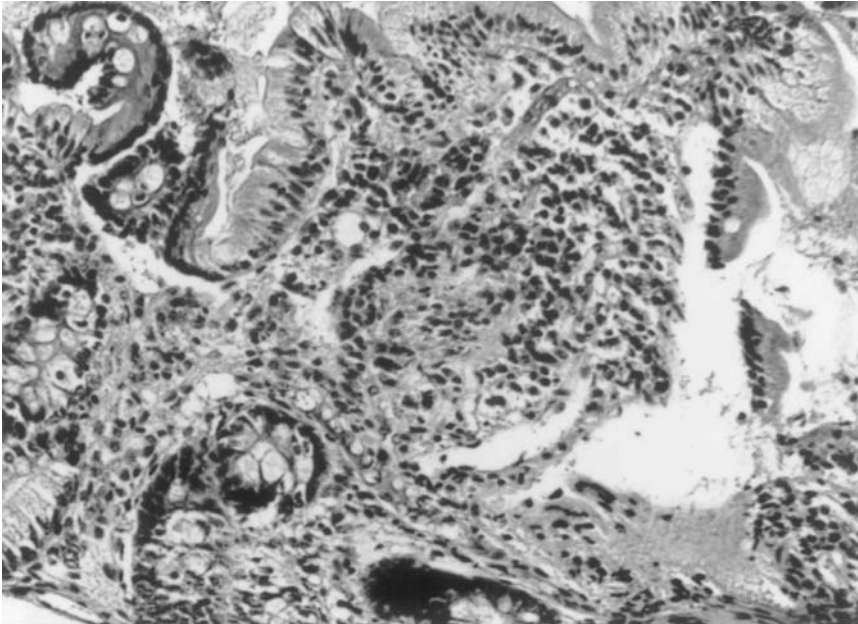
Symptomatic giardiasis is characterized by malabsorption of nutrients from the small intestine and diarrhea. Although the exact mechanism by which *Giardia* causes diarrhea and malabsorption is unknown, the characteristic intestinal lesion associated with symptomatic giardiasis is the presence of trophozoites on the intestinal wall, often in high numbers. Pathologically, the infection is characterized by varying degrees of villous atrophy (Fig. 3). When trophozoites attach to the intestinal epithelium, there are resulting microvillous abnormalities that can be demonstrated by electron microscopy (68). The findings in symptomatic patients vary from normal villi total atrophy in a small portion of patients. The prevalence of histological abnormalities in patients with giardiasis is not known, but a large study of the histological findings of giardiasis found abnormalities in only 4% of 480 duodenal organisms (69). Deep tissue invasion and extraintestinal infection have not been reported.

Functionally, reduced activities of the disaccharidases, lactase, sucrase, and maltase have also been reported and may contribute to the diarrhea. These decreased enzyme activities have been described in humans and in animal models of giardiasis. In general, the reduced disaccharidase activity has been associated with villous abnormalities in humans and in certain animal models. Along with the reduced enzyme activities, water, sodium, and chloride transport are impaired. Studies in animal models have shown that the impairment primarily affects stimulated rather than basal transport, although basal transport can also be impaired so that a true secretory diarrhea results (70). The effect on stimulated transport fits well with the clinical observation that malabsorption is a very common manifestation of giardiasis, while true secretory diarrhea is rare.

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

At least two major genotypes of *G. lamblia* infect humans, which may differ in their ability to cause human disease. A neonatal rat model of giardiasis using three different *G. lamblia* isolates showed differences among the three isolates in generation of villous abnormalities and in intestinal transport abnormalities, but the villous abnormalities were more common with the WB and PI isolates (both genotype A-1; Nash Group 1), while the transport abnormalities were more common with the VNB3 isolate (genotype not stated) (71). The dissociation of the morphological and functional abnormalities suggests not only a difference in virulence for different isolates, but also that different pathogenic mechanisms may be involved.

Differences among isolates have also been suggested by the results of epidemiological studies and experimental human infections. When human volunteers were inoculated with the ISR isolate (Genotype A-1), none of five were infected, while five of five were infected with the GS isolate (Genotype B), and three developed symptoms (24). These results suggest the possibility that Genotype B is more virulent for humans than Genotype A. There is a recent report indicating a higher percentage of illness associated with Genotype B than A (72). However, it is also possible that the difference in virulence was related to a difference in surface antigen type. Trophozoites undergo antigenic variation of a family of approximately 150 cysteine-rich surface antigens (variant-specific surface antigens, VSPs). Only one VSP is expressed at a time on a single trophozoite, and expression changes from one to another at a frequency of approximately 10-3 to 10-4 (73). The function of VSPs is not known, and the reason for antigenic variation is not well understood. Human and animal studies suggest that antigenic variation plays a role in evasion of the host immune response. In the human volunteer studies of antigenic variation, the timing of the initial VSP switch was 2 weeks after the initiation of infection, consistent with what would be expected with adaptive immunity



**FIGURE 3** (Top) Jejunal biopsy from a patient with giardiasis demonstrates flattening of the intestinal villi and a mononuclear inflammatory response. (Bottom) High-power magnification from the same biopsy demonstrates a *G. lamblia* trophozoite (arrow) attached to the intestinal epithelium. (From Ref. 67. Photographs courtesy Richard Sobonya.)

(74). However, the VSP type did not continue to change after that initial switch. A similar effect has been shown in adult (75,76) and neonatal mice (77), but one study showed a more complex switching pattern consistent with a change selected by adaptive immunity (78).

These observations suggest that antigenic variation may play a role in avoidance of the host immune response, but that there may be other reasons for antigenic variation. One additional reason is suggested by the observation that isolates of the same genotype expressing different VSPs have markedly different protease susceptibility patterns. This difference in protease susceptibility suggests the possibility that different VSPs may allow the parasite to adapt to different intestinal environments (79). If so, antigenic variation may provide a way for *G. lamblia* to adapt to its numerous mammalian hosts and may also provide a way of adapting to a changing intestinal environment within the same host. If this hypothesis is correct, the inconsistent results with cross-species transmission experiments could be a result of the VSP type of the isolate used rather than an effect of the species specificity of the isolate. Caution should also be used in interpreting differences in virulence among different isolates. These differences may be the result of genetic differences among the isolates, but may also be due to phenotypic differences resulting from VSP type. This latter possibility is suggested by the observation that infections resulted in 4 of 4 humans inoculated with one VSP type of the GS isolate, but only 1 of 13 inoculated with another VSP type. It may be that both VSP type and genotype are important in determining virulence, but further studies will be necessary to determine their relative importance.

## VIII. CONTROL MEASURES

### A. Prevention

Since *G. lamblia* cysts are spread by the fecal-oral route, efforts at prevention center around interrupting this cycle of transmission. In the United States, the most common sources for *Giardia* infection are drinking water or exposure to small children in daycare centers. Foodborne infections are less common but well documented. Direct person-to-person transmission can be reduced substantially by effective hand-washing with soap and water, especially after changing diapers.

*Giardia* cysts are the most stable when kept at 4–8°C in a moist environment. Infectivity is markedly decreased with freezing and with desiccation (80). Cysts are inactivated at temperatures of 20°C for 3 days, 37°C for 1 day, 55°C for 5 minutes, and instantly by boiling. Cysts can also be inactivated by chlorination of water. However, the efficiency of inactivation is highly dependent on pH and temperature of the water. The effectiveness of chlorine decreases slightly with increasing pH and markedly with decreasing temperature. Low temperatures also impair the efficacy of iodine for inactivating cysts.

The types of filtration used for purifying municipal water supplies are highly effective for removing cysts and include diatomaceous earth, slow sand, and coagulation filtration.

Tourists to areas with inadequately treated water can avoid exposure by filtering their water with a filter that has a 1 µm or smaller pore size, by bringing the water to a boil, or by drinking carbonated beverages. Similarly, backpackers and campers should filter or boil water taken from rivers or streams, even in areas where human contamination is unlikely. Iodination is usually effective, but since inactivation depends on water temperature, pH, and particulate matter, it is not as reliable as the other methods for eliminating cysts (81,82).

Foodborne infections occur through contamination by food handlers or by washing food with contaminated water. Fresh vegetables and fruits (except those with thick peels) should be avoided in areas with poor water supplies. Food workers should wash their hands with soap after using the toilet, and workers with diarrhea should be excluded from handling food. If giardiasis is diagnosed in a food worker, that worker should be excluded from handling food until the infection is resolved.

In addition to interrupting transmission, acquisition of giardiasis and other gastrointestinal illnesses by infants can be reduced by breast feeding (83–85). This not only provides passively ac-

quired protective immunity to infection, it also reduces exposure to potentially contaminated water or food.

## B. Treatment

Symptomatic giardiasis should be treated with one of several potential antimicrobial agents (86,87) (Table 2). Quinacrine was formerly the treatment of choice for giardiasis but is no longer commercially available in the United States. Even before quinacrine became unavailable, metronidazole was the most commonly used drug for treating giardiasis in the United States and can now be considered the drug of choice. Tinidazole is a nitroimidazole related to metronidazole and is highly effective when given as a single 2 g dose, but it has not been approved by the Food and Drug Administration for use in the United States. Treatment of giardiasis during pregnancy presents a special problem, since none of the available agents have been confirmed to be safe during pregnancy. Frequently, paromomycin is used in mild cases, since it is a nonabsorbed aminoglycoside and should have minimal toxicity. However, metronidazole is used extensively in pregnancy after the first trimester; therefore, severe cases later in pregnancy should probably be treated with metronidazole, because paromomycin is less effective.

Response rates of approximately 90% have been seen in the studies of the better agents. Therefore, it is not uncommon that retreatment is required. There have been suggestions in the literature that some of the treatment failures are due to antimicrobial resistance; however, because of the difficulty of axenizing *Giardia* and the lack of standard susceptibility testing, these proposals of drug resistance have not been proved or disproved. Frequently, higher doses (e.g., metronidazole) or more prolonged therapy is used in cases failing the initial course of therapy. Truly refractory cases have usually responded to a combination of metronidazole and quinacrine (31).

The role for treatment of asymptomatic giardiasis is not as clear. In areas with a high prevalence and high rates of reinfection, there is probably little benefit to treatment of asymptomatic cases. However, in developed nations with a low prevalence of giardiasis, asymptomatic cases, especially

**TABLE 2** Treatment of Giardiasis

Drug	Adult dose	Pediatric dose	Duration	Comments
Metronidazole	250 mg tid	5 mg/kg tid (max 750 mg/d)	5–7 days	Most commonly used treatment in United States
Quinacrine	100 mg tid	2 mg/kg tid (max 300 mg/d)	5 days	Not commercially available in United States
Furazolidone	100 mg qid	2 mg/kg qid (max 400 mg/d)	7–10 days	Only drug FDA-approved for giardiasis; somewhat less effective than other agents, but commonly used in children because of the available liquid form
Paromomycin	8–10 mg/kg tid	same as adult	5–10 days	Less effective, but commonly used in pregnancy because it is not absorbed
Albendazole	400 mg qd	same as adult	5 days	As effective and better tolerated than metronidazole in recent studies, but relatively little clinical experience to date
Tinidazole	2 gm	50 mg/kg (max 2 g)	single dose	Not approved by the FDA for use in United States



children in daycare centers, pose a risk of transmitting symptomatic infections to others in the community and should be treated.

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## *Cryptosporidium parvum*

Saul Tzipori

Tufts University School of Veterinary Medicine, North Grafton, Massachusetts, U.S.A.

### I. BACKGROUND

Although *Cryptosporidium* was first described in the laboratory mouse by Tyzzer in 1907 (1), the medical and veterinary significance of this protozoan was not fully appreciated for another 70 years. The interest in *Cryptosporidium* has escalated tremendously over the last two decades as reflected in the number of publications, which increased from 80 in 1983 to 3150 citations in 2002 listed in MEDLINE. The early history of *Cryptosporidium* is extensively documented in several review articles and book chapters published recently (2–4). Taxonomically, *C. parvum* belongs to the phylum Apicomplexa (possessing apical complex), class Sporozoasida (reproduce by asexual and sexual cycles, with oocyst formation), subclass Coccidiasina (life cycle involving merogony, gametogony, and sporogony), order Eucoccidiida (schizogony occurs), suborder Eimeriina (independent micro- and macrogamy develop), family *Cryptosporidiae* (4 naked sporozoites within oocysts—no sporocyst) (5). Like other enteric coccidia of vertebrates, *Cryptosporidium* has a monoxenous life cycle that is primarily completed within the gastrointestinal tract of a single host. It has, however, many unique features which distinguishes *Cryptosporidium* from other coccidia, of which the most obvious are the lack of host and organ specificity, resistance to antimicrobial agents, ability for autoinfection, and the curious location it occupies within the host cell membrane (6).

Between 1980 and 1993 three broad entities of cryptosporidiosis became recognized. The first involved the revelation in 1980 that *Cryptosporidium* was in fact a common, serious primary cause of outbreaks as well as sporadic cases of diarrhea in certain mammals (7). From 1980 onward *Cryptosporidium* emerged, with the acquired immunodeficiency syndrome (AIDS), as a life-threatening disease in this subpopulation, and in 1993 it reached the public domain when it became widely recognized as the most serious, and difficult to control, cause of waterborne-related diarrhea (8). The first glimpse of the seriousness of *Cryptosporidium* in mammals, namely in calves, was provided in the late 1970s (9,10). The later investigators successfully transmitted for the first time the infection between calves, but the resulting diarrhea was complicated by the presence of other enteric agents. Pohlenz et al. (10) were the first to show that cryptosporidiosis in calves could be diagnosed by detection of the relatively small oocysts (~4 µm) in Giemsa-stained fecal smears, the same oocysts first described by Tyzzer in 1907 and 1912 (1,11). Until then *Cryptosporidium* was mostly identified histologically in infected gut sections or in biopsy specimens (12,13). In 1980, *Cryptosporidium*, which was thought to be highly host specific, was successfully transmitted from calves, lambs, deer, and humans to newborn mice, rats, guinea pigs, chicks, piglets, and lambs. Of the experimentally infected mammalian species, acute diarrhea was induced in only piglets, calves, and lambs, and a mild diarrhea was observed in infant rats (14,15). Investigations of outbreaks of acute diarrhea in domestic herds in Scotland and the United States in 1980 led to the recognition of *Cryptosporidium* as a serious, highly contagious, primary cause of sporadic and outbreaks of acute diarrhea of calves, lambs, red deer, and goat kids (7,16,17). Serological tests conducted at the same time gave the first

indication of a widespread infection in all 10 mammalian species tested, which included humans (7,18).

The first reports linking *Cryptosporidium* with AIDS appeared shortly thereafter (19–23). Almost simultaneously, the first two reports incriminating *Cryptosporidium* as a common cause of acute diarrhea in a cluster of normal adults (24) and in children (25) were published. Although it was known as early as 1985 (26) that cryptosporidiosis can be contracted from drinking contaminated water, it was not until a major outbreak in Milwaukee in 1993 (8) that it became recognized by government agencies and water utilities as a serious waterborne pathogen.

## II. CHARACTERISTICS

Many aspects of the biology and nature of *Cryptosporidium* interaction with the host remain unclear. The taxonomy of this parasite continues to be a serious challenge to biologists and molecular epidemiologists. While there appear to be clear differences among isolates of *Cryptosporidium* obtained from different sources, these differences at present are difficult to fully characterize or define phenotypically for the purpose of speciation. The two major obstacles that hinder progress in this area are the inability to continuously propagate the parasite in vitro and the inability to cryopreserve the parasite, as is done with the majority of microorganisms. These technical problems are reflected in the absence of well-characterized reference strains of *Cryptosporidium* representing different vertebrate classes, species, or genotypes. The few *C. parvum* isolates currently being used in laboratory investigations are maintained by passage through animals, mostly calves. There are only a handful of *C. parvum* isolates, which are widely used as partially characterized genetically and phenotypically.

### A. Life Cycle

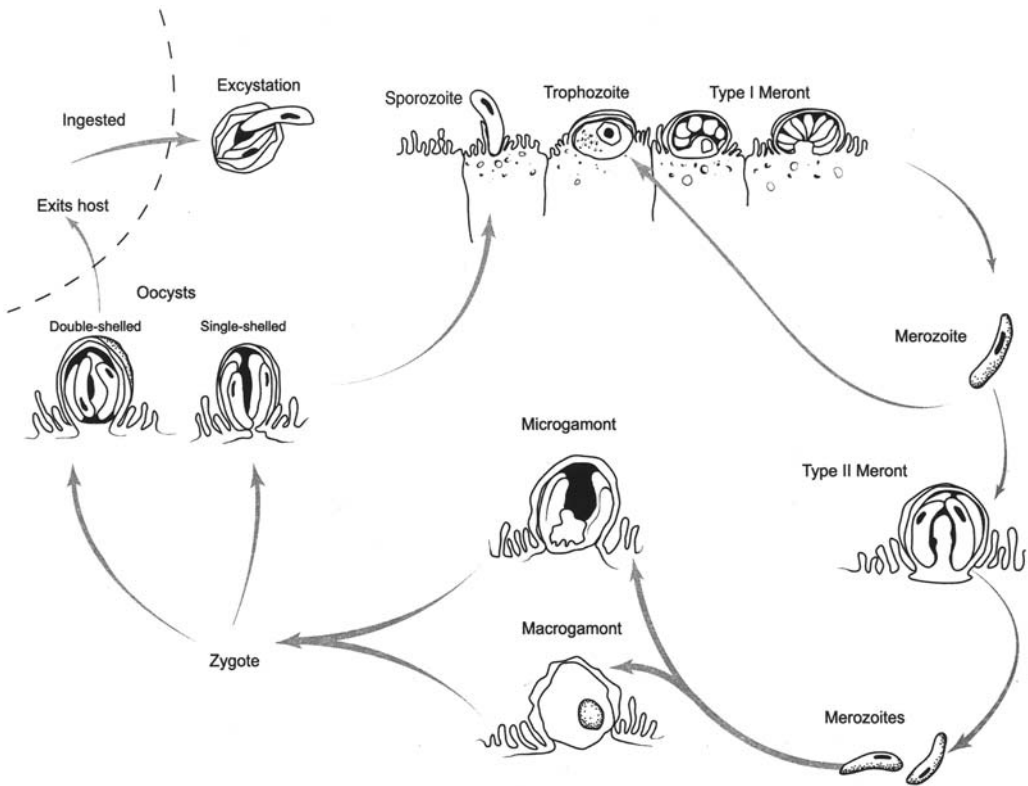
The life cycle of *C. parvum* has been outlined in most reviews (2,27), and a simple schematic diagram is presented in [Figure 1](#). Studies on ultrastructure and morphogenesis of *C. parvum* have been few in recent years (28–32). Few such studies have been published since the seminal and detailed observations made in 1986 on *C. parvum* and *C. baileyi* (33,34).

The life cycle begins with the ingestion of oocysts by the host, and the four naked sporozoites are released in the gut, which then infect epithelial cells and initiate asexual development. They become internalized and undergo two successive generations of merogony, releasing eight and four merozoites, respectively. The four merozoites released from the second merogony give rise to the sexual developmental stages, the micro- and macrogamonts. The release of microgametes and their union with macrogametes give rise to the zygote, which, after two asexual divisions, forms the environmentally resistant oocyst containing four sporozoites, often while still inside the parasitophorous membrane (Fig. 1).

The ability of the parasite to persist inside a single host is attributed to repeated first-generation merogony (not clear how many) and the production of sporulated thin-walled oocysts, a characteristic quite distinct from other coccidia. The fact that most oocysts are sporulated upon release is probably the most important attribute, as thick wall oocysts, by far the predominant type (33), readily excyst at body temperature and are therefore likely to contribute considerably to autoinfection, a phenomena observed by Tyzzer (11). It is assumed that in the normal host the infection remains localized to the gastrointestinal tract. Extraintestinal phases, however, should not be ruled out, as oocysts injected into the blood stream of mice (35), or sporozoites into the peritoneal cavity (S. Tzipori, unpublished), leads to gut infection. The migration course of sporozoites from these sites into the gut is intriguing. An extragastrointestinal phase has been observed in *Eimeria tenella* and *E. maxima* (36,37).

### B. Intracellular Development

A detailed account of the invasion and internal development of *Cryptosporidium* as compared with other intracellular parasites, including other Apicomplexa, has been published (6). The degree of



**FIGURE 1** *Cryptosporidium parvum* life cycle: after ingestion, 4 sporozoites are released from a single oocyst inside the host. The free sporozoite reaches and attaches to the epithelial microvillus border, and after internalization by the host cell membrane it forms the trophozoite. After three nuclear divisions, eight first-generation merozoites are formed within the type I meront. Merozoites either recycle to form more type I meronts or go on to form type II meronts, with each containing four second-generation merozoites. Presumably half of the second-generation merozoites go on to form a microgamont, which may contain up to 16 microgametes, or the macrogamont, which contains a macrogamete. The fertilization of the macrogamete by the released microgametes from the microgamont results in the formation of the zygote. Two types of sporulated oocysts are developed from the fertilized zygote, still within the host. The majority are double-shelled, environmentally resistant oocysts, which are released in the feces, and a minority of single-shell oocysts, which excyst within the host, releasing four sporozoites that initiate the life cycle within the same host. This, and recycling of the first-generation merozoites, is believed to be the basis for autoinfection and persistence of infection within the same host. (From Ref. 90.)

host tissue invasion by enteric pathogens range from noninvasive bacteria such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, which cause no morphological changes to the microvillus border of enterocytes, to cellular invasion by pathogens such as enteric viruses, *Shigella*, *Salmonella*, *Coccidium*, and *Eimeria* spp., to deep tissue invasion as seen in *Yersinia* spp., and *Salmonella typhi* and *S. paratyphi*. No organism other than *Cryptosporidium*, however, so extensively alters the cell membrane to create a niche for itself between the cell membrane and the cell cytoplasm. Neither the mechanisms of this process, nor the implications in terms of accessibility to the parasite in this unusual location, are understood. The location and nature of this dual sequestration from the gut and from the cell cytoplasm may hold the key to its enigmatic resistance to chemotherapy.

*Cryptosporidium*, like other coccidia, sequesters itself inside the host cell during development. It is protected from the host immune response and the hostile environment of the gut while accessing the nutritional and energy reservoirs of the host cell (38). Again, like other coccidia, it lies within a parasitophorous vacuole (PV) bounded by a parasitophorous vacuolar membrane (PVM), which in other coccidians is the portal through which nutrients from the host cytoplasm enter the parasite. Unlike any other organism, however, *Cryptosporidium* has in addition a unique structure known as the feeder organelle membrane (FOM), which directly separates the cell and parasite cytoplasm. It is assumed that the PVM in *Cryptosporidium* provides only a protective function, while the FOM is the site for nutrient and energy uptake from the host cell. It is conceivable however, that the PVM is also selectively permeable to certain molecules from the gut lumen. This is based on the fact that the PVM, originally derived from the host cell membrane, may retain some of its absorptive and other functional activities.

### III. DISEASE AND PATHOGENESIS

Cryptosporidiosis is one of the most common human enteric infections in developed and developing countries. It is ubiquitous, zoonotic in nature, occurring in most if not all species of vertebrate, and can induce infection with as few as 10 oocysts or less in adult human volunteers (39). How hazardous to human health are oocysts from species of animals other than ruminants is not clear but probably considerable. Children acquire the infection mostly during or after weaning, and episodic disease occurs throughout life.

Exposure to *C. parvum* oocysts, either directly through contact with infected humans or animals or indirectly by drinking or eating food washed with contaminated water, may lead to acute diarrhea. *C. parvum* causes an acute, self-limiting infection and diarrheal disease in immunocompetent people, in whom the onset may be rapid (3–7 days), depending on a combination of host (age, presence of maternal antibodies or previous exposure, infectious dose) and parasite factors (origin and age of oocysts, species/genotype). Infection presumably begins in the small intestine, where the emerging sporozoites infect enterocytes, and after amplification endogenous forms spread throughout the epithelial surfaces of both villi and crypts. The infection may spread throughout the gut, including the gastric mucosa and the small and the large intestines, or it may remain localized in segments of the small and or large intestine. The extent of spread and the sites involved determine whether the infection is clinical or subclinical as well as the overall intensity of the disease. Generally, the more proximal in the small intestine the location is, the more severe and watery the manifestation of diarrhea. Infections confined to the distal ileum and/or the large bowel can often result in intermittent diarrhea or even be asymptomatic. Infections may often involve the pyloric region of the gastric mucosa. Parasite forms displace the microvillus border and eventually lead to the loss of the surface epithelium. The rapid loss of surface epithelium causes marked shortening and fusion of the villi and lengthening of the crypts due to acceleration of cell division to compensate for the loss of cells. The combined loss of microvillus border and villus height diminish the absorptive intestinal surface and reduce uptake of fluids, electrolytes, and nutrients from the gut lumen. The loss of the microvillus border in the proximal small intestine also leads to loss of membrane-bound digestive enzymes, whose role in children in particular is crucial, and contributes to a marked maldigestion in addition to the malabsorption. Diarrhea lasting 7–10 days results in serious dehydration and loss of body weight. Specific antibodies are not considered to be a major factor in recovery from infection, although they may play a role in protection against reinfection. Although the immune factors that contribute to recovery from cryptosporidiosis in the immunocompetent host are not well understood, clearly the absence of optimal number of circulating or mucosal CD4 T lymphocytes, or interferon-gamma (IFN $\gamma$ ), are critical (40). Studies have shown that other cytokines and immune cells may play a significant role in recovery and protection against reinfection (40).

Cryptosporidiosis is considered to be one of the most serious opportunistic infections that complicate AIDS. Individuals with CD4 T-cell counts of <150 mL who are exposed to *C. parvum*



invariably develop persistent infection with profound and life-threatening diarrhea (41). Although the prevalence in people with AIDS is not high—5–15% in developed countries—the lack of effective treatment makes this infection extremely troublesome. Prolonged infections lasting several months or years in people with acquired (42) or congenital (43) immunodeficiencies often spread from the gut to the hepatobiliary and the pancreatic ducts, causing cholangiohepatitis, cholecystitis, choledochitis, or pancreatitis. In chronically infected gut the mucosal architecture undergoes gradual but profound disorganization, which includes disrupted epithelial surface, fibrosis, cellular infiltration, and crypt abscessation (44).

#### IV. EPIDEMIOLOGY

The prevalence of cryptosporidiosis in diarrheal illness ranges from 1 to 3% in adults and 4 to 7% in children (45), depending on climatic conditions. The prevalence is higher (5–10%) in warmer, less developed countries, and it can be as high as 20–27% among children aged 6–48 months in Africa and Latin America (45). Prevalence can also be high in developed countries among children in daycare centers, where outbreaks involved 50–60% of children below the age of 2 years have been documented (46). Griffiths (45) provides a comprehensive summary of prospective studies on the incidence and prevalence of cryptosporidiosis in people with and without HIV/AIDS reported in the literature between 1990 and 1998. The link between malnutrition and chronic cryptosporidiosis in children is strongly suspected but is not systematically documented.

The significance of *C. parvum* infection in domestic animals, newborn calves in particular, became evident in the early 1980s. The course of the infection and the disease it induces in a variety of small ruminants was reproduced experimentally, reported extensively, and reviewed many times over (3,7). Infections of domestic and wild ruminants provide the biggest source of environmental contamination. While *C. parvum* is probably present in every domestic cattle herd worldwide, the incidence of diarrhea varies considerably from none (47) to more than 59% in a nationwide U.S. survey involving 1103 farms (48). The incidence of diarrhea in calves varies among farms and geographic locations, and between the ages of 1–6 weeks it can reach up to 75% of calves (49), with individual calves excreting in excess of  $10^{10}$  oocysts over the course of the infection. Apparently healthy calves can also become subclinically infected and contribute to oocyst excretion in feces (50). Asymptomatic infections and prolonged oocyst excretion by adult cattle (51,52) have become recognized as another major and continuous source of environmental contamination and clearly the source from which newborn calves contract the infection at a very young age. *C. parvum* is also common in sheep and goat herds (2,7,17), but prevalence is not as well documented. Newborn lambs (7,17,53) and goat kids (7) are susceptible to *C. parvum*-induced diarrhea, which undoubtedly contributes to oocyst dissemination, the extent of which is not clear.

Serological evidence suggests that the infection is widespread among swine herds (54), although diarrhea is not a serious problem in young piglets. Infections in domestic pets, such as dogs, cats, and horses, have also been reported, and therefore are considered a potential source for human infection. However, *C. parvum* is not known to cause diarrhea in these species of animals, a condition that is normally responsible for massive production and environmental dissemination of oocysts. The prevalence of cryptosporidiosis in these species of animals is not extensively documented. Oocysts have been detected in dog feces of some populations (55), but not others (56,57). Oocyst excretion by domestic pets is risky because of the shared proximity with people, particularly children. It is not clear, however, whether dogs and cats are mostly infected with *C. parvum* or with other *Cryptosporidium* spp. that were reported to at least be infectious to individuals with AIDS (see below).

Some 80 animal species, the majority of which are in the wild, are known to be naturally susceptible to *Cryptosporidium*, (2). Until we have methods to identify key virulence factors associated with infectivity and pathogenicity of *Cryptosporidium* for humans, and until we are able to confidently speciate clinical isolates, *Cryptosporidium* from all sources including birds and lower

vertebrate animals should be regarded as potentially hazardous to public health. Wild animals, whether clinically or subclinically infected with *Cryptosporidium*, contribute to the perpetuation of infections among mammals as well as to the risk of environmental contamination in general. A report suggests that some animals may even disseminate oocysts by acting as mechanical vectors (58).

## V. ISOLATION AND IDENTIFICATION

Several methods are currently available for the detection of *C. parvum* infection in people. Until 1980, diagnosis was confirmed by examination of endoscopically obtained intestinal biopsies. Diagnosis is made by demonstrating the presence of oocysts in stool samples. *C. parvum* oocysts in infected persons can be identified by staining of fecal smears with modified acid-fast (MAF) stain. Oocysts measure 4–5  $\mu\text{m}$ , stain bright red with refractile round thick capsules, and are observed under high-power light microscopy. The size, round shape, and structure of the oocysts make their detection by an experienced technician easy. They may be confused with oocysts of *Cyclospora* spp., which are larger (8  $\mu\text{m}$ ). They can also be detected by direct or indirect immunofluorescence (IF), using commercially available kits with specific monoclonal antibodies, or by polymerase chain reaction (PCR)(59). Several investigators have described the use of ELISA or flow cytometry (59). The latter technique is used in laboratory investigations rather than in clinical diagnosis. Of these methods, the MAF staining technique is by far the quickest and the least expensive, and it is as sensitive as detection by IF. IF is highly sensitive and specific and is convenient to use for technicians who do not encounter oocysts regularly. The commercially available kits, however, are expensive. For diagnostic purposes, ELISA can be more sensitive in formalin-fixed samples or in poorly preserved specimens in which residual antigens derived from oocysts as well as shared antigens from other stages of the life cycle are present in feces. ELISAs need to be developed by each laboratory since they are not available as commercial kits. PCR is more sensitive for detecting mild *C. parvum* infections in fixed, in frozen, or often in poorly preserved fecal samples. However, extraction of DNA from feces is a lengthy procedure and without proper controls may produce false-negative or false-positive results. Detection of waterborne oocysts is more difficult because they need to undergo a concentration step, which is not simple (60).

## VI. GENETIC FACTORS INVOLVED IN SPECIATION

In 1980 *Cryptosporidium* isolates obtained from calves, lambs, and a human adult with severe diarrhea readily infected seven other species of animals (14,15). The transmission of the human isolate, which induced acute diarrhea in lambs indistinguishable from that caused by other animal isolates, strongly indicated the potential zoonotic nature of *Cryptosporidium* (22). Based on these early observations, the naming of *Cryptosporidium* species after their respective animal host (61), seemed questionable (14). Subsequent studies extending over the last 2 decades however, indicated that other species may exist. *C. parvum*, however, the named mammalian species (11), remains the single most important species perpetuating in mammals. The exact number of additional species is still unclear, but molecular methods are improving our understanding of the taxonomy of this genus. Of the original 21 different *Cryptosporidium* species listed in 1980, the majority became invalid as a consequence of the transmission experiments described above. Six (2) or more *Cryptosporidium* species are currently recognized, based largely on molecular genetic studies and/or on very limited number of transmission experiments. The currently recognized six species include two mammalian, *C. parvum* and *C. muris*; two avian, *C. meleagridis* and *C. baileyi*; a reptilian, *C. serpentis*; and a fish, *C. nesorum*. Other less clearly defined species include those from guinea pigs (*C. warii*), cats (*C. felis*), dogs (*C. canis*), and marsupials (2). The identification of *C. felis* and *C. canis* from patients with AIDS who had diarrhea apparently indistinguishable from that caused by *C. parvum* (62), and

the remarkable genetic homology between *C. parvum* and *C. meleagridis* (63), cast further doubt on the merit of the current system of speciation.

Of the several proposed mammalian *Cryptosporidium* species, *C. parvum* appears to be associated with the majority of human disease. The recent identification of *C. felis* and *C. canis* in HIV/AIDS patients with chronic diarrhea (62) suggests that other *Cryptosporidium* species may also infect humans. It is not known how common *C. felis* and *C. canis* infections are in humans or whether they are likely to occur only in individuals with immunodeficiencies. *C. muris*, the first mammalian species to be described (1), was observed in the stomachs of a few animals including mice, rats, cats, dogs, cattle, and camels. Oocysts of *C. muris* are larger than those of *C. parvum*, and the infection is asymptomatic (2). *C. muris* has not so far been identified in humans.

Most of the evidence presented on host specificity in support of speciation are based on cross-transmission experiments performed with a limited number of *Cryptosporidium* isolates, often tested in a single representative host, mostly the infant mouse (64). The mouse, however, cannot be regarded as a representative of all mammals, since it seems to be resistant to infection with *C. parvum* isolates belonging to genotype 1 (65–67). Nor presumably does a single *Cryptosporidium* isolate represent all isolates that perpetuate in the class of vertebrates from which it was obtained. The epidemiological and epizootiological implications of conclusions based on limited and anecdotal observations carry a major risk to public health, which can lead to a complacent view that humans are safe from exposure to *Cryptosporidium* isolates other than *C. parvum*, including those from lower vertebrates.

The host of the *Cryptosporidium*, the site of infection and oocyst size, and the phenotypic attributes used to differentiate species are probably not the most critical features, largely because of the ubiquitous nature of this protozoa. It is more than likely that speciation in the future will not necessarily follow along classes of vertebrates, but rather be determined by tangible virulence attributes with defined host range that can be linked to genetic markers. Demonstration of significantly greater sequence homologies within species than among species at multiple unlinked loci in isolates obtained from a large and diverse range of host species and locations could provide in the future a solid genetic basis for elucidating the taxonomy of the genus *Cryptosporidium*. Confirmatory evidence would require an experimental system to test whether putative species are distinct reproductive entities.

As mentioned earlier, considerable phenotypic and genotypic variations occur among and within *C. parvum* isolates. As the number of characterized isolates increases, and as new tools for differentiation become available, more such differences will be identified. Differences have been recognized based on infectivity to other animals (1,7), pathogenicity (68–70), protein banding (71–73); antigenicity (74–76), isoenzyme typing (77,78), and genotyping (79–84). Phenotypic (64) and genotypic (see below) differences have been used to distinguish between human and animal (calf) isolates. There is clear evidence that among *C. parvum* isolates there is a strong preference to one host or a group of hosts. Some isolates have a clear preference for and readily infect adult rodents in which they perpetuate (11,85). In contrast, most *C. parvum* genotype 2 isolated from calves visibly infects only newborn rodents or immunodeficient adult rodents. *C. parvum* of genotype 1 isolated from humans and macaques does not appear to infect mice at all. The same probably applies to *Cryptosporidium* found in guinea pigs, which currently is considered by some to be a separate species.

Research recently has focused on understanding the genotypic heterogeneity within the species and defining the taxonomic relationship with other species and related genera using molecular markers. The most productive approach has relied on restriction fragment length polymorphism (RFLP) of PCR-amplified loci known to harbor polymorphic restriction sites. Most loci analyzed in this manner lie within coding regions, because many coding regions have been sequenced as part of other research activities or were included in the *C. parvum*-expressed sequence tags (EST) database. In many cases, PCR-based methods are the only practical avenue because *C. parvum* cannot be cultivated in vitro. This limitation restricts the amount of parasite material available in feces to mostly genetic analysis. Because *C. parvum* is primarily recovered from stool, and in some cases from water samples, obtaining oocysts free of other microorganisms and contaminants is a labor-

intensive procedure. The ability of PCR to amplify a selected DNA target from complex mixtures of nucleic acid makes this technique an ideal choice for detection and genetic fingerprinting. The popularity of this method is reflected in the significant number of articles recently appearing in the literature relying on PCR for the characterization of *C. parvum* (65–67,77,79,80,86).

Work in several laboratories has demonstrated the occurrence of two subgroups within the species *C. parvum*. This conclusion is based mainly on genotypic data, primarily RFLP. These genotypes have been designated 1 and 2 (65), H and C (67), human and bovine (79), human and animal (66), or human-derived and calf-derived (81). Other genotyping methods, such as random amplified polymorphic DNA (RAPD) (66,70,81) and sequence analysis (83,84), have confirmed the existence of two subgroups.

Because of the dimorphic nature of most RFLP markers, this typing method has generally not provided any insights into the heterogeneity within 1 and 2 subgroups. Sequence alignments of PCR clones amplified from multiple isolates were performed on a few loci (61,82,84). These comparisons have demonstrated that the 1 and 2 subgroups are not homogeneous and that individual isolates can carry multiple alleles. The examination of noncoding regions, such as the ribosomal internal transcribed spacers and the  $\beta$ -tubulin intron (84), have documented genotypic differences not detected by RFLP. It is expected that intergenic regions, which are less constrained in their evolution than coding regions, will reveal highly polymorphic sequences. In particular, sequences incorporating simple repeats (microsatellites) are expected, in analogy to other *Apicomplexa* (87,88), to be highly polymorphic. Preliminary data on length polymorphisms of a polythreonine repeat in *C. parvum* supports this assumption. Length polymorphisms have the advantage of being relatively easy to detect without requiring sequencing.

The broader implications of the observed phenotypic and genotypic diversity are difficult to predict. They may in the future provide a genetic basis to help explain the movement of *Cryptosporidium* among mammalian, or even vertebrate species, and shed new light on the speciation of the genus. Linking clearly recognized virulence attributes to stable genetic markers is a better way to define a species, which, for *Cryptosporidium*, one suspects will not necessarily be confined to a particular animal host, or even to a class of vertebrates.

The clinical significance of the observed *C. parvum* subtypes in human cryptosporidiosis is unknown. Of particular interest is the question of whether isolates belonging to different genotypes manifest different disease patterns. Limited observations suggest that at least with regard to *C. parvum* genotypes 1 and 2, *C. canis* and *C. felis*, in individuals with HIV/AIDS, no apparent differences in terms of clinical manifestations were reported (62). However, large-scale epidemiological studies are required to conclusively address this issue. The nature and extent of polymorphism in developing countries, where *C. parvum* remains a significant public health problem (89), is unknown. Animal surrogates susceptible to all known *C. parvum* genotypes represent an additional method of determining genetic markers associated with virulence. With the currently known *C. parvum* 1 and 2 genotypes, the piglet model appears so far to be the only candidate available for such studies (90).

Reports on the genotypic and phenotypic dimorphism of *C. parvum* led to the assumption of distinct anthroponotic and a zoonotic transmission cycles (65,66). Analysis of multiple, unlinked genetic markers in isolates from various hosts and geographic origin failed to identify recombinant genotypes (66,80). One possible explanation of this finding is that 1 and 2 genotype populations are reproductively separated. Since both types can infect the same host—humans—this separation is not due to the physical barrier of propagation in different host species. Moreover, a low-level infection with type 1 parasite in a calf-propagated laboratory isolate was observed, indicating that 1 and 2 genotypes might share other host species besides humans. Mixed infections in humans have also been documented (77).

A survey of published data on *C. parvum* genotypes in humans with known HIV status identified 215 cases assigned to one of two types mostly based on RFLP data. Of the total reported cases, 15% were identified as genotype 2, the remaining as genotype 1. The breakdown for isolates from HIV-positive and HIV-negative individuals was 90% and 84% type 1 and 2, respectively, a statisti-

cally nonsignificant difference (90). From this limited and geographically skewed sample one concludes that genotype 1 and 2 occur with equal frequency in immunocompetent and immunodeficient individuals. This suggests that people with AIDS and immunocompetent individuals contract cryptosporidiosis from the same source, the most likely being drinking water. Based on these data one can also postulate that there is no difference in the relative importance of anthroponotic versus zoonotic transmission in immunocompromised and immunocompetent individuals.

Typically, assumptions of the origin of *C. parvum* causing outbreaks or sporadic infections are based on the genotype recovered from infected individuals. Although from a technical point of view this is often the only feasible approach, one should bear in mind that between the initial contamination of a host and excretion of oocysts, the parasite has undergone multiple cycles of asexual reproduction and presumably one cycle of sexual reproduction. This may impact the profile of the excreted oocysts, which may no longer match that of the infecting oocysts. Experimental observations suggesting that the population makeup of *C. parvum* isolates can change following passage through different hosts were recently observed in our laboratory (91).

The absence of type 1 and 2 recombinants so far suggests, as mentioned, that these genotypes, reproductively, are probably separate species. If such reproductive separation can be experimentally confirmed, 1 and 2, like other genotypes that behave similarly, should be considered as different species. Experimental analysis of sexual reproduction in *C. parvum* is hampered by the absence of an in vitro culture systems and cloned strains. Propagation studies with single sporozoites of genotype 2 to establish clonal populations of *C. parvum* in gamma knockout mice will help address some of these issues.

## VII. LABORATORY PROPAGATION OF *C. PARVUM*

Methods for laboratory propagation and maintenance of *Cryptosporidium* isolates other than *C. parvum* are not available. Recent literature sources provide a comprehensive outline of techniques used for laboratory investigations (3,4). The inability to continuously propagate *C. parvum* in vitro remains the most important obstacle to performing the kind of studies possible with so many other medically important pathogens. This limitation is reflected in the lack of access to stages of the life cycle other than oocysts, which contain the sporozoites, and the need to use animals to generate oocysts for laboratory investigations. Furthermore, oocysts generated in animals have a limited viability span, which infectivity markedly diminished within 6–8 weeks for in vitro and in vivo infectivity studies. Because there are as yet no methods that allow an indefinite storage of infectious material, isolates have to be continuously passaged through animals, usually calves. A limited parasite growth in cell culture, largely confined to merogony, is available and provides a restricted scope.

Oocysts and sporozoites are the only stages of the life cycle that can be produced in large quantities in experimentally infected calves. Oocysts are readily purified and concentrated from calf feces. Oocysts can also be obtained from experimentally infected small ruminants immediately after birth (92,93) or in small quantities from rodents (94,95). Typically, laboratory research is performed with calf-propagated oocysts only, which do not include the genotype most frequently found in human infections. There is clearly a risk that properties derived from commercially available type 2 oocysts might not apply to the entire species. Indications that oocysts of type 1 and 2 display different phenotypic properties were recently obtained in our laboratory (91). Some type 1 oocysts were found to have a half-life at room temperature shorter than those of oocysts originating from calves. Differences in buoyant density in CsCl were also observed (91).

### A. Propagation in Cell Culture

In the decade since the first report of *C. parvum* growth in cell culture (96), there have been more than 30 publications describing in vitro cultivation techniques (97). Although there has been some improvement, the overall extent of parasite growth remains limited largely to the asexual phase.

Cells from a variety of sources can be infected with *C. parvum* in which growth peaks at 48–72 hours, after which it gradually declines. Several investigators reported the production of a small number of oocysts using Caco-2 cells (98), bovine fallopian tube epithelial cells (99), and MDBK cell line (100). However, the amount of oocysts produced is smaller than the inoculum. Upton and coworkers compared the growth of *C. parvum* in various cell lines, improved some of the parameters required to enhance in vitro excystation, and identified a supplemental medium formulation that is said to further enhance parasite growth (101–103). Current and Haynes (96) described the growth cycle of *C. parvum* in cell culture in detail. With all the limitations of cell culture propagation, the two merogony stages of the life cycle have been useful for developing in vitro assays for screening compounds and immune reagents for *C. parvum*.

## B. Propagation in Animals

In addition to parasite propagation and production of oocysts, animal models for *C. parvum* have been used to help understand the clinical course of the infection and the disease it induces in humans, the pathology and pathophysiology of digestion, immunology and immunopathology, and for drug testing and evaluation. For each one of those aspects of the host-pathogen interaction, different animal models are used. For instance, clinically affected species such as lambs, calves, and piglets were used to acquire insight, not only into the specific effect of infection in the respective species, but into the clinical course and pathogenesis of acute cryptosporidiosis in neonates in general. Because of the significance of cryptosporidiosis in other mammals, namely calves and other small ruminants, studies have been conducted since 1980 to investigate the infection and disease that are unique to those economically important domestic animals. These and many other aspects of animal models used for biomedical research are detailed elsewhere (59).

## VIII. TREATMENT AND CONTROL

Immunologically healthy individuals normally recover uneventfully after a short illness of diarrhea, and generally they require no treatment other than supportive therapy if needed. The situation is different in immunodeficient people, particularly those with AIDS, who would benefit tremendously from an effective treatment. Unfortunately, there is no consistently effective treatment against cryptosporidiosis. There appears to be little incentive in the private sector to invest in the development of specific therapy against *C. parvum*. The tendency instead is to test compounds against *C. parvum* that are intended for other important protozoa, e.g., malaria.

Since the identification of cryptosporidiosis as a serious disease of humans in the early 1980s, almost every available antimicrobial and antiparasitic agent has been tested in one form or another against the disease in humans and by many investigators under laboratory conditions. Of those that have been tried, the macrolides, spiramycin in particular, showed some promise early in the AIDS epidemic, but the response to therapy was inconsistent at best. Over the last decade hyperimmune bovine colostrum (HBC), which is produced by immunizing pregnant cows with parasite antigen, was tested in individuals with immunodeficiencies and cryptosporidiosis with partial success (104). While there was some symptomatic improvement, the treatment did not eliminate the infection of any of the patients. After cessation of treatment a relapse invariably followed.

Paromomycin is another drug that showed promise and was extensively used to treat chronic cryptosporidiosis in AIDS. Again the results were mixed; there was symptomatic improvement in some, no improvement in others, but paromomycin also did not eliminate the infection in any of the cases at doses of 2 g/day (105,106). Paromomycin, despite its beneficial effect, is considered to be toxic when used in high doses over a long period.

More recently, in the United States and elsewhere, nitazoxanide (NTZ), a nitrothiazole benzamide that has a wide range of activity against protozoans, helminths, and bacterial pathogens, was tested in human clinical trials. NTZ was shown to be highly effective in cell culture against *C.*

*parvum* and partially effective in the piglet diarrhea model (107). Studies from one clinical trial in the United States involving 22 patient with AIDS and cryptosporidiosis were assigned to receive between 500 and 2000 mg/day orally for 4 weeks. Preliminary analysis revealed that 15 (68%) had reduced stool frequency at doses >1000 mg/day, 4 had complete resolution of diarrhea, and 4 others lacked detectable oocysts in the stool. These investigators concluded that NTZ appeared to have a favorable clinical but less pronounced parasitological effect on *C. parvum* (108). The use of NTZ, like other earlier promising drugs, was discontinued.

In contrast to other related pathogenic protozoa, *C. parvum* displays a most unusual resistance to antimicrobial and antiprotozoal agents, which may relate to the unique intracellular extracytoplasmic location it occupies in the host cell. Although it remains located outside the cell cytoplasm, it is surrounded and protected by the cell membrane. This novel intracellular niche, and the manner in which its own membranes fuse with those of the host cell, creates a curious interface, known as the feeder organelle. It is possible that this structure, which controls the flow of nutrients from the cell cytoplasm into the parasite, only permits the passage of molecules essential for parasite growth and development. Indeed, a recent report by Perkins et al. (109) has identified ATP-binding cassette (ABC) proteins thought to be localized in the feeder organelle with a structural similarity to the multidrug-resistance (MDR) proteins subfamily. This is consistent with our own studies in which the partially active drug paromomycin enters the parasite not through the cell cytoplasm and the feeder organelle but through the parasitophorous membrane that isolates the parasite from the outside milieu (110).

In the absence of effective therapy, prevention may be the best option for protection against contracting the infection. It is particularly crucial in individuals with immunodeficiencies. Since water is now considered a major source of infection, drinking water and water used to wash vegetables and fruit should be boiled. Contact with domestic animals, particularly young ruminants (calves, lambs, kid goats), should be avoided, as should contact with children and adults with diarrhea.

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## *Cyclospora cayetanensis*

Michael J. Arrowood

*Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A.*

### I. BACKGROUND

*Cyclospora cayetanensis* is a protozoan parasite of humans that causes diarrheal illness after colonizing the mucosal epithelium of the small intestine. In retrospect, this coccidian parasite was first described in persons from Papua New Guinea in 1979 (1), but the specific identification as a *Cyclospora* species would not occur until 1993 (2). In the intervening years the parasite was alternately described as either a large variant of *Cryptosporidium*, a blue-green alga (cyanobacterium-like), or an unidentified coccidian. The actual nature was not discerned until stool specimens were stored without a fixative for sufficient time to allow the undifferentiated oocysts to sporulate and reveal both sporocysts and sporozoites (2,3).

Particular attention was focused on this newly recognized pathogen as a result of several large foodborne outbreaks of diarrheal illness in the United States (4–9). Cyclosporiasis had already been described in international travelers and indigenous populations with linkage to water and food consumption. A cosmopolitan parasite, *C. cayetanensis* has been described worldwide (10–12) and identified in water and food (13–18). Much like cryptosporidial diarrhea, patients frequently report gastrointestinal flu-like symptoms, including profound diarrhea lasting 1–3 weeks, often with accompanying weight loss, nausea, anorexia, vomiting, and abdominal cramping (12). These variable clinical signs are not pathognomonic, i.e., they do not provide sufficient information to incriminate *Cyclospora*, thus necessitating parasitological identification in stool.

While a number of morphologically similar species have been observed in primates, including *C. cercopitheci*, *C. colobi*, and *C. papionis* from vervet monkeys, colobus monkeys, and baboons, respectively (19), only *C. cayetanensis* has been described in humans. Attempts to establish an animal model of cyclosporiasis using human-derived oocysts has not yet been achieved (20). Likewise, recent attempts to cross-infect nonhuman primates with oocysts from other nonhuman primates have failed (unpublished data) as have attempts to infect human volunteers with *Cyclospora* isolates collected from individuals involved in foodborne outbreaks (M. Eberhard, personal communication, 2002).

DNA sequences obtained from rRNA-coding regions revealed the close phylogenetic relationship between *Cyclospora* and *Eimeria* (21,22). Further molecular analyses of geographically diverse human isolates and isolates from nonhuman primates revealed distinct characteristics of isolates from each host species (19,23–25). That is, the *Cyclospora* isolates from each host species appear unique and show no signs of cross-infection. This is consistent with the general observation that *Eimeria*-like parasites are host specific. If host specificity is inherent in *Cyclospora*, then epidemiological questions regarding environmental sources and potential reservoir hosts may be restricted to humans and not to feral or domesticated animals.

## II. CHARACTERISTICS

As a coccidian parasite, *Cyclospora cayetanensis* has a complex life cycle, including asexual and sexual stages occurring in the same host (26,27). It has a thick-walled oocyst like those of *Eimeria*, *Toxoplasma*, and *Isospora*, but has only two sporozoites in each of two sporocysts per oocyst, which is characteristic of the genus. Following ingestion of sporulated oocysts (or free sporocysts?), sporozoites are released, which invade the enterocytes of the small intestine. The timing and sequence of the asexual and sexual stages have not been fully described, but development has been observed in the apical (supranuclear) cytoplasm of enterocytes. Examination of duodenal and jejunal biopsies clearly demonstrated trophozoites, type 1 meronts (8 to 12 merozoites), type 2 meronts (4 merozoites), and sexual stages (26,27). Oocysts produced following fertilization of macrogametocytes by microgametes are approximately 8–10 µm in diameter and are nearly spherical. Unlike *Cryptosporidium* oocysts and *Giardia* cysts, *Cyclospora* oocysts are not infectious when passed in the stool. In this respect, *C. cayetanensis* is more like *Isospora* or *Toxoplasma*, but has a longer sporulation period (10–14 days vs. 1–2 days for *Isospora* or *Toxoplasma*). Because most sporulation times have been calculated under laboratory conditions, it is not known whether this time frame is consistent with sporulation under natural conditions. Since no laboratory models exist to evaluate infectivity, it is not known whether oocysts sporulated under laboratory conditions are truly infectious, even though motile sporozoites are evident after excystation in the laboratory (2,20). *Cyclospora* oocyst walls are autofluorescent under UV excitation, a property exploited in diagnostic laboratories (see below). This feature may be a defense mechanism, protecting the parasite nucleic acids from UV damage caused by exposure to the sun while the oocysts are sporulating.

## III. DISEASES

Clinical signs associated with cyclosporiasis in immunocompetent persons range from asymptomatic to rampant diarrhea with accompanying risk of dehydration and weight loss. Symptoms may also include anorexia, nausea, vomiting, abdominal bloating, cramping, fatigue, body aches, and occasional low-grade fever. The incubation period preceding the onset of symptoms averages 7 days, and a cycle of remitting and relapsing symptoms lasting several weeks to months is not uncommon among untreated individuals (this includes those individuals that cannot be treated due to sulfa drug allergies). The median duration of diarrheal illness among individuals with outbreak-associated cyclosporiasis ranged from approximately 1.5 to 3 weeks. Oocyst shedding may persist for several weeks after symptoms have abated, but typically disappears within days of the initiation of drug therapy (12). Among immunocompromised individuals (e.g., HIV-infected), cyclosporiasis symptoms may be exacerbated. However, drug treatment is generally effective in controlling infection, and relapse (or reinfection) can be averted by maintenance or prophylactic drug dosages (28,29).

## IV. EPIDEMIOLOGY

*Cyclospora cayetanensis* has been reported worldwide in both developing and industrialized countries. Excluding recent outbreak-associated infections in North America, most infections are observed in tropical and subtropical environments. This is probably related to the potential for endemic transmission and the long-term survival of the organism in the environment. A marked seasonality is associated with infections in Nepal, Guatemala, Peru, and Haiti (10,17,30,31), but temporal (annual period of transmission) and environmental conditions (temperature, humidity, rainfall, elevation) follow no consistent pattern between these sites. The lack of adequate water treatment (e.g., filtration) and sanitation (sewage treatment) is perhaps key to maintaining disease transmission in endemic settings.

In North America and other developed countries, nonoutbreak rates of *Cyclospora* infection (stool positivity) generally fall below 0.5% (12). Conversely, the vast majority of infection events in

North America have been tied to outbreaks linked to international travel or consumption of imported produce. The low prevalence rate (excluding outbreaks) may be attributable to high water quality, sanitation, and, to a lesser extent, hygiene. Without a mechanism for contamination of water or produce, conditions conducive to the establishment of endemic, entrenched infection are limited.

Outbreaks of cyclosporiasis in North America have been linked to imported produce, including raspberries, mesclun (baby) lettuce, and basil (7,9,12,18,32,33). The sources of oocyst contamination were not identified, but given the imported source of the produce and the identification of *Cyclospora* oocysts in river or tap water from developing countries (14,17), an irrigation-related contamination source may be speculated.

Our understanding of the epidemiology of *C. cayetanensis* in endemic and nonendemic settings is yet in its infancy, and much additional data will need to be collected to elucidate the relationship between this parasite, its host, and the environment.

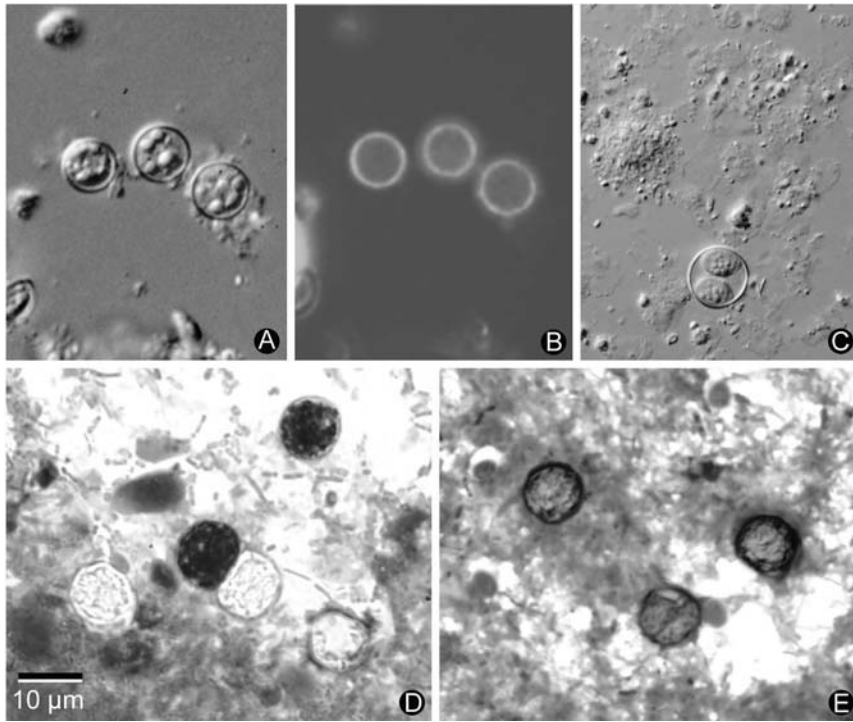
## V. ISOLATION AND IDENTIFICATION

Diagnosing infections caused by *C. cayetanensis* requires laboratory tests to detect the parasite in the feces of the host. With difficulty, the parasite has been identified in histological sections of biopsies from the small intestine, but this is of limited value in routine diagnosis. As mentioned previously, the 8–10  $\mu\text{m}$ , nearly spherical oocysts passed in the stool are unsporulated, with a cytoplasm exhibiting an amorphous, globular character under brightfield microscopy (Fig. 1). While some stool samples are heavily laden with oocysts, making direct microscopy of wet mounts a possibility, it is more common to find stools with few to moderate numbers of oocysts, necessitating a concentration step.

Stools samples are routinely collected into 10% buffered formalin and subsequently subjected to a formalin–ethyl acetate sedimentation procedure (34). The process removes debris and fats and concentrates oocysts. The concentrate may be prepared as a dried smear and stained using a modified acid-fast technique (35,36) or a hot safranin technique (37); the former yields acid-fast variable oocysts (some oocysts may not stain at all, with a gradation of stain intensity occurring in others from faint pink to intense red), while the latter technique yields oocysts that are nearly uniformly bright red to orange, albeit with a tendency for the background to be more reddish in character (Fig. 1D and E, respectively). Traditional parasitological stains that do *not* work well with *Cyclospora* include iodine, trichrome, Giemsa, hematoxylin, Grocott-Gomori methenamine–silver nitrate, periodic acid–Schiff, chromotrope, or Gram-chromotrope stains.

Wet mount examination of stool concentrates using brightfield (especially phase contrast) microscopy or, preferably, UV fluorescence microscopy is a great alternative to staining techniques (35,38). Microscope filter sets with an excitation range of 330–380 nm, dichroic mirror of 400 nm, and barrier filter of 420 nm work well, but comparable filter sets (narrowband or wideband) will also work. With experience, *Cyclospora* oocysts are readily distinguished from fecal debris when mindful of the size, nearly spherical shape, and especially the brilliant blue autofluorescent appearance (Fig. 1B). A negative consideration is that formalin fixation will inactivate the oocysts, preventing the oocysts from maturing and forming the characteristic paired sporocysts with their paired sporozoites within.

A definitive diagnosis can be obtained by sporulating the oocysts in the fecal sample or by performing a molecular diagnosis. In either case, the sample should be preserved in a manner that facilitates sporulation or does not inhibit the molecular assay. Oocyst sporulation occurs during storage at room temperature (approximately 18–20°C). If the fecal specimen is not diluted with sufficient water or blended with a stabilizer that inactivates the bacteria in the fecal matter, the sample will quickly become anaerobic and inhibit the sporulation process. Ideally, the diarrheic fecal sample should be blended with an equal volume of 5% aqueous potassium dichromate (2.5% if the stool is formed). This process inactivates the bacteria but does not interfere with parasite differentiation. A side benefit is that fecal odor is largely neutralized by the dichromate treatment.



**FIGURE 1** *Cyclospora cayetanensis* oocysts in wet mount and stained preparations. (A) Differential interference contrast view of unsporulated oocysts (note amorphous, globular cytoplasm). (B) Autofluorescence of oocysts under UV illumination (matched view of panel A). (C) Differential interference contrast view of sporulated oocyst (note paired sporocysts). (D) Acid-fast stained oocysts under brightfield illumination showing variable staining pattern. (E) Safranin-stained oocysts under brightfield illumination showing uniform staining pattern.

After approximately 2 weeks, oocysts should be observed, each containing two sporocysts (Fig. 1C). Counting individual sporozoites is more difficult and best accomplished with purified oocysts [following excystation (2,3) or nuclear staining of frozen-thawed oocysts with 4',6-diamidino-2-phenylindole (DAPI) (39,40)]. It is important to wash out the dichromate before performing UV microscopy, otherwise the autofluorescence of the oocysts will be obscured. Dichromate-stabilized stool samples are suitable for molecular analysis after the dichromate is washed out. Alternatively, stool can be stored frozen and later subjected to molecular analyses (35).

*Cyclospora* oocyst detection in environmental samples is confounded by the lack of parasite-specific antibody reagents. Unlike *Cryptosporidium* and *Giardia*, which have excellent antibody reagents suitable for both identification and immunomagnetic separation/isolation, no such reagents exist for *Cyclospora*. Nevertheless, the intrinsic autofluorescent character of the oocysts greatly facilitates identification in complex samples (16,17). Water sample analyses have been accomplished using a calcium carbonate flocculation technique combined with UV fluorescence microscopy and PCR analysis (17). Alternatively, water may be concentrated using routine techniques associated with *Cryptosporidium* and *Giardia* analyses (41) (<http://www.epa.gov/nerlcwww/1623.pdf>) with the caveat that commercial antibody reagents are unavailable to facilitate oocyst isolation from water concentrates.



Identification of *Cyclospora* in food is more complex given the challenge of recovering oocysts from the wide range of potential food characteristics (e.g., fresh vegetables and fruits vs. processed foods like chicken pasta salad) (7,15,18). Nevertheless, the difficulties of isolating and identifying oocysts using microscopic methods have been complemented by rapidly progressing molecular biological methods (21,42–45) with advantages in sample processing (especially of complex samples) and assay sensitivity.

## VI. PATHOGENICITY

The pathogenesis of diarrhea associated with cyclosporiasis is poorly understood. Histological examination of small bowel biopsies from clinical cases revealed morphological changes ranging from essentially no alteration of epithelial cell architecture to overt villous blunting, epithelial cell disarray, inflammation, and crypt hyperplasia (27,46). Intestinal malabsorption among individuals with cyclosporiasis was indicated by impaired D-xylose uptake (46,47). Such malabsorption would account for the remarkable diarrhea often accompanying *Cyclospora* infections. Infections have not been associated with intestinal ulceration or invasion of tissues beneath the epithelium, and bleeding does not accompany the watery diarrheic symptoms (48). Symptoms may be exacerbated in immunocompromised individuals, including the potential for biliary tract involvement (49).

Little is known about specific immune responses following *Cyclospora* infections, and the significance of humoral versus cellular immunity remains obscure. The observation that infections in endemic environments are often associated with mild symptoms or are asymptomatic suggests repeated exposure leads to functional, if partial, immune responses (31). Such responses may minimize infection-related symptoms even if they cannot prevent reinfection. In naive populations, infections appear to be more frequently symptomatic and severe. This reinforces the role of immunity in ameliorating the clinical signs of infection.

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

The molecular biology of *Cyclospora* is in its infancy, and little is known about potential virulence, pathogenicity, or drug susceptibility differences among isolates involved in outbreaks and in endemic environments. Until an infectivity model is available that supports the entire life cycle of the parasite, many of these characteristics will remain obscure.

## VIII. CONTROL MEASURES

### A. Prevention

*Cyclospora cayetanensis* is transmitted via the fecal-oral route. Immature, noninfectious oocysts are shed from the infected host and require an extended period in the environment to sporulate and become infectious. Consequently, even with the potential for immediate fecal contamination, direct person-to-person transmission is unlikely. Contaminated food and water are the most likely vehicles for disease transmission, as evidenced by the outbreaks in the United States and elsewhere. Controlling the potential for exposure to contaminated water during produce production should be a priority. Consumption of uncooked, fresh foods is clearly a risk, depending on the likelihood that the produce is contaminated. In the United States, where foodborne vehicles have been strongly implicated or actually identified, most of the outbreaks have been associated with imported produce.

*Cyclospora* oocysts are thought to be quite hardy, much like other coccidian oocysts. If oocyst sporulation is used as a surrogate for an infectivity model, existing data show that elevated temperatures, freezing, anaerobic conditions, and desiccation prevents sporulation. Whether sporulated oocysts are likewise sensitive to these conditions is not yet apparent. Exposure to routine disinfecting

agents (e.g., chlorine: dilute bleach) does not inhibit sporulation and is not likely to serve as a useful approach to control these tough organisms. Until large-scale physical disinfection methods (e.g., gamma irradiation) are validated and in place, it should be expected that contaminated produce remains infectious, especially with storage under refrigeration. Efforts to wash foods will help, but will not necessarily remove contaminants with intended efficiency (12). Freezing or cooking foods may be alternatives, but these are not always options chosen or available during food preparation.

Protecting water is probably best accomplished by eliminating sources of contamination. Given that humans are thought to be the primary source of oocysts (likely the only source), controlling fecal/sewage contamination is paramount. Treatment of contaminated water is best achieved using effective filtration (removal of oocysts) since chemical disinfection is unlikely to be accomplished using routine disinfection approaches (i.e., chlorination). Successful purification of municipal scale water supplies requires filtration using diatomaceous earth, slow sand and coagulation filtration, or comparable filtration technologies. However, in environments without endemic infection, significant fecal contamination is unlikely and filtration may be irrelevant in removing *Cyclospora* oocysts.

Travelers, especially international travelers, can reduce the risk of exposure to these parasites by controlling water consumption (i.e., drinking water filtered through a 1 µm filter or by drinking water previously brought to a boil). Although bottled water or carbonated beverages are generally thought to be safe, mixing with ice prepared from unsafe water sources can compromise this safety. Consumption of fresh, uncooked foods is a perennial risk for travelers. The general approach of requiring food workers to wash their hands after lavatory use is a must for public health reasons, but probably is not as relevant for *Cyclospora* since it is not infectious until well after the parasite is shed in the stool.

## B. Treatment

Symptomatic cyclosporiasis is treated with trimethoprim-sulfamethoxazole (TMP-SMZ) for 7–10 days at an adult dosage of 160 mg TMP plus 800 mg SMZ twice a day (50). Although not rigorously tested, the following drugs appear to have little or no activity against *Cyclospora*: albendazole, TMP alone, azithromycin, nalidixic acid, norfloxacin, tinidazole, metronidazole, quinacrine, tetracycline, diloxanide furoate (12). Among individuals that cannot be treated with TMP-SMZ (e.g., sulfa drug allergies), treatment options are limited and infections may be prolonged. An alternative that demonstrated moderate activity in HIV-infected patients was ciprofloxacin at a 500 mg dose administered twice daily (29). While active, ciprofloxacin was not as effective as TMP-SMZ in this population and has not shown comparable activity in immunocompetent patients (12).

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# 30

## *Toxoplasma gondii*

**Miles B. Markus**

*Biomedical Analysis International, London, England*

### **I. BACKGROUND**

*Toxoplasma gondii* is a protozoan organism that is currently becoming a popular model for researching cellular, immunological, and molecular aspects of intracellular parasitism. *Toxoplasma* was first isolated in 1908 from a North African rodent, the *gondi*—hence the species name. The generic appellation is derived from the Greek word *toxon*, for bow, which refers to the crescentic shape of tissue zoites of the parasite. *Toxoplasma* can be harbored by many vertebrates. Most infections in humans are subclinical, but *Toxoplasma* is an important opportunistic pathogen in persons who are immunocompromised. It can be a particular danger to the fetus. One of the most important sources of human toxoplasmosis is food.

### **II. CHARACTERISTICS**

*Toxoplasma* belongs to the group of protozoan parasites that are classified together as the Sporozoa (synonym: Apicomplexa). As a result of advances in molecular phylogenetics and morphological cladistics, the Sporozoa have recently been placed in a new clade, the Alveolata (1).

Although the extraintestinal stages of *Toxoplasma* have been known for almost a century, knowledge of the life cycle was incomplete until nearly 35 years ago, when coccidian asexual (schizogonic) and sexual (gametogonic) stages were seen in the intestinal epithelium of experimental domestic cats. Wild members of the family Felidae can also act as definitive hosts. The sexual cycle results in the formation of oocysts (containing sporozoites), which are shed in the cat's feces, making it easy to understand how many human infections and most of those in noncarnivorous vertebrates are acquired.

It is widely assumed that postnatally, humans or animals usually become infected with *Toxoplasma* by swallowing food or water contaminated with oocysts or by inadvertently eating tissue cysts in uncooked meat or offal (viscera). After ingestion, sporozoites or the organisms from tissue cysts convert to a rapidly multiplying stage that has been given the name tachyzoite (or endozoite). This replication takes place intracellularly by endodyogeny (formation of daughter cells, each surrounded by its own membrane, while still in the mother cell). *Toxoplasma* reproduces asexually in this way in a wide range of cells in the body. At a later stage of the infection, slowly dividing, encysted bradyzoites (or cystozoites) are characteristically present in muscle and in the central nervous system (2).

### **III. DISEASES**

Recent and detailed discussions of clinical toxoplasmosis have been published (3,4). Postnatally acquired human toxoplasmosis, although usually asymptomatic, can mimic several conditions.

Lymphadenopathy is the most usual symptom of clinical disease, the involvement varying from enlargement of a single lymph node to generalized lymphadenopathy. Enlargement of lymph glands may or may not be accompanied by one or more of the following: fatigue, fever, malaise, muscular pain, hepatomegaly, splenomegaly, abdominal pain, headache, rash, or sore throat. Toxoplasmosis is today recognized as being a common cause of lymphadenopathy in cases where the Paul-Bunnell test for glandular fever is negative.

In more severe cases, such as in immunodeficient individuals, including those receiving immunosuppressive therapy, there may be encephalitis, hepatitis, pneumonitis, myocarditis, or pericarditis. The consequences of reactivation of quiescent *Toxoplasma* infection in immunologically compromised patients where the defense mechanisms have been rendered ineffective by underlying disease and/or treatment can be lethal, and the protozoon has emerged as an important opportunistic parasite in cases of acquired immunodeficiency syndrome (AIDS), malignancy, and in transplantation recipients. Early therapy may prove life-saving in such patients, and thus toxoplasmosis should always be promptly considered when there are symptoms referable to brain (in particular), heart, liver, or lungs.

Congenital toxoplasmosis has generally been thought to result from primary infection of the mother during pregnancy, but this concept is still being debated (3,5). Some authors consider *Toxoplasma* to be an important cause of repeated human abortion, but the possibility requires further investigation. In addition to abortion of the fetus (probably rare), the effects of transplacental infection include cerebral calcification, hydrocephaly, microcephaly, and seizure disorders in the infant. Apart from damage to the central nervous system, there may be signs and symptoms such as fever, generalized edema, pneumonitis, rash, hepatosplenomegaly, anemia, jaundice, lymphadenopathy, or myocarditis. Retinochoroiditis (6) occurs in a large number of cases, most frequently in the young adult and often in the absence of other symptoms. Subclinical congenital toxoplasmosis in neonates may not be uncommon and could be an important cause of ocular or central nervous system problems that only become apparent in later infancy or childhood (3,7).

Whether ocular toxoplasmosis can also result from postnatally acquired infection has, historically, been uncertain, but it is becoming increasingly clear that this must happen frequently (8–13).

#### IV. EPIDEMIOLOGY

As indicated in Sec. II, the most common ways in which humans acquire *Toxoplasma* infection are probably by consuming undercooked meat, raw meat, or offal containing tissue cysts, and by accidentally ingesting oocysts that have contaminated unwashed fruit and vegetables or water supplies (14,15).

*Toxoplasma* is present in approximately 30% of the human population worldwide. The prevalence of infection in any given region increases with age but varies from country to country and among different ethnic groups inhabiting the same area (16). For instance, in parts of continental Europe where a lot of uncooked, lightly cooked, and cured meat is eaten, up to 80% of adults in some communities are thought to be infected with *Toxoplasma*. Estimates of congenital toxoplasmosis per 1000 live births generally range between 0.25 and 6–7 in different countries.

Pork, lamb, and mutton, along with a range of game meats, are important sources of *Toxoplasma* infection. However, the prevalence of occurrence of the protozoon in domestic animals in various countries (16) has declined markedly following the introduction of intensive methods of farming. Infection can also be acquired from goat meat. Products like raw sausages and even cured meats are to be regarded as potential sources of *Toxoplasma* infection (17). Further studies involving both beef and poultry are required in order to clarify their role in the epidemiology of human toxoplasmosis (16,18).

Apart from the two main routes of infection referred to above, transmission of *Toxoplasma* can occur via blood products, particularly leukocyte transfusion; in transplantation involving heart, kidney, liver, and bone marrow; and by accidental infection of laboratory workers (some cases

having been illustrative of transmission by wound contamination). Considering that there is only a transient peripheral parasitemia in toxoplasmosis, the risk of infection from normal blood transfusion may be low.

*Toxoplasma* occurs in the milk of various animals, including that of cows, goats, and sheep. Therefore, unpasteurized milk and some other dairy products are also likely sources of infection (16, 19–23). In addition to milk and blood, tachyzoites of *Toxoplasma* have been found in saliva, semen, tears, and urine. There is, as yet, no evidence of transmission to humans from these four body fluids. On the other hand, there are reports of human toxoplasmosis associated with evisceration and skinning of animals; and in certain surveys, antibody prevalences have been higher in slaughterhouse workers than in control subjects. How most of these infections were acquired is not clear (24,25).

Tachyzoites of *Toxoplasma* have been reported from chicken eggs, but this is an unlikely source of human infection in the western world, considering that infection in chickens is rare and that even light cooking or salting will kill the labile tachyzoites (16).

Cats per se are not considered to be a risk factor (17,26–28). To date, there is no evidence that oocysts, which are noninfective when shed by cats, adhere to fur (27) or that they would sporulate if they were to do so (29). Rather, it is soil in which oocysts have sporulated that is a source of infection. The resistant oocysts from cats can remain viable in moist soil for long periods (30,31). Oocysts can be disseminated by transport hosts such as cockroaches, earthworms (thereby infecting birds that eat the worms), flies, and dogs (29,32–34). The enteroepithelial cycle of *Toxoplasma* does not take place in dogs, but they are often coprophagous and ingest cat feces, resulting in viable oocysts being shed in their excrement (29,34). Moreover, dogs might swallow oocysts, albeit in much smaller numbers, by snapping at and consuming flies that have visited *Toxoplasma* oocyst-containing canine or feline feces (35).

## V. ISOLATION AND IDENTIFICATION

An authoritative and comprehensive account of approaches to the diagnosis of toxoplasmosis has recently been updated (3), and a summary of the use of key laboratory tests in particular situations has been published (36).

*Toxoplasma* infections can be diagnosed in four main ways: isolation of the organism in tissue culture or by mouse inoculation (3,37,38); histological detection of the protozoon in tissue or identification of zoites on impression smears (3); recovery of *Toxoplasma* DNA from various body fluids by use of the polymerase chain reaction (PCR) (3,39–56); and immunological investigation (3,36,57–68). Of these, the last-mentioned is employed most widely. It has the greatest limitations, however. Inter alia, test results can be falsely positive or falsely negative (3,36). Common antigenicity with other organisms is minimal, but there might be some cross-reaction with *Neospora*, depending on the antigen used (69–77). An understanding of the characteristics of available diagnostic procedures is crucial to their application and interpretation in different clinical situations, notably in cases of immunodeficiency, ocular toxoplasmosis (78–83), and pregnancy (3,36,84–91). Historically, serological tests have included the Sabin-Feldman dye test, complement fixation test (CFT), and the indirect fluorescent antibody test (IFAT), all of which measure IgG anti-*Toxoplasma* antibodies. The IgM IFAT has also been widely used. More recent methodologies have included agglutination tests (3). Enzyme-linked immunosorbent assay (ELISA) technology has largely replaced other immunological diagnostic techniques. In HIV-infected patients, neuroimaging investigations are carried out to search for lesions that may be suggestive of toxoplasmic encephalitis. There are no pathognomonic features in neuroradiological imaging to definitively diagnose the condition, however.

In immunocompromised individuals with possible cerebral toxoplasmosis, detection of IgM anti-*Toxoplasma* antibodies is not a routine part of the diagnostic workup. This is because recrudescence of toxoplasmosis that arises from latent, chronic infection is usually the cause. In other persons such as pregnant women, the implications of the presence of IgM antibodies are to be considered carefully. Because IgM antibodies may persist for months or even years after the acute infection,

their greatest value lies in determining that a pregnant woman was possibly not infected during her pregnancy. A negative result often rules out the likelihood of such an early infection, depending on the test used. If IgM-related results are positive, there are various additional tests, including some for the detection of IgA, and/or IgE, and/or IgG antibodies, which can help to differentiate between recently acquired and less recently acquired infection. In this regard, a differential agglutination test is particularly helpful (92). A number of tests for avidity of IgG antibodies to *Toxoplasma* have been introduced to assist in distinguishing between distant and recently acquired infection (63).

Whereas routine pathology laboratories tend to use assays that are commercially available, specialist laboratories are more often able to use in-house assays. The challenges of interpreting the results of tests for *Toxoplasma* infection in pregnancy, immunocompromised persons, and ocular disease, are best left to specialist laboratories. Specimens from cases such as these can be either redirected to a specialist laboratory or first screened using a commercial assay to assess whether or not to refer a specimen for further testing. Examples of specialist centers are the *Toxoplasma* Serology Laboratory at the Palo Alto Medical Foundation Research Institute (TSL-PAMFRI) in the United States (93), the Public Health Laboratory Service *Toxoplasma* Reference Unit, which serves England and Wales (36), and the Scottish *Toxoplasma* Reference Laboratory (94,95).

A technique for isolating oocysts from drinking water has been described (37,96). Oocysts of *Toxoplasma* in feline feces are normally detected by centrifugal flotation and microscopy, but they may well prove to be acid-fast, like oocysts of other coccidia, if stained in direct fecal smear preparations (97,98).

## VI. PATHOGENICITY

Pathogenicity is determined by the virulence of the strain of *Toxoplasma* concerned and by the susceptibility of the host species and individual. Most human infections are subclinical.

*Toxoplasma* does not appear to produce a toxin. The available evidence suggests that tissue pathology associated with toxoplasmosis is the result of lytic destruction of individual host cells. Tachyzoites that have multiplied in a cell exit from it and invade adjacent cells. Molecular mechanisms used for both invasion of and egress from cells may be similar (99). The original host cell dies. This necrotic cell death leads, directly or indirectly (via the resultant inflammatory reaction), to the pathology that has been observed and which is particularly clear in a sensitive site like the brain. The process of tachyzoite-bradyzoite interconversion is central to the pathogenicity and longevity of infection (100–102). Initially, *Toxoplasma* reproduces in a variety of tissues. The ability of the organism to evade host immune mechanisms leads to chronicity of infection and ensures that there is frequently a reservoir of parasites in the body (thought to be mainly in muscle and the brain and spinal cord) that can give rise to recrudescence of clinical toxoplasmosis if the host become immunodeficient.

Immunological mechanisms involved in pathogenesis and host resistance are slowly being elucidated (103–115).

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

Virulent strains of *Toxoplasma* have been distinguished from avirulent strains on the basis of immunological techniques such as their reactivity with certain monoclonal antibodies. Likewise, a relationship between isoenzyme patterns and virulence has been documented. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses have also been used to type isolates as avirulent or virulent. Most strains of *Toxoplasma* fall into one of three clonal groups, which have been designated as types I, II, and III. Type I lineage organisms are highly virulent in mice and, possibly, in humans. Strains of groups II and III, on the other hand, are relatively avirulent (116–118). Microsatellite analysis has revealed two clonal lineages (119).

The genetic and other host and parasite factors that contribute to virulence of *Toxoplasma* are



poorly understood at present and are the subject of ongoing investigations. Among the mechanisms to which virulence of *Toxoplasma* has been related are the following: expression of high levels of heat shock proteins; de novo pyrimidine biosynthesis as a requirement for virulence (120); increased DNA polymerase activity; higher numbers of a repeat sequence in the promoter region of the SAG-1 gene; deficient humoral responses in the host (121,122); greater dense granule protein GRA-7 synthesis (123); superior migratory capacity of virulent parasites (124); and major histocompatibility complex (MHC) genetic factors (125–127).

Classical genetic linkage mapping has revealed that genes which mediate acute virulence are associated with chromosome VII in *Toxoplasma*. To enhance the resolution of gene mapping studies, single-nucleotide polymorphisms are being developed based on an extensive database of expressed sequence tags (ESTs) from *Toxoplasma*. In addition, DNA microarray investigations are being employed to research the expression of host and parasite genes during infection (128). Together, these approaches may improve understanding of virulence in toxoplasmosis.

Murine models are proving to be extremely useful for studying host and parasite genetic factors related to the virulence of *Toxoplasma*. However, this type of research is still in its infancy as far as the human host is concerned. Methods that use both single gene and comprehensive approaches to human genetics are needed to define the underlying genetic mechanisms related to virulence.

## VIII. CONTROL MEASURES

### A. Prevention of Infection: Domestic Animals

Basic control strategies to reduce the frequency of transmission of *Toxoplasma* to humans are the implementation of appropriate farming practices and the theoretical possibility of vaccination (see below) of cats and domestic meat animals. Free-ranging livestock will obviously be associated with acquisition of *Toxoplasma* infection, but when animals or poultry are kept indoors, control is feasible. Where possible, rodent populations should be kept down because they can be an important source of infection for cats (129). Contamination of food and water with cat feces, either directly or indirectly, is to be avoided. This can be largely achieved by using sterilized feed, preventing pet animals from having access to food stores and sheds, and keeping sheds free of birds, insects, and rodents (16).

### B. Prevention of Infection: Humans

Effective strategies for prevention of infection in risk groups are based on the most likely sources of infection in a given population, but the main recommendations for prevention of infection are universally applicable. Since toxoplasmosis is primarily a danger to the fetus and someone who is immunocompromised, pregnant women or immunodeficient persons, irrespective of whether seronegative or seropositive for *Toxoplasma*, should take care when handling raw meat. Apart from keeping fingers away from the mouth and eyes while doing so, they should not prepare any food that is not going to be cooked, e.g., cold salad, without first washing the hands. They should avoid unnecessarily close contact with the household cat's litter tray, particularly if the animal is fed on raw meat or is in the habit of catching rodents or birds. The cat should preferably be given only commercial or thoroughly cooked food and kept indoors. If it is the person potentially at risk who has to clean the litter tray, gloves should be worn. The litter box should be emptied twice daily. Boiling water should be poured over the box if the bottom or sides have come into contact with feces. Flies should be kept away from food; and only milk that has been pasteurized should be drunk. Hands should be washed after soil contact and before eating. Likewise, vegetables or fruit that may have become contaminated by soil containing cat feces should be well rinsed. Dogs that have eaten or rolled on cat feces could theoretically transport viable oocysts (29,34), so general hygiene should also be observed in relation to dogs.

Tissue cysts of *Toxoplasma* in meat sometimes survive freezing, and cured meat may or may

not contain infective organisms (16). Only well-cooked meat or other edible parts of animals should be consumed by someone who is pregnant or immunocompromised. During cooking, the temperature must reach 67°C. Survival of cysts at lower temperatures depends on the duration of cooking (16). Microwaving will not necessarily inactivate bradyzoites in all tissue cysts because the meat is heated unevenly. Gamma irradiation can render cysts in meat nonviable (16).

Screening of pregnant women for toxoplasmosis can cause anxiety. If advice is given on how to avoid becoming infected, the need for screening during pregnancy can be reduced (90,130).

### C. Treatment

Much remains to be learned about the treatment of human toxoplasmosis and prevention thereof in immunodeficiency. New information is becoming available (131–139). Despite the large number of studies that have been carried out during the past 30 years, it is still not clear to what extent antenatal treatment in women with presumed toxoplasmosis can prevent congenital transmission (140–142). However, the possibility that it does so cannot be ruled out (143). The absence of an effect of prenatal treatment (144) might be due to intrauterine transmission having taken place before the commencement of therapy. There is a lack of evidence to support the routine use of antibiotics for the treatment of acute toxoplasmic retinochoroiditis. Moreover, the evidence that long-term antibiotic treatment reduces the chances of recurrence in patients with chronic, recurrent toxoplasmic retinochoroiditis is weak (145). The subject of treatment for toxoplasmosis has recently been reviewed (3,146,147). (For suggested treatment protocols, see Ref. 4.)

### D. Vaccines

Parasites have achieved a unique evolutionary balance with mammalian immune systems. This defeats simple vaccine strategies that have been successful against viruses and bacteria. No vaccine against toxoplasmosis is available for humans. In designing vaccines, the heterogeneity of human responses to individual recombinant *Toxoplasma* antigens would have to be taken into account (148). However, it is debatable whether a vaccine is needed for use in humans. Vaccine-related research has been carried out with the possibility of vaccinating cats and farm animals in mind (149–152). The objectives would be to reduce human exposure to infected meat and to *Toxoplasma* oocysts and to reduce the number of abortions that occur primarily in sheep and goats as a result of transplacental infection of fetuses.

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# 31

## *Taenia solium*

**Oscar H. Del Brutto**

*Hospital-Clínica Kennedy, Guayaquil, Ecuador*

**Julio Sotelo**

*National Institute of Neurology and Neurosurgery, Mexico City, Mexico*

### I. BACKGROUND

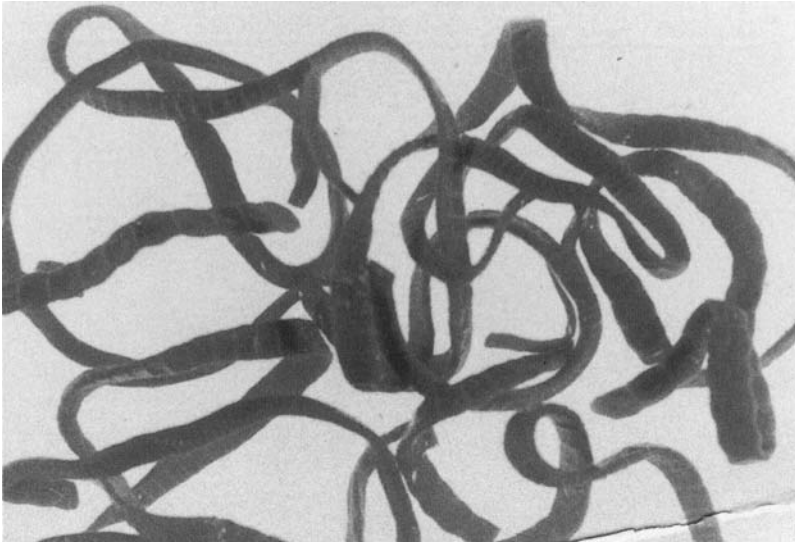
*Taenia* spp. are common parasites of humans, affecting more than 50 million people worldwide. These cestodes (tapeworms) belong to the phylum Platyhelminthes, class Cestoidea, order Cyclophylloidea, family Taeniidae (1). The family includes three parasites, *Taenia solium* (the pork tapeworm), *Taenia saginata* (the beef tapeworm), and a third form called Asian *Taenia* that is morphologically similar to *T. saginata* but biologically akin to *T. solium* (2). While *T. saginata* is more common and widespread than *T. solium*, the latter is more relevant as a human pathogen since infection with its larval form causes cysticercosis, a serious disease (3).

### II. CHARACTERISTICS

*T. solium* has a head (scolex) that consists of four suckers and a double crown of hooks, an unsegmented narrow neck, and a large body (strobila) formed by several hundred proglottids (Fig. 1). Proglottids located proximal to the scolex are immature and lack sexual organs, those in the middle of the strobila contain both male and female sexual organs, and those in the distal end are gravid and contain thousands of fertile eggs, which are accumulated within a branched uterus. The adult *T. solium* inhabits the small intestine of humans, where it is attached to the intestinal wall by its potent suckers and hooks. Every day, a few gravid proglottids are detached from the distal end of the strobila and are passed with the feces, liberating eggs which consist of two main elements, an inner part called the oncosphere (hexacant embryo) and a surrounding coat or embryophore (4). Eggs are resistant to adverse environments and can remain viable for several months in water, soil, and vegetation (5).

In places with poor sanitation, pigs may ingest human feces containing *T. solium* eggs and become intermediate hosts in the life cycle of *T. solium*. Once in the intestinal tract, eggs lose their coat, liberating oncospheres that, in turn, cross the intestinal wall, enter the bloodstream, and are carried to the tissues of the pork where embryos evolve forming larvae (cysticerci) (6–8).

*T. solium* cysticercus is a vesicle consisting of a vesicular wall and an invaginated scolex. The vesicular wall is a membranous structure composed of an outer layer called the cuticular mantle, a middle cellular layer with pseudoepithelial structure, and an inner layer formed by circular muscle and reticular fibers (9). The invaginated scolex has a similar structure to that of the adult *T. solium*, including a head armed with suckers and hooks, an elongated neck, and a rudimentary body (Fig. 2). As the body grows, it bends within the vesicle, originating the so-called spiral canal, which leads



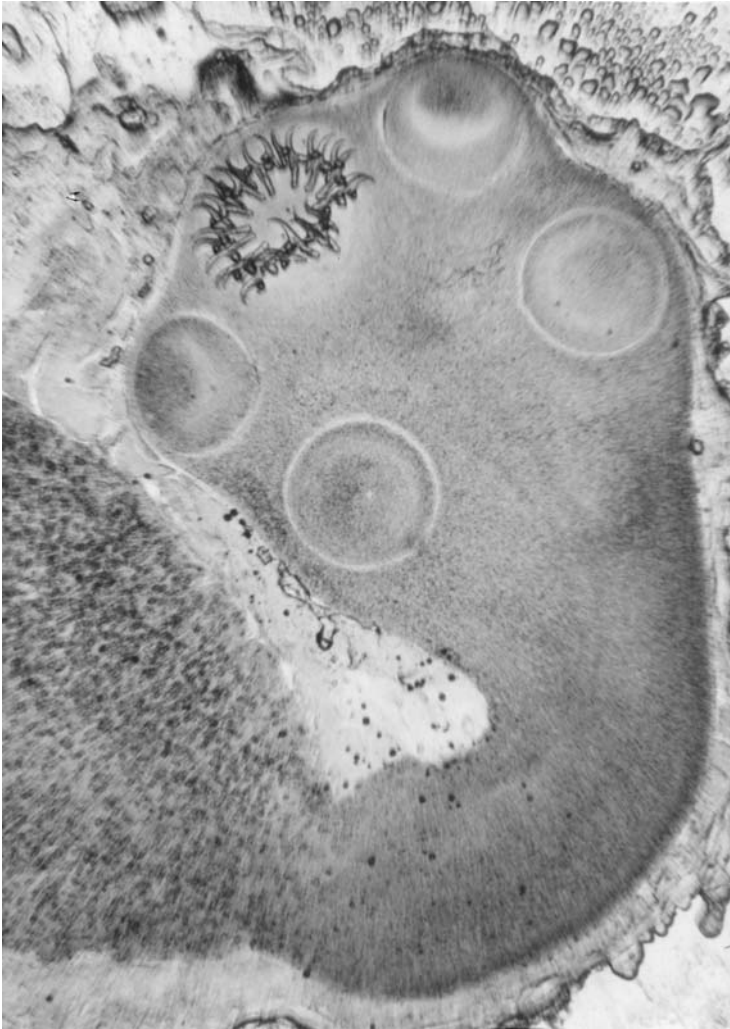
**FIGURE 1** Adult *Taenia solium* showing the ribbon-like appearance of the strobila. (From Ref. 3.)

from the bladder wall to the scolex. The tegument of the body is similar to the vesicular wall, except that the cuticular mantle is thicker, and the reticular layer has numerous calcareous corpuscles (10).

Human consumption of improperly cooked infected pork meat results in the release of cysticerci in the small intestine, where, by the action of digestive enzymes, the metacestode evaginates and attaches to the intestinal wall. After the scolex is attached, proglottids multiply and become the adult cestode. Humans can also act as intermediate hosts for *T. solium* after ingesting its eggs. Under these circumstances, human cysticercosis develops. The mechanism by which eggs cross the intestinal wall and lodge in human tissues is the same as that described in the pork. The two main sources from which humans acquire cysticercosis are ingestion of water or food contaminated with *T. solium* eggs and the fecal-oral route in patients harboring the adult parasite in the intestine. While the former was considered the most common form of transmission, recent epidemiological evidence stresses the importance of *Taenia* carriers in the dissemination of the disease (11). Indeed, nonhygienic handling of food by *T. solium* carriers is currently considered a frequent form of transmission of human cysticercosis (12,13).

### III. DISEASE

*T. solium* may produce two main diseases in humans according to whether the infection is caused by the adult worm or by its larval form. The former, called taeniosis, is a benign condition that may cause loss of appetite, nausea, abdominal discomfort, constipation, or diarrhea, but it is frequently asymptomatic (14). In contrast, cysticercosis may be a severe disease due to the tendency of cysticerci to lodge in the central nervous system (15). Clinical manifestations of neurocysticercosis depend on the number and location of lesions as well as the severity of the host's immune reaction against the parasites (Table 1). Epilepsy is the most common manifestation of the disease, occurring in more than 70% of patients (16). Seizures related to cysticerci usually occur in otherwise healthy middle-aged individuals coming from endemic areas (17,18). Indeed, according to the International League Against Epilepsy, neurocysticercosis is the single most common cause to explain the increased prevalence of active epilepsy in developing compared to developed countries (19). A variety of focal neurological signs (pyramidal tract signs, sensory deficits, cerebellar ataxia, signs of brain-



**FIGURE 2** Cysticercus of *Taenia solium* showing the scolex with four suckers and a double crown of hooks. (From Ref. 3.)

stem dysfunction, and involuntary movements) have been described in patients with neurocysticercosis (20). These manifestations usually follow a subacute or chronic course, making the differential diagnosis with neoplasms or other infections of the nervous system difficult on clinical grounds. However, focal signs may occur abruptly in patients who develop a cerebral infarct as a complication of subarachnoid neurocysticercosis (21). Some patients present with increased intracranial pressure or dementia related to hydrocephalus or massive cysticercal infection of the brain parenchyma (15,22). A particularly severe form of neurocysticercosis, cysticercotic encephalitis, occurs when cysticerci are acutely rejected after entering the nervous system and is characterized by cloudiness of consciousness, seizures, signs of intracranial hypertension, and blindness (23). Clinical manifestations of spinal neurocysticercosis are nonspecific, and the differential diagnosis with other diseases of the spinal cord is difficult on clinical grounds. Spinal arachnoiditis is characterized by root pain and weakness of subacute onset, and cysts in the cord parenchyma usually course with motor and

**TABLE 1** Common Clinical Manifestations of Neurocysticercosis According to Location of Parasites

Location/Form of the disease	Usual clinical manifestations
Brain parenchyma	
Vesicular cysts	Seizures; may be asymptomatic
Colloidal cysts	Seizures; headache; vomiting; focal signs
Granulomas/calcification	Recurrent seizures
Cysticercotic encephalitis	Coma; seizures; intracranial hypertension
Subarachnoid space	
Giant cysts in CSF cisterns	Seizures; intracranial hypertension; focal signs
Diffuse arachnoiditis	Focal signs; intracranial hypertension
Angiitis	Acute stroke syndromes
Ventricular system	
Ventricular cysts	Focal signs; intracranial hypertension
Ependymitis	Seizures; intracranial hypertension
Spinal cord	
Arachnoiditis	Root pain; weakness
Parenchymal cysts	Motor and sensory signs

sensory deficits that vary according to the level of the lesion (24). Cysticercosis outside the central nervous system is usually asymptomatic, although ocular cysticerci may cause sudden blindness, and massive infestation of skeletal muscles may produce muscular pseudohypertrophy (25,26).

#### IV. EPIDEMIOLOGY

Neurocysticercosis is the most common helminthic disease of the central nervous system and a rather frequent neurological disorder in most of the developing world (Fig. 3). Massive immigration of people from endemic to nonendemic areas has caused an increase in the number of cases of neurocysticercosis in some developed countries where this condition was rare in the past (27,28). According to the World Health Organization (WHO), more than 2 million people harbor the adult *T. solium* and many more are infected with cysticerci (29). *Taenia* carriers are contagious sources of cysticercosis, endangering themselves and everyone coming into contact with them (30). Indeed, most patients with neurocysticercosis have been infected by someone harboring a *T. solium* in the patient's close environment (11–13). Neurocysticercosis accounts for 10% of all admissions to neurological hospitals in endemic areas. It is estimated that 50,000 new deaths due to neurocysticercosis occur every year, and many times that number of patients survive but are left with irreversible brain damage. The latter is an important public health problem since most affected people are at productive ages (29).

Most Latin American countries are endemic for cysticercosis. Among these, Mexico, Ecuador, and Peru are the countries where the disease has been more extensively studied. Necropsy studies from general hospitals have shown that the prevalence of neurocysticercosis in the general population may be as high as 3.6% in some areas (31). The endemia of cysticercosis in Latin America is related to the poor socioeconomic conditions of their inhabitants and to the lack of knowledge about the nature of the disease and its form of transmission (32). The endemia of cysticercosis in Asia and Africa is mainly related to the religious practices of their inhabitants. Because the Koran prohibits the consumption of pork, taeniosis and cysticercosis are almost unknown conditions in Moslem countries. On the other hand, these conditions are endemic in areas where pork is massively consumed under poor hygienic conditions, such as in most sub-Saharan African countries, in the Indian subcontinent, and in several other countries of Southeast Asia (33,34). The prevalence of neurocys-



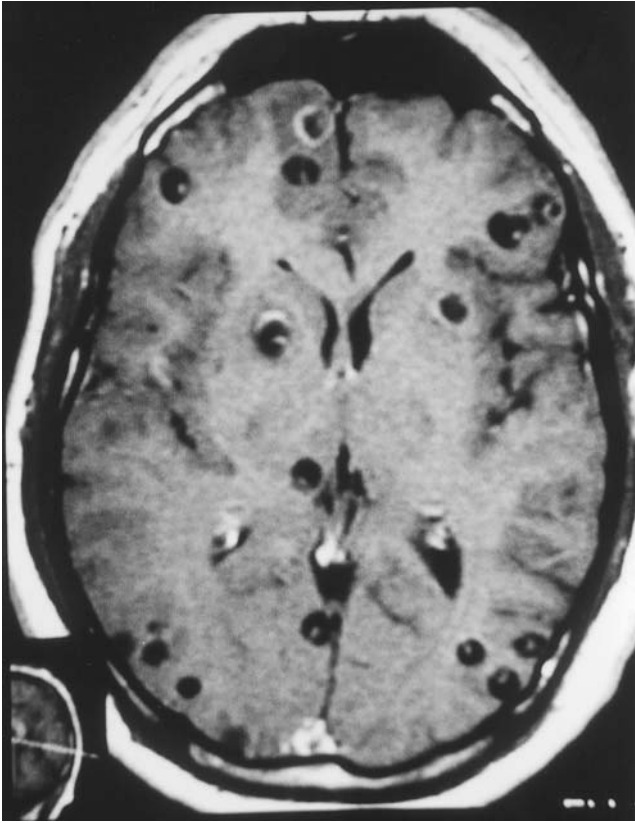
**FIGURE 3** World map showing areas where *Taenia solium* cysticercosis is endemic. (From Ref. 3.)

ticercosis in these countries is still underestimated because of the lack of diagnostic facilities in rural areas where the reported cases actually represent the tip of the iceberg.

By the turn of the twentieth century, taeniosis and cysticercosis were endemic in several countries of Central Europe. Nevertheless, improved public sanitation and careful inspection of pork meat before public distribution promptly reduced its prevalence. Transient increases in the prevalence of cysticercosis were noted in some of these countries after the return of soldiers from endemic areas (35). In contrast, the disease is still present in some countries of Eastern Europe as well as in Spain and Portugal. In the United States, obligatory inspection of pork meat caused a large impact on the prevalence of cysticercosis, with fewer than 150 reported cases until 1979. Nevertheless, together with the growing number of immigrants from endemic areas, there has been an increase in the number of patients with neurocysticercosis during the past few years. Most cases have been reported from Texas and California where there are more than 10 million immigrants from endemic countries (27,28). While more than 90% of neurocysticercosis patients in the United States are Latin American immigrants, some cases have been recognized in American citizens with no history of travel to endemic areas. Many of these patients acquired the disease through a household contact harboring a *T. solium* (36).

## V. ISOLATION AND IDENTIFICATION

In patients with taeniosis, mild eosinophilia and elevated serum IgE levels are common but nonspecific findings. Recognition of *Taenia* eggs is not easy, and many patients may escape detection when a single coproparasitological study is performed. Therefore, several stool specimens must be examined before the patient is considered negative. More sensitive than routine stool examination is the perianal cellophane tape test, which has the advantage of detecting *Taenia* eggs adhered to the buttocks and is positive in up to 85% of infected individuals (37). It must be noted, however, that recognition of *Taenia* eggs in feces does not allow differentiation between infection with *T. saginata* and *T. solium*. Microscopic examination of a gravid proglottid is needed to identify *Taenia*

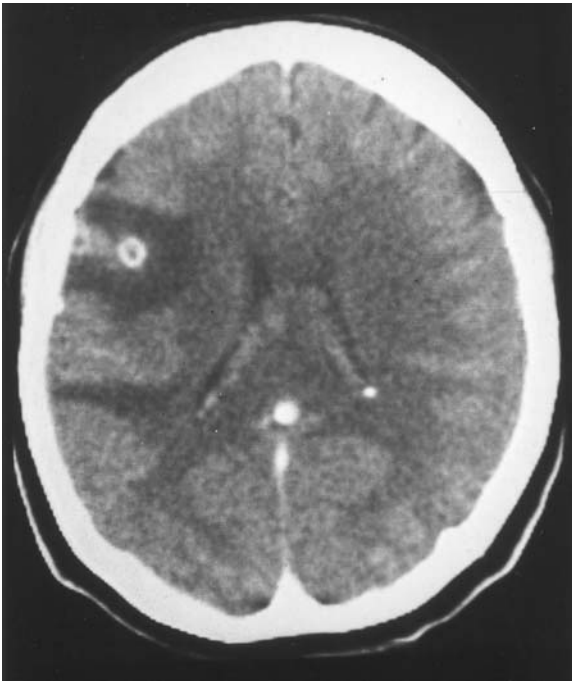


(a)

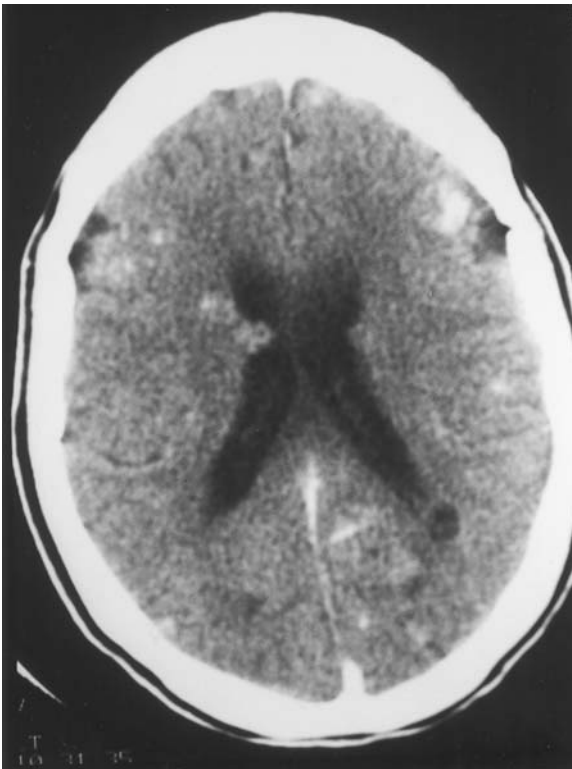
**FIGURE 4** Common neuroimaging findings in neurocysticercosis: (a) vesicular cysts showing the scolex; (b) single enhancing lesion; (c) parenchymal lesions in different evolutive stages; and (d) large subarachnoid cysts.

spp., since the uterus of *T. saginata* has 15–20 lateral branches while that of *T. solium* has 7–13 (4,14). More recently, ELISA using *Taenia*-specific antibodies for coproantigen detection and DNA hybridization techniques for egg identification have improved the diagnosis of taeniosis, allowing better screening for *T. solium* carriers in endemic areas (38).

Diagnosis of neurocysticercosis is a challenge. Its most common clinical manifestations are also caused by a broad range of neurological conditions. Neuroimaging studies are usually abnormal but, in most cases, not pathognomonic (Fig. 4). A number of serological tests have been developed to support the diagnosis. However, older tests had low specificity and current assays have decreased sensitivity in patients with single lesions (39,40). A set of diagnostic criteria based on the objective evaluation of clinical, radiological, immunological, and epidemiological data has recently been proposed (41). This set includes four categories of criteria—absolute, major, minor, and epidemiological—stratified on the basis of their individual diagnostic strength. Absolute criteria allow unequivocal diagnosis of neurocysticercosis, major criteria strongly suggest the diagnosis but cannot be used alone to confirm the disease, minor criteria are frequent but nonspecific manifestations of the disease, and epidemiological criteria refer to circumstantial evidence that favor the diagnosis of cysticercosis. Interpretation of these criteria result in two categories of diagnostic certainty—definitive and probable—according to the likelihood that neurocysticercosis is present in a given patient (Table 2).



(b)



(c)

**FIGURE 4** Continued





(d)

FIGURE 4 Continued

## VI. PATHOGENICITY

With the exception of local inflammatory changes in the intestinal mucosa, the adult *T. solium* does not produce pathological changes in the tissues of the hosts. In contrast, cysticerci induce a myriad of pathological changes in the nervous system that, together with the host's immune response against the parasite, are the main determinants for the clinical pleomorphism of this disease (42,43). After entering the nervous system, cysticerci are viable and elicit few inflammatory changes in the surrounding tissues (vesicular stage). Cysticerci may remain for decades in this stage when the host falls in a state of immune tolerance or may enter, as the result of a complex immunological attack from the host, in a process of degeneration that destroys the parasite. The orderly stages by which cysticerci pass through in this process of degeneration are the colloidal, granular, and calcified stages (44). These stages are characterized by specific changes within the parasite and in the surrounding brain parenchyma (Table 3).

Both the humoral and the cellular immune response of the host play fundamental roles in the severity of disease expression in patients with neurocysticercosis (Fig. 5). Some cysticercal antigens stimulate the production of specific antibodies that form the basis for the immunological diagnosis of cysticercosis, while others play a role in the mechanisms for evasion of the immune surveillance against cysticerci. One of these antigens, called antigen B, inhibits the classical pathway of complement activation and could play a role in the protection of cysticerci against the host's immunological attack (43). Despite the presence of specific antibodies against cysticerci, there is no correlation between the severity of parasite destruction and the overall titers of such antibodies. Moreover,

**TABLE 2** Diagnostic Criteria and Degrees of Diagnostic Certainty for Neurocysticercosis

## Diagnostic Criteria

## Absolute

- Histological demonstration of the parasite from biopsy of a brain or spinal cord lesion
- Evidence of cystic lesions showing the scolex on neuroimaging studies
- Direct visualization of subretinal parasites by fundoscopic examination
- Spontaneous resolution of small single enhancing lesions

## Major

- Evidence of lesions highly suggestive of neurocysticercosis on neuroimaging studies
- Positive serum immunoblot for the detection of anticysticercal antibodies
- Resolution of intracranial cystic lesions after therapy with albendazole or praziquantel

## Minor

- Evidence of lesions suggestive of neurocysticercosis on neuroimaging studies
- Presence of clinical manifestations suggestive of neurocysticercosis
- Positive CSF ELISA for detection of anticysticercal antibodies or cysticercal antigens
- Evidence of cysticercosis outside the central nervous system

## Epidemiological

- Individuals coming from or living in an area where cysticercosis is endemic
- History of frequent travel to disease-endemic areas
- Evidence of household a contact with *T. solium* infection

## Degrees of Diagnostic Certainty

## Definitive

- Presence of one absolute criterion
- Presence of two major plus one minor or one epidemiological criteria

## Probable

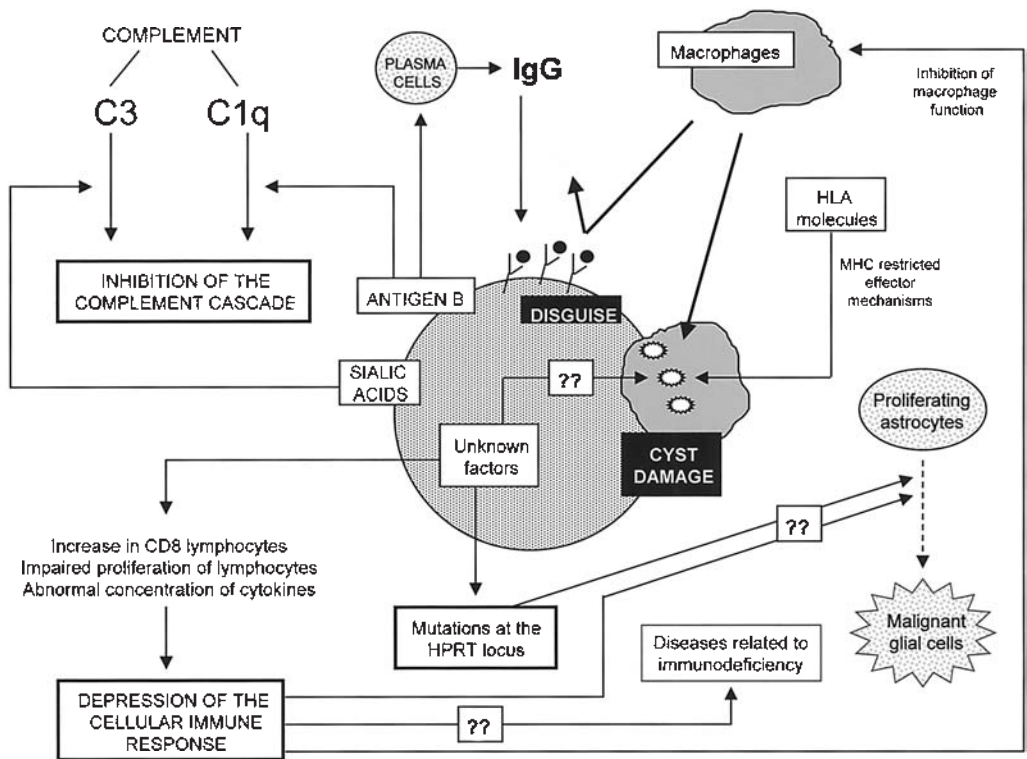
- Presence of one major plus two minor criteria
- Presence of one major plus one minor and one epidemiological criteria
- Presence of three minor plus one epidemiological criteria

Source: Ref. 41.

immunoglobulins are more frequently found around living parasites than surrounding dead cysts, suggesting that cysticerci use these molecules as a screen to avoid recognition from the immune system (45). Impairment of cellular immunity resulting from an increase in the subpopulations of CD8 T lymphocytes, impaired proliferation of lymphocytes, and abnormal concentration of cytokines has also been documented in some patients with neurocysticercosis (5). This may be responsi-

**TABLE 3** Correlation Between Stage of Involution of Cysticerci, Their Morphological Appearance, and Presence of Inflammatory Changes in Nervous System

Stage	Appearance of parasite	Pathological changes
Vesicular cysts	Translucent vesicular wall and fluid; viable scolex	Scarce inflammation in the surrounding brain
Colloidal cysts	Thick vesicular wall; turbid fluid; degenerated scolex	Intense inflammatory reaction; thick collagen capsule around the cyst
Granulomas/Calcifications	Mineralized nodules; no scolex	Intense gliosis; giant cells



**FIGURE 5** Schematic representation of the complex host-parasite relationship showing the different mechanisms used by cysticerci to evade the host's immune response. (From Ref. 3.)

ble for the reported association of this parasitic disease with conditions resulting from immunodeficiency states such as leukemia, ataxia-telangiectasia, lymphoma, and brain tumors (46–49).

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

The distribution of HLA class I and class II determinants was investigated in patients with neurocysticercosis and compared with healthy controls from the same ethnic group (50). The two antigens showing significant differences between patients and controls were HLA-A28 and HLA-DQw2; the first was found to be increased (39.6% vs. 15.6%,  $p = 0.008$ ) and the second was decreased (4.2% vs. 31.7%,  $p = 0.004$ ) in patients with neurocysticercosis when compared with controls. In this study, the relative risk for developing neurocysticercosis in HLA-A28-positive subjects was 3.55. These findings suggested that susceptibility and resistance to neurocysticercosis are partially related to genetic influences through molecules of the major histocompatibility complex system, a phenomenon that has also been observed in other infectious diseases of the central nervous system.

## VIII. CONTROL MEASURES

Taeniosis and cysticercosis are linked to poverty and low education and are common in developing countries where conditions favoring the transmission of *T. solium* may be found, including deficient disposal of human feces, low levels of education, slaughtering of swine without veterinary control, and presence of free-roaming pigs around households. Cysticercosis is a potentially eradicable dis-

**TABLE 4** Targets of Control Measures to Consider in Programs Attempting to Eradicate Taeniosis and Cysticercosis in Populations

Community level	Mass chemotherapy Education
Infected pigs	Improved husbandry Meat inspection Freezing of pork meat Mass chemotherapy Vaccination
Environment	Improved sanitation

ease (51). To be effective, however, eradication programs must be directed to all the targets for control, particularly human carriers of the adult tapeworm, infected pigs, and eggs in the environment (Table 4). Inasmuch as these targets represent interrelated steps in the life cycle of *T. solium*, inadequate coverage of one of them may result in a rebound in the prevalence of taeniosis/cysticercosis after the program has been completed.

*T. solium* is highly sensitive to praziquantel given as a single dose of 5–20 mg/kg (4,14). Niclosamide, 2 g in two divided doses given one hour apart, is an alternative to praziquantel. In patients with neurocysticercosis, treatment must be individualized according to the location of the parasites and the degree of disease activity (52,53). Patients with viable parenchymal brain or sub-arachnoid cysts must be treated with albendazole, 15 mg/kg/day for 8 days (54), or praziquantel, 75–100 mg/kg given in three divided doses every 2 hours (single-day course of therapy) (55,56). There is recent evidence supporting the use of longer courses of albendazole in patients with huge subarachnoid cysts (57). The use of cysticidal drugs results in the disappearance of most cysts on neuroimaging studies and improvement of the clinical manifestations in most patients (16,58). Patients with calcified lesions should receive only symptomatic treatment, i.e., antiepileptic drugs for seizures. Patients with ventricular cysts may also be treated with cysticidal drugs; however, the usual approach to these lesions is surgical resection through endoscopic devices (59). Cysticidal drugs should be cautiously used in patients with cysticercotic encephalitis, because their use may exacerbate the brain edema associated with this condition; in this form of the disease, corticosteroids and osmotic diuretics are advised for the management of intracranial hypertension (23). In patients who have mixed forms of the disease, therapeutic priorities (e.g., shunt placement for hydrocephalus) must be considered before starting cysticidal treatment (15,52).

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## *Fasciola hepatica*

Ana M. Espino

*Medical School Campus, University of Puerto Rico, San Juan, Puerto Rico*

Blanca E. Dumenigo

*Instituto de Medicina Tropical "Pedro Kouri," Ciudad de la Habana, Havana, Cuba*

### I. BACKGROUND

Fasciolosis is a disease caused by digenetic trematodes of the genus *Fasciola*, of which *Fasciola hepatica* is the most common. Nevertheless, there are a number of related trematodes that infect mammals, including *Fasciola gigantica*, *Fascioloides magna*, and some lesser-known species.

Fasciolosis is considered an economically important disease due to significant losses incurred by the animal husbandry industry (1,2). The infection not only renders the livers unsuitable for human consumption, but also reduces the quality of milk and meat produced (1,3). A recent economic analysis at a local slaughterhouse in Cuba found in 1991, 600,689 livers were condemned. The losses increased annually, and in 1995 the rate of livers condemned reached 276 tons (4). In Peru, similar analysis during the last decade demonstrated that losses by mortality, diminution of milk production, and liver condemnation are numerous, costing \$10 million annually. Thus fascioliasis is considered the second most important disease affecting economy (5). A recent economical analysis of treatment practices in cattle herds in five subtropical areas of Florida demonstrated that of 134 producers with 164,000 cattle, 42% applied treatment to all herds in the spring, 31% in the fall, and 16% in both spring and fall; 5% treated herds three to four times per year (6). A slaughter survey of 25,000 animals (mainly beef cows) in 1984 revealed a mean liver condemnation rate of 64%.

*F. hepatica* is prevalent worldwide. There is a high percentage of infection in herbivorous animals in America, Europe, Asia, and Africa. In the Americas it is found in Cuba, the United States (including Hawaii), Mexico, Costa Rica, Puerto Rico, Columbia, Peru, Argentina, Uruguay, Bolivia, Venezuela, and Chile. It is found in Russia, and in Europe it is found in Hungary, Germany, Spain, Holland, England, and France. In Africa it is found in Algiers, Egypt, French Somaliland, and South Africa. In Asia it is found in China, Tibet, Syria, Siberia, and the Middle East. Traces are also found in Australia. *F. gigantica* is the common liver fluke in the Far East, Africa, the Indian subcontinent, and some Pacific Islands. In some areas infections with *F. hepatica* and *F. gigantica* coexist. *Fascioloides magna* is a parasite of the mouse, elk, and deer and is primarily found in North America, where it is known as "the large American fluke" (7,8).

*F. hepatica* has been more intensively studied than the other species because of its impact on the health of domestic animals and the search for effective means of control. More recently its importance is increasing due to the escalating number of sporadic human cases reported, especially in Latin America. In addition, serious outbreaks have been reported in Cuba, France, and England.

### II. CHARACTERISTICS

*F. hepatica* is a flattened, leaf-shaped hermaphroditic trematode. The parasite is found in the biliary passages of the livers of herbivorous animals and humans, resulting in a variety of designations,



such as “liver rot,” “maggot,” or “wormy liver.” The adult parasite measures up to 35 mm in length by 15 mm in width. Its anterior end is a conical projection, terminating anteriorly in an oral sucker. On the ventral surface near the base of the cone there is a ventral sucker. The posterior end is broadly pointed. On the surface of its body are integumentary spines, directed posteriorly. The eggs are large, ovoid, operculate, and yellowish-brown. They measure 130–150  $\mu\text{m}$  in length by 63–90  $\mu\text{m}$  in width.

The life cycle of *F. hepatica* is complex and involves more than one host. The eggs are laid in the biliary passages, proceed to the intestine with the bile, and are evacuated in the feces in immature states. The eggs must be deposited in freshwater for the cycle to continue. After an incubation period of 9–21 days at an optimal temperature of 15–25°C the miracidium emerges from the operculated egg, it swims, and in order to survive, it must penetrate a snail of the Lymnaeidae family within a few hours. Shortly thereafter, miracidium penetrates the body wall of the snail host, enters the hepatopancreas, and transforms into sporocyst. Three weeks later the sporocyst produces the first-generation rediae (mother rediae) and during the fourth week produces a second-generation rediae (daughter rediae) in which the cercariae develop after 6 weeks of penetrating the intermediary host. The mature cercariae emerge from the snail and swim about a few hours until they come in contact with and adhere to the surface of aquatic plants, mainly wild watercress, where they shed their tails and form cysts as metacercariae, which is the infective stage.

Herbivorous mammals including humans contract infection after ingestion of plants or water contaminated with metacercariae. After ingestion by the host, metacercariae excyst in the intestine and the juvenile flukes pass through the intestinal wall into peritoneal cavity (9). They penetrate the capsule of Glisson and reach the liver parenchyma a few days later. The time taken for the juvenile flukes to reach the peritoneal cavity varies with the host: in mice it takes under 24 hours (9), in rats 24–48 hours (10), in rabbits 48 hours (11), and in sheep 72 hours (12). The flukes then move through the peritoneal cavity to the liver, which occurs within 48 hours of infection in rodents and 90 hours in sheep. They penetrate the liver and move through the parenchyma, creating tunnels behind them, into which they deposit their excretions and eggs. The immature flukes migrate through liver parenchyma for 5–6 weeks, feeding on hepatocytes and blood. The duration of the parenchymal stage of infection also varies with the host and with the intensity of the infection: the parasite appear in the bile ducts after 24 days in mice (13), after approximately 28 days in rats (10), after around 40–42 days in sheep (12,14) and after 49 days in calves (15). On the other hand, in heavy infection the rate of growth and time of entry into the bile duct is retarded for some flukes (16,17). Finally immature flukes reach the bile ducts, where they become adults 3–4 months after infection. Once the mature fluke starts to shed eggs, a new cycle of life is initiated.

The *F. hepatica* adult worm lives in the bile duct for many years in permanent contact with the bile stream, from which it obtains nutrients in unlimited amounts. The parasite lives to perpetuate the species, and its mechanism to produce eggs is continuously functioning during its whole life, producing about 20,000 eggs per day (18). It has been estimated that each parasite sheds 4 eggs per second, with a probability of 1/1,000,000 of finding the intermediate host. This enormous number of eggs is almost totally destroyed by the bacterial toxins in the gastrointestinal tract, which is probably the cause of intermittent shedding of eggs and its rare finding in fecal samples by single coprological analysis.

### III. DISEASE

Fascioliasis in humans is characterized by its unspecificity, polymorphism, and variable time of latency. Thus, the epidemiological suspicion is fundamental to establish clinical diagnosis. Once the parasite has infected the human organism, clinical manifestation can be found after a period of time ranging between 2 weeks to several months. After this lapse of time it is possible that clinical manifestation is notable, or, by contrast, it may be unspecific or so slight that individuals are considered asymptomatic. Fascioliasis can be highly pathogenic and can lead to severe morbidity of the

infection depending on the host species, the stage of the infection, and the number of flukes infecting the liver. Thus, fascioliasis may produce a group of symptoms and nonpathognomonic signs that simulate other diseases.

## A. Phases of Infection

Following the characterization described by Chen and Mott (19), three phases can be identified during clinical infection: acute, latent, and chronic.

### 1. Acute Phase

The acute phase of fascioliasis usually presents as a transitory period of dyspepsia followed by an abrupt onset of high fever and abdominal pain, localized to the right hypocondrium or epigastrium, and of variable intensity. This phase is related to the period in which immature flukes are actively migrating through the peritoneal cavity and reach the liver parenchyma through the Glisson capsule. The symptomatology produced in this phase is mainly due to the destructive action of flukes during their migration through this tissue, producing toxic and allergic reactions (20). Other symptoms such as postulation, anorexia, sweating, myalgias, joint and bone pains, violent headache, nausea, and vomiting may also develop. Occasionally, marked asthenia and wasting are observed. Thus, the acute phase of the disease is characterized by allergic, immunological, and toxic reactions. A painful enlargement of the liver ensues, which, together with fever and marked eosinophilia (80–90%), forms a triad of symptoms of diagnostic importance. Leukocytosis up to 35,000 is observed, the erythrocyte sedimentation rate is increased, and there may be anemia. There is alteration of thymol turbidity test, bilirubin may be normal or altered, and alkaline phosphatase and transaminases may be increased as a manifestation of hepatic dysfunction. Peripheral blood eosinophilia, which appears soon after infection, increases rapidly during the acute stage of infection and persists at a high level after the flukes enter the bile ducts (21–23). Other white blood cell populations that have been observed to increase are lymphocyte neutrophils and occasionally monocytes and basophils.

The entry of newly excysted metacercariae into the liver produces tissue destruction, mainly traumatic, the severity of which depends on the number of parasites. The latter burrow into the liver substance, producing tunnels with ragged walls of damaged parenchyma, bleeding, and leukocytic infiltration. The liver is soft, friable, and may be markedly enlarged with distention of the capsule of Glisson. The acute phase lasts 2–4 months. However, in endemic areas where reinfection with *F. hepatica* is common, acute lesions may overlap with the chronic phase; thus the acute phase may be prolonged and overlapping with other phases of infection.

### 2. Latent Phase

When the fluke reaches the lumen of the bile ducts and becomes mature, the latent phase of the disease begins. This may last several months or even years. In this phase symptoms are very few and are usually of obstructive biliary or inflammatory nature. This explains why the chronic forms of the diseases, although more frequent, have gone unrecognized in endemic zones. During this phase an unexplained and increased eosinophilia may suggest as helminthic infection. In addition, patients may complain of a diffuse abdominal pain predominantly in the right hypochondrium and epigastrium, nausea, vomiting, dyspepsia, and diarrhea. Infrequently, hepatomegaly, jaundice, and paleness may be detected.

### 3. Chronic Phase

The presence of an adult parasite in the bile ducts causes inflammation and hyperplasia of the biliary epithelium. There is mechanical obstruction and irritation of biliary channels by the parasite and its spines, there is hyperplasia of the ductal epithelium, and there is dilatation, fibrosis of the walls, and calcification. Some consider the parasite to be a factor in formation of stones, and it is not infrequent to find its coexistence with cholelithiasis and, in a few cases, choledocholithiasis. The obstruction, irritation, and fibrous thickening of the biliary tree, together with secondary bacterial

infections, may possibly lead to biliary cirrhosis (24). However, although this finding is frequent in herbivorous animals, it has not been demonstrated in humans in massive reinfection. The relationship between fascioliasis and primary biliary cancer suggested by some authors (25) has not yet been proven. This hypothesis has been suggested because of the marked adenomatous hyperplasia of the biliary duct epithelium and neof ormation of biliary ducts in this disease (26–28).

The anemia that develops in all host species during chronic fascioliasis is probably the single most important factor contributing to morbidity and mortality of the host. The anemia due to hemorrhage is attributed to the feeding activities of the flukes in the bile ducts, which is exacerbated by hyperplasia of the bile duct's epithelium (29). The rate of blood loss in the alimentary tract due to each adult fluke has been estimated at 0.2–0.5 mL per day (29,30).

Albumin and immunoglobulins are the major protein components of plasma. Albumin is only synthesized in the liver, and immunoglobulins are synthesized in the liver and in a variety of other sites in the body. Both hypoalbuminemia and hyperglobulinemia commonly occur in *Fasciola* infections in all host species. During the acute stage of the infection, plasma concentration decline is due to liver dysfunction caused by the migrating flukes. During the chronic stage the loss of blood in the intestine is so extensive that the synthetic capacity of the liver is unable to replace the lost albumin. Consequently, a progressive loss of plasma albumin occurs in all infected host species, starting from around the time the flukes commence blood feeding. In calves the loss of albumin declines late in the infection, probably due to the ability of calves to resolve the infection at the adult stage (31).

Increased immunoglobulin synthesis commences in the infected host within several weeks of infection and persists throughout the infection (21,32,33). The increase occurs in IgM, IgG1, IgG2a, and IgE fractions. The rate of turnover of immunoglobulins in normal animals is generally more rapid than that of albumin; in sheep the half-life of IgG is around 250 hours (34).

The release of hepatic enzymes into the blood as a result of damage to liver tissue has been used to monitor the progress of the infection in a variety of hosts under experimental conditions, as well as a diagnostic aid in field infection. Serum activity of the hepatocyte enzymes glutamate dehydrogenase and glutamate-oxaloacetate aminotransferase is elevated from early in the infection, reaching a peak towards the end of the parenchymal stage of the infection (15,35,36). Release into the blood of  $\delta$ -glutamyl transferase, an enzyme present in the bile duct epithelium, is an indicator of damage to the bile ducts, and the peak of enzyme activity in the serum also appears towards the end of the parenchymal stage of the infection (35,37).

One of the most important functions of the liver is the regulation of the concentration of blood glucose. The liver converts 3-carbon precursors into glucose or glycogen by the process of gluconeogenesis. Under hormonal control, glucose is released from the liver into the blood to maintain blood glucose levels. Studies have assessed that glycogen content in infected rats is significantly lower throughout infection than in normal rats (38). A lower glycogen concentration would mean that under conditions where glycogenolysis is required to maintain blood glucose levels, the rate of glucose release could be reduced and the duration of glucose release would be shorter in infected animals.

Lipid metabolism is also affected during infection. It has been demonstrated that the concentrations of both lipid and phospholipid components of hepatic homogenates declines in infected rats (39,40). In the case of the total lipid fraction, the decline commences in the fourth week postinfection, reaching about 50% of control values in the sixth week. The decline in phospholipid concentration is more extreme, commencing in the second week postinfection and declining to about 25–30% of control values in the sixth week.

It might be expected that the flow and composition of bile would be affected during liver fluke infection, but only a few studies in calves and rats have addressed this question. In rats, the flow of bile declined during the acute stage of infection (41), after which it increased during the chronic phase. In infected calves bile flow also increased from 10 weeks postinfection (42). Although the mechanical obstruction of biliary ducts due to *F. hepatica* have been reported in many cases (43,44), the proportion of individuals whose infection develops to the chronic phase is still unknown.

## B. Diagnosis

The symptoms in all phases are general and nonpathognomonic; thus, in the absence of eggs, the symptoms and lesions described above cannot constitute specific diagnosis for fascioliasis, they only constitute observations that contribute to the understanding of the disease.

The only way to diagnosis *F. hepatica* infection is by finding the fluke eggs in feces or biliary dressage. However, parasitological diagnosis alone is inadequate in the latent period of infection since flukes require a period of at least 3–4 months to attain sexual maturity and release eggs (19). Thus, infected humans have significant clinical symptoms long before eggs are found in the stools. In addition, in many patients the fluke eggs are often not found in the feces, even after multiple stool examinations. For example, Hillyer et al. had to perform multiple coprological assays on the same patient to find one sample positive for *F. hepatica* eggs (45). Similarly, Espino et al. had to perform multiple stool examinations over a period of 6 weeks on 59 patients with antecedents of ingestion of contaminated watercress to demonstrate parasite eggs in stools (46).

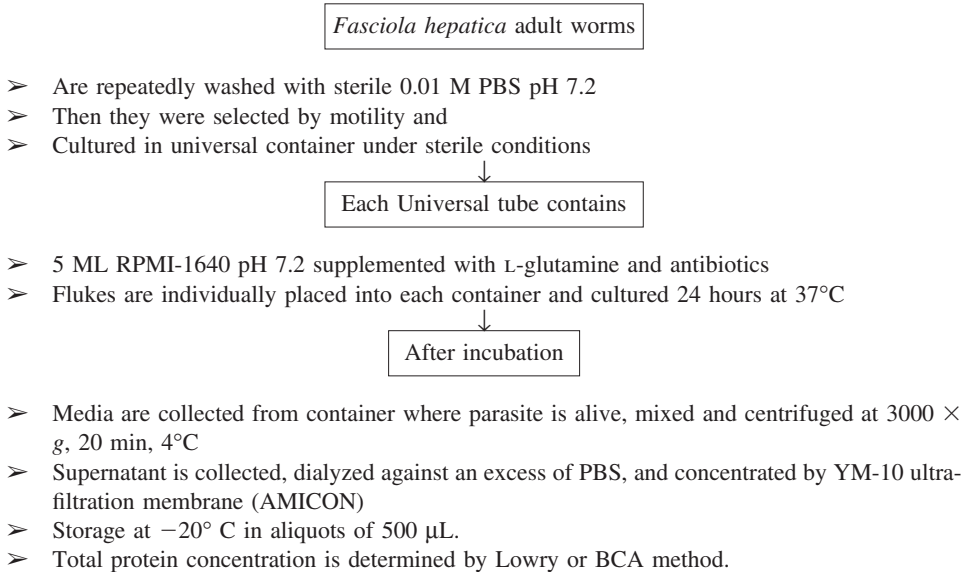
Parasitological techniques used to demonstrate *F. hepatica* eggs during the latent or the chronic phase of disease encompass a simple gravity sedimentation technique (47) to procedures based on stool concentration by centrifugation or quantitative techniques such as Kato-Katz (48). In general, all these procedures are insensitive mainly in light infection and more than one stool examination is required to demonstrate the infection. Therefore, use of the above-mentioned parasitological technique in epidemiological studies and even in individual diagnosis has serious limitations.

## C. Immunodiagnosis

During the last two decades, studies on fascioliasis have used several immunodiagnostic procedures as excellent alternatives for diagnosis. Overall, the need for immunodiagnostic tests for fascioliasis is as necessary for humans as for their livestock. Their use in seroepidemiology studies is obvious, but there is a real need in individual patients either from endemic areas or after visiting such areas before specific chemotherapy can be prescribed or to predict success or failure of this chemotherapy. On the other hand, domestic animals are generally dealt with on a herd basis, and diagnosis is usually performed by simple coprological tests using several grams of feces. Nonetheless, immunodiagnostic tests for fascioliasis in animals are also needed during the prelatent phase to avoid liver damage and the subsequently economic losses, for experimental purposes including challenge infections and vaccine trials, and monitoring the effects of chemotherapy of herds. The immunodiagnosis of fascioliasis has been reviewed elsewhere (49,50).

All immunodiagnostic procedures are based on measurement of one or more components of specific immune response (either cell-mediated or antibody-mediated) directed to parasite antigens or based on the detection of parasite antigens in body fluids. Evaluation of specific cellular responses mostly requires complex and expensive techniques. Therefore, only a few studies using these procedures have been published. The intradermal assay constitutes the exception to this rule, and it has seldom been used with crude or partially purified extract of adult *F. hepatica* (51,52). However, although simple and sensitive enough, intradermal assay is highly unspecific and, therefore, rarely used (51). On the other hand, immunodiagnostic procedures based on the detection of antibodies to parasitic antigens are inexpensive, faster, and simpler for use in routine investigations. These include hemagglutination assays (53), indirect immunofluorescence (54), gel immunodiffusion (55), counterimmunoelectrophoresis (56), enzyme-linked immunosorbent assays (ELISA) (57–59), and Western blot (60).

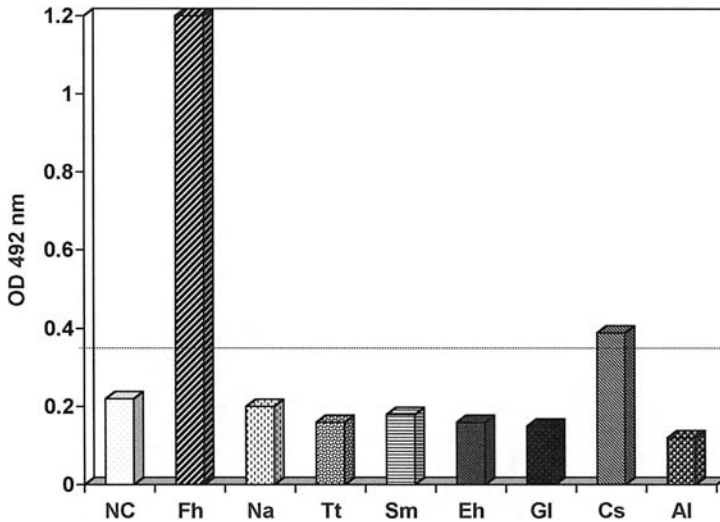
The antigen preparation used by most laboratories for antibody detection is *F. hepatica* excretory secretory antigen (FhES antigen). The steps in its isolation differ slightly from laboratory to laboratory. Adult worm incubation times at 37°C of 3 hours (61) or 18 hours (62) or 24 hours (63) with or without cell culture medium, and with or without protease inhibitors have been reported. One example is seen in [Figure 1](#). Hillyer and Soler de Galanes used a FAST-ELISA with FhES antigens from a 3-hour culture and found that mice with fascioliasis had increased antibody levels by 1 week



**FIGURE 1** Procedure to obtain excretory secretory antigens of *Fasciola hepatica* adult worms with high diagnostic specificity.

of infection, sheep and cattle by 2 weeks of infection, and rabbits by 2–3 weeks of infection (59). Mice infected with *Schistosoma mansoni* had their anti-FhES antibody levels rise by 6 weeks, reinforcing their own previous observation that FhES has cross-reactive antigens with *Schistosoma* (61). Espino et al. found all 20 patients with confirmed fascioliasis with ELISA using FhES antigens. In this study no cross-reactivity was observed using sera from patients with other parasitic infections including shistosomiasis (63). In a previous paper, the authors studied the optimal maintenance in vitro conditions of adult parasites in terms of temperature, culture medium, pH, and parasite rate/medium. They concluded that a culture of 24 hours at 37°C in RPMI-1640 medium supplement with L-glutamine and antibiotics in which the parasite was individually cultured at a rate of 1 parasite per each 5 mL of medium is optimal to obtain adequate amounts of ES antigens with high specificity. The key to the specificity could be that 100% of the parasites must be alive (64,65). In fact, the antigens obtained that way exhibit little or no cross-reactivity with serum from individuals with other parasitic infections when they were tested in highly sensitive ultramicroanalytic ELISA (UMELISA), which uses an anti-human IgG alkaline phosphatase conjugate and a fluorogenic substrate (66). Evaluation of 100 sera from patients with *Ascaris lumbricoides*, *Schistosoma mansoni*, *Necator americanus*, *Trichuris trichiura*, *Entamoeba histolytica*, *Giardia lamblia*, and *Clonorchis sinensis* only revealed one positive serum (*C. sinensis*) cross-reactive with FhES antigens (Fig. 2). Using either ELISA or UME-*Fasciola*, a positive result was considered when absorbance or fluorescence of the serum sample of the patient exceeded the mean OD plus 2 standard deviations of the negative control group (generally  $\geq 100$  controls). A highly significant difference ( $p < 0.001$ ) was observed between sera from patients with fascioliasis, controls, and sera from other diseases. No intermediate absorbance values (between positive and negative) were seen with sera from patients with infections other than *F. hepatica* (63,66).

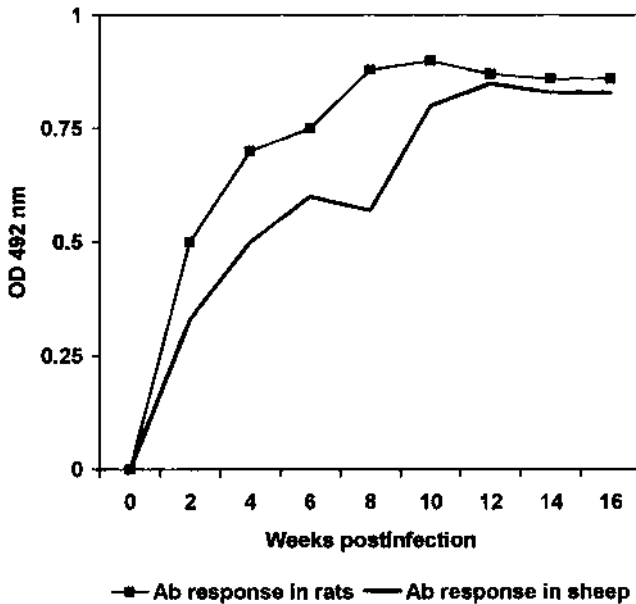
Other studies using FhES antigens and ELISA have demonstrated detectable antibody levels in experimentally infected rats by 2 weeks postinfection, with absorbance values peaking at 6–10 weeks of infection (Fig. 3) (67). In cattle, antibody to FhES antigens were also detected early, by 2 weeks postinfection, with absorbance peaks at weeks 8–10 postinfection (68). In sheep the increase was slightly slower by 4 weeks.



**FIGURE 2** ELISA specificity using FhES antigens obtained from 24-hour culture. A highly statistical difference ( $p < 0.001$ ) was obtained between sera from patients with *Fasciola hepatica*, sera from controls and sera from patients infected with other parasites. NC, negative control group ( $n = 100$ ), Fh, sera from patients with *Fasciola hepatica* ( $n = 25$ ), Na, sera from patients with *Necator americanus* ( $n = 20$ ), Tt, sera from patients with *Trichuris trichiura* ( $n = 20$ ), Sm, sera from patients with *Schistosoma mansoni* ( $n = 20$ ), Eh, sera from patients with *Entamoeba histolytica* ( $n = 20$ ), GI, sera from patients with *Giardia lamblia* ( $n = 10$ ), Cs, sera from patients with *Clonorchis sinensis* ( $n = 5$ ); Al, sera from patients with *Ascaris lumbricoides* ( $n = 20$ ). (....), cut-off value (mean OD of negative control + 2 standard deviations).

A 17 kDa antigenic polypeptide (from ES antigens) has been identified by Western blot in sera from humans, rabbits, cattle, and sheep as an excellent candidate for the immunodiagnosis of acute and chronic fascioliasis (61). The combination of ELISA and Western blot has been recommended as an accurate method of immunodiagnosis of fascioliasis. In a study to determine the prevalence of human fascioliasis in a community in the Bolivian Altiplano, the FAST-ELISA using FhES antigens was compared with parasitological examination. The study revealed that 53 of 100 sera tested were positive by FAST-ELISA, while only 20 individuals were positive parasitologically. Within this group 19 (95%) of 20 confirmed individuals were positive by FAST-ELISA. The Western blot analysis of the sera from the confirmed infected individuals revealed three *F. hepatica* bands with molecular weights of 12, 17, and 63 kDa. The 79% of the 53 persons who were positive by FAST-ELISA were confirmed as infected by their banding pattern. Thus, FAST-ELISA and Western blot combined suggested a prevalence of 42% in the population studied (60). Although performing of FAST-ELISA followed by Western blot is time-consuming, both procedures constitute a powerful tool for diagnosis of *F. hepatica* infection.

It is well known that in fascioliasis relevant antibody response is mostly of the IgG type; therefore, the vast majority of the above-mentioned techniques have been designed to detect this immunoglobulin class in the serum of patients or animals infected. Only a few publications on fascioliasis have addressed detection of IgM (69,70). Detection of IgG antibodies has a major limitation because the presence of antibodies indicates previous exposure to the parasite rather than the existence of a current infection. In fact, it has been well demonstrated that many people who live in endemic areas can develop detectable antibody levels in their serum without developing fascioliasis (46,59,72). In addition, after an effective cure, most patients and animals retain detectable antibody levels for periods ranging between 6 months and one year or more (46,72,59). Taking into account such



**FIGURE 3** Antibody response to FhES antigens during a primary experimental infection in rats and sheep. Absorbance value peak was observed between weeks 6 and 10 postinfection in rats. In sheep absorbance value peaked at weeks 8–10.

limitations, during the last decade the antigen-detection assay has been developed as a new approach to diagnosis *F. hepatica* infection. In contrast to the detection of antibody, antigen detection may indicate active current infection in that the presence of antigens in body samples indicates that the parasite is alive in the host.

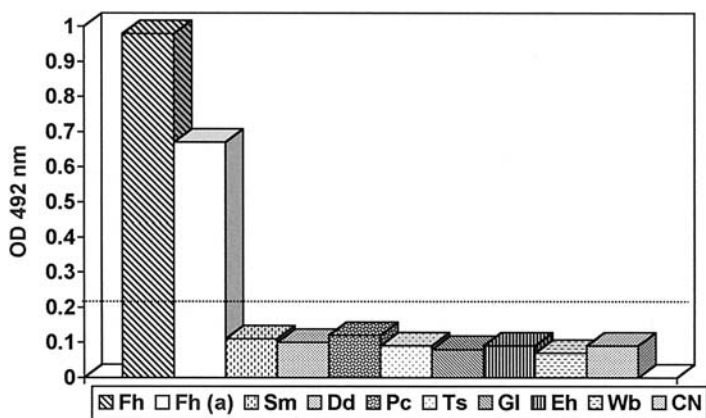
The first studies on antigen detection in fascioliasis were published by Ambroise-Thomas et al. and Knoblock in 1980 and 1985, respectively (73,74). These authors developed a capture enzyme-linked immunosorbent assay using a polyclonal antibody to *F. hepatica* antigens to detect circulating antigens in sera of humans and animals with fascioliasis. However, in both studies sensitivity of the assay was low as individuals with proven *F. hepatica* infection were recorded as negative for antigen detection in serum. Langley and Hillyer (75) developed a two-site ELISA using polyclonal antibody to FhES antigens to detect circulating antigen in sera from mice infected with *F. hepatica* during weeks 1–5 postinfection. The assay had a sensitivity of 25 ng/mL and diagnosed infection at 1 week postinfection with maximal circulating antigen levels at 3 weeks postinfection. Rodriguez-Pérez and Hillyer found circulating antigen in three out of five sheep experimentally infected at 2 weeks postinfection, whereas in the other two sheep FhES antigens were first detected at 6 or 8 weeks postinfection (76). Other authors using polyclonal antibody prepared for antigenic fractions of *F. gigantica* were able to detect antigens in stools of patients with fascioliasis using counterimmunoelectrophoresis (77,78). The most important findings of this report were that (a) the assay was positive for all patients with confirmed fascioliasis, (b) there was no cross-reactivity with stool samples from patients with schistosomiasis, and (c) antigen detection in stool was more sensitive than parasitological egg detection.

Espino developed monoclonal antibodies (Mab) to *F. hepatica* ES antigens and selected for diagnosis purposes the Mab denoted ES78 (mouse IgG 2a) (79). The criteria used to select this Mab were (a) high reactivity with *F. hepatica* antigens, (b) no cross-reactivity with antigens from parasites other than *F. hepatica*, (c) high capture capacity in enzyme-linked immunosorbent assays, and (d) that epitope recognized by Mab ES78 is present on ES antigens from either adult or immature flukes.

ES78 is the only Mab developed for diagnostic purposes that has been extensively used by the authors and others in detecting circulating FhES antigens or coproantigens in humans and animals infected with *F. hepatica*. The assay in which Mab ES78 is used is a capture enzyme immunoassay (sandwich ELISA). In this assay, microtiter plates were coated with Mab ES78 to capture circulating antigen in serum or coproantigens in stools of patients and animals infected. The antigen was then identified using an IgG anti-FHES antigen peroxidase conjugate. The assay had a sensitivity of 10 ng/mL in detecting circulating antigen (79) and a sensitivity of 15 ng/mL in detecting coproantigens (80,81). All patients with chronic fascioliasis were positive in this assay, with antigens levels in sera ranging from 14 to 82 ng/mL. Mean antigen concentration in stool eluates of patients with fascioliasis was 250 ng/mL, approximately 10 times higher than in sera. Stool antigen concentration in cattle were lower than in human and ranged from 25 to 130 ng/mL, with a mean value of 100 ng/mL (81). Mean coproantigen level in sheep was 50 ng/mL (82). Sera and stool samples from humans and animals infected with *Schistosoma mansoni*, *Trichinella spiralis*, *Giardia lamblia*, *Entamoeba histolytica*, *Wuchereria bancrofti*, *Dicrocoelium dendriticum*, *Paramphistomum cervi*, and more than 100 healthy subjects as controls were uniformly negative (Fig. 4). ES78-sandwich ELISA has been, until now, the only procedure designated to detect active infection that has been successfully evaluated during human *F. hepatica* outbreak (46) and in the large bovine and ovine herds studies (81–83). A brief description of the assay is shown in Figure 5.

A comparison in terms of sensitivity, specificity, and predictive values of the antibody detection ELISA and ES78 sandwich ELISA is shown in Table 1. For this analysis, repeated parasitological diagnosis was considered as the gold standard. The 34 persons included as negative controls were all healthy subjects with repeated negative stool examination. They lived in the same endemic area as the patients. Therefore, the presence of antibodies in these persons may indicate previous exposure to parasite or a past infection.

The course of a primary *F. hepatica* infection has also been studied in experimentally infected animals and in humans using ES78 sandwich ELISA. Circulating antigens were detected as early as 1–3 weeks postinfection in rats (67), and from 1 to 5 weeks postinfection in sheep. Eventually

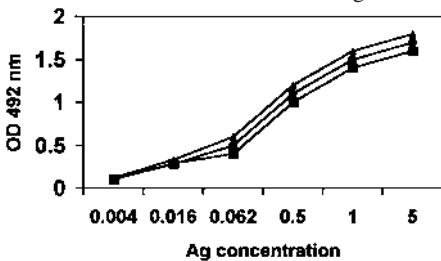


**FIGURE 4** Diagnostic specificity of the sandwich ELISA using Mab ES78 as capture antibody. Fh, stool specimens from humans with *Fasciola hepatica* ( $n = 25$ ); Fh(a), stool specimens from calves with *F. hepatica* ( $n = 70$ ); Sm, stool specimens from patients with *Schistosoma mansoni* ( $n = 20$ ); Dd, stool specimens from patients with *Dicrocoelium dendriticum* ( $n = 5$ ); Pc, stool specimens from patients with *Paramphistomum cervi* ( $n = 50$ ); Ts, stool specimens from patients with *Trichinella spiralis* ( $n = 50$ ); GI, stool specimens from patients with *Giardia lamblia* ( $n = 20$ ); Eh, stool specimens from patients with *Entamoeba histolytica* ( $n = 20$ ); Wb, stool specimens from patients with *Wuchereria bancrofti* ( $n = 10$ ); CN, stool specimen from healthy and parasitologically affected persons and calves ( $n = 150$ ); (.....), cut-off value (mean OD from negative control group + 3 standard deviations).



Antigen detection by sandwich ELISA. A capture diagnostic assay is as follows:

- 1-Polystyrene 96-well plates were used
- 2-All application to the wells were done in 100  $\mu$ L volumes
- 3-Mab ES78 capture antibody at a protein concentration of 5  $\mu$ g/mL in carbonate buffer pH 9.6 was used to coat plates overnight at 4°C
- 4-Plates were washed three times with PBS/0.05% Tween 20 (wash buffer)
- 5-Plates were then blocked by incubation with wash buffer + 5% nonfat dry milk for 1 hour at 37°C
- 6-Plates are washed as in 4
- 7-Application of serum sample or stool eluate and defined antigen standard was as follows:
  - 100  $\mu$ L aliquots of human/animal sera or stool eluates were applied to the wells undiluted and tested in duplicate.
  - wash buffer was used as blank
  - normal/negative control was serum and stool eluate without any added FhES antigen
  - the standard curve was done in duplicate in each plate by adding defined amounts of FhES antigens to an undiluted normal/positive control beginning at 5  $\mu$ g/mL protein and diluted fourfold down to a 3.9 ng/mL
  - each plate had a positive control well contained 1  $\mu$ g FhES antigens
- 8-Plates were incubated 2 hours at room temperature and washed as in 4
- 9-The second antibody was a rabbit IgG anti-FhES antigen peroxidase conjugate and was used at 1:1000 dilution
- 10-Plates were incubated 1 hour at 37°C and washed as in 4 prior to adding the substrate
- 11-*o*-phenylenediamine (Sigma) was used 20 mg + 50 mL 0.05 M phosphate-citrate buffer pH 5.0 + 20  $\mu$ L H<sub>2</sub>O<sub>2</sub> (30% w/v)
- 12-After 20 min, reaction was stopped with 50  $\mu$ L 12.5% sulfuric acid
- 13-Plates were read at 492 nm using a MR 700 Microplate ELISA READER (Dynatech Prod)



**FIGURE 5** ES78 sandwich ELISA procedure. The figure shows the absorbance values of the FhES78 antigens added to normal stool eluate with fourfold dilutions beginning at 5  $\mu$ g to 3.9 ng/mL protein in three separate plates. The lower sensitivity of the assay was 15 ng/mL.

those sheep with a higher worm burden also may develop detectable antigen levels at weeks 11–12 postinfection (84). In humans, circulating antigens were detected in patients 40–81 days postinfection, which reflects a prolonged antigenemia compared to those in sheep and rat studies. However, the dynamics observed were similar since the levels of antigen detected in patients' serum diminished and became undetectable early during the infection. Unfortunately, no patients with infection times < 40 days were included in the study. Because in human fascioliasis symptoms are nonpathognomonic or absent, patients seldom seek medical attention during the prepatent period of infection. For this reason, the probability of detecting infected patients with early infection by means of circulating antigen determination is extremely low. This is also the reason that it is not possible to determine when mean circulating antigens became negative in humans. Since in a primary infection by *F. hepatica* the antigenemia period is only attributable to the early phase of infection, the presence

**TABLE 1** Results of Antibody ELISA AND ES78 Sandwich ELISA for Antigen Detection at Beginning of Outbreak Study

Patients	No.	IT days	Antibody		Circulating Ag		Copro-Ag	
			P	%	P	%	P	%
Patent phase	14	94–238 × = 136	14	100	0	0	14	100
Prepatent phase	64	60–152 × = 96	63	98.4	19	29.6	52	81.3
Healthy subject	34	None	16	47	0	0	0	0

P: Number of positive patients; IT: time after infection.

of circulating antigen in patients or animals with chronic infection may be due to reinfection. In this case antigen detected in serum would be a consequence of the overlapping of subsequent infections with a primary chronic infection.

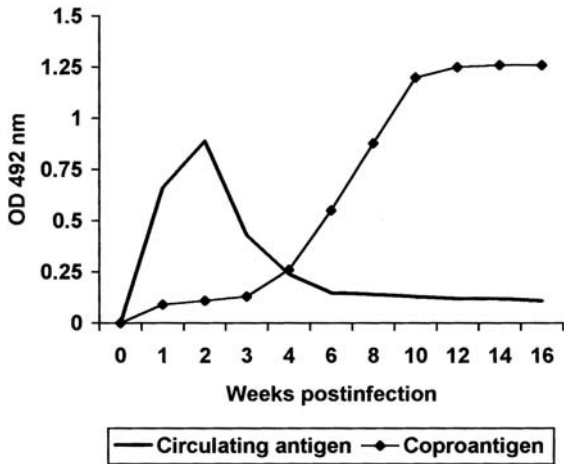
Coproantigens were detected in patients 60 or more days postinfection. In rats and sheep experimentally infected with *F. hepatica*, coproantigens were detected from the fourth week after infection. Coproantigens were detected in both humans and animals several weeks before *Fasciola* egg excretion was initiated and remained at detectable levels during the entire infection (67,84) (Table 2). Circulating antigens are related to the time in which immature flukes are actively migrating throughout liver parenchyma, excreting large amounts of antigens into the bloodstream, while coproantigens are related to the sexual maturity process of the parasite in the bile ducts. Therefore, using ES78 sandwich ELISA, *F. hepatica* infection could be detected in all phases of infection. Figure 6 shows the dynamics of circulating antigens and coproantigens for infected sheep and rats.

It has been demonstrated that coproantigens are correlated with egg count (65,80) (Fig. 7). In addition, a correlation between coproantigens and parasite burden has also been demonstrated (81). These results suggest that coproantigens may be used as a measurement of the intensity of infection (Table 3).

Antigen detection by means of ES78 sandwich ELISA have also been used to predict the success or failure of chemotherapy with bithionol, praziquantel, and triclabendazol (65,72,80,85). Figure 8 shows a typical dynamic for coproantigens in a group of 78 patients treated with triclabendazol (two doses of 10 mg/kg), 70 of whom were cured (65). Thirty days after an effective cure, all patients became coproantigen negative, while those who did not respond to the treatment remained positive. It was demonstrated that coproantigens can become negative as early as 1 week after cure. However, because patients who failed to respond to treatment also became negative during week

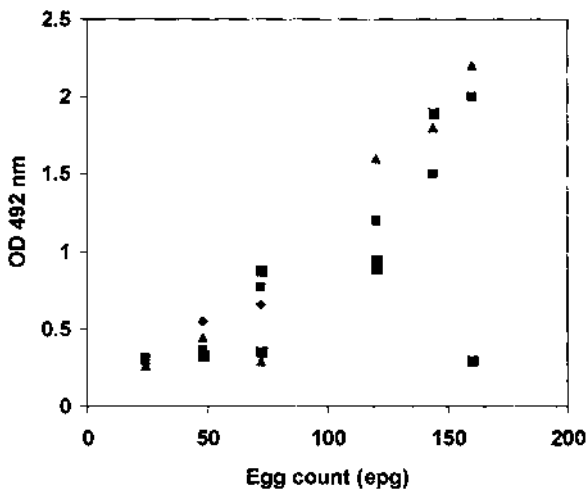
**TABLE 2** Follow-Up Study Performed Weekly for 6 Weeks with 64 Patients with Prepatent *F. hepatica* Infection

Determination	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	P	%	P	%	P	%	P	%	P	%	P	%
Circulating Ag	19	29.7	3	4.7	0	0	0	0	0	0	0	0
Coproantigen Stool examination	52	81.3	57	89	60	93.8	60	93.8	60	93.8	60	93.8
	0	0	19	29.7	40	62.5	40	62.5	44	69	64	100



**FIGURE 6** Dynamics of antigenemia and coproantigen during a primary *Fasciola hepatica* infection in rats and sheep.

1–2 posttreatment (probably due to a temporal diminution in the metabolic cycle of the parasite), a conservative time period for assessing successful therapy in humans was estimated to be 30 days (65). Similar results were seen in sheep experimentally infected with *F. hepatica* when they were treated with fasinex (85). In this study no therapy failure was found and coproantigen become negative in all animals from the first week posttreatment. An antibody assay was also used to follow therapeutic response, but no difference was seen in antibody response between cure and noncure patients (65). The above results demonstrate that the ES78-sandwich ELISA is a better tool than parasitological examination and antibody detection assay for diagnosis of active early infection and could potentially replace the parasitological examination during outbreaks, herd studies, and even in individual diagnosis. The assay permits one to perform a total of 180 single antigen determinations



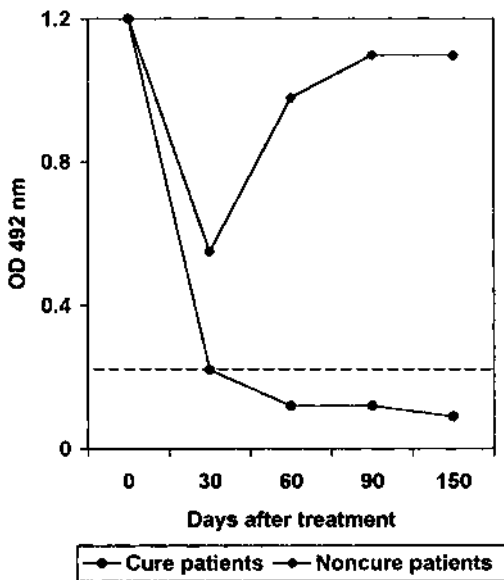
**FIGURE 7** Correlation observed between absorbance values of stool eluate from patients with *Fasciola hepatica* (measured by ES78 sandwich ELISA) and the egg count measured by Kato-Katz.

**TABLE 3** Sensitivity, Specificity, and Predictive Values from Antigen Detection and Antibody Assay During *F. hepatica* Outbreak

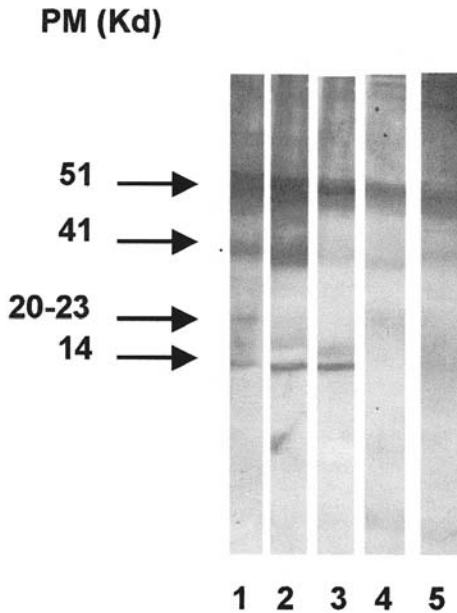
Stool examination	Antibody		Coproantigen	
	Positive	Negative	Positive	Negative
Positive	77	1	74	4
Negative	16	18	0	34
Specificity	53%		100%	
Sensibility	98.7%		94.9%	
Predictive				
Positive value	82.8%		100%	
Predictive				
Negative value	94.7%		89.5%	

at the same time on sera or stool samples from either humans or animals, and the time required to perform the diagnosis has recently been reduced to a maximum of 4.5 hours. The assay is now commercially available in Cuba. It was found to have a sensitivity of 95% and specificity of 99% for the detection of antigen in stools in cattle herds, and a specificity of 100% and sensitivity of 98% in sheep herds (83).

Characterization studies performed on the epitope recognized by Mab ES78 demonstrated that it is a partly protein/party carbohydrate antigenic determinant. It is found on several ES antigenic molecules of adult and immature flukes with relative molecular weight of 23–27, 17, 11, 10, and 6 kDa and contains at least one disulfide bond and  $\beta$ -galactose, probably as galactose- $\beta$ (1-3)-*N*-



**FIGURE 8** Dynamics of coproantigen after treatment with Triclabendazol in humans with fasciolosis. Triclabendazol was applied as 10 m/kg (two doses). Cure patients became negative from 30 days posttreatment; noncure patients remain highly positive to coproantigen. (—), cut-off value of ES78 sandwich ELISA (OD > 0.22).



**FIGURE 9** Polypeptides identified by Western blot in stool of patients with *Fasciola hepatica* using a rabbit hyperimmune serum to FHES antigens. 1,2: patients in which all polypeptides are represented, 3: patient in which the 51 kDa and 14 kDa polypeptides were detected. 4,4: patients in which only the polypeptide of 51 kDa was detected.

acetylgalactosamine disaccharide. These peculiar characteristics found in the epitope could be responsible for the high specificity of the Mab ES78 sandwich ELISA used to diagnose humans and animal fascioliasis (86).

Other studies using Western blot demonstrated the presence of a 26 kDa stable antigen in the feces of six calves with patent infections of *F. hepatica* (87). Polypeptides of 51, 41, 23, 20, and 14 kDa were also found when stool samples from chronic patients were tested using a rabbit hyperimmune serum to FhES antigens and sera from infected patients (Fig. 9). From these polypeptides of 51, 23, and 14 kDa were isolated using affinity chromatography from stool samples of rats 6–12 weeks postinfection (88).

#### IV. EPIDEMIOLOGY

Development of *F. hepatica* infection is determined by the presence of snails as the intermediate hosts, herbivorous animals, and diet habits of people. The epidemiology of fascioliasis is very closely related to the ecology of snails, depending upon the topography and meteorological conditions involved in the appearance of the disease. In regions with high temperatures and low humidity, few fascioliasis cases have been reported. In contrast, the high humidity related to frequent rains and moderate temperatures can predict a hyperendemic infection in herbivorous animals, because the number of animals likely to ingest water and vegetables contaminated with metacercariae of *F. hepatica* is greater. Human infections occurred most frequently in years with strong and repeated rainfall. On the other hand, the dietary habits of people are very closely related to the prevalence of fascioliasis, since watercress and other aquatic plants eaten raw constitute the main infection source. Taking into account these and other factors, such as consumption of contaminated water,

inappropriate excretion elimination, and lack of knowledge of the disease, it may be possible that the prevalence of this zoonosis presently constitutes a serious problem for animal and human health.

Human fascioliasis has been reported in more than 60 countries on all continents. However, the real prevalence has until now been unknown. In one review only some 100 human infections were collected in a 20-year span involving cases from Egypt, Cuba, Peru, France, Portugal, Spain, and the former Soviet Union (19). However, they were considerably underrepresented. For example, in that review, Bolivia was not even listed as having human infection. Yet today it is known to have one of the highest endemic rates in the world. A recent seroprevalence study found over 40% prevalence in Aymara Indians from Corapata in the Altiplano of Bolivia (60). A second study reported on an outbreak of acute fascioliasis in Calasaya and Santa Ana also in the Altiplano of Bolivia; consumption of *kjosco*, an aquatic plant, was associated with acute fasciolosis in humans. A recent report, which employed ELISA (89), confirmed the high level of human fascioliasis in this region. In children, it may even be more significant as it was found the almost 28% of 558 school children from four communities in the Northern Bolivian Altiplano were found to be positive for *F. hepatica* in stools (90). Since approximately 1.8 million subsistence farmers live in the Department of La Paz in the Altiplano, a conservative estimate of 20% of humans with fascioliasis would suggest that as many as 360,000 people are infected in this region (91). In cattle and sheep in this same region, antibody levels to *F. hepatica* are 58% and 89%, respectively. Prevalence of 67% has been found in hyperendemic subzones of Bolivian Altiplano (92).

Studies performed in region VII of Chile, which includes Curico, Talca, and Linares, estimated that a minimum of 2000 persons are infected with *F. hepatica* in that region (93). In the Sharkia Governorate of Egypt, 5% of 13,509 school children had *F. hepatica* eggs in their stools (94).

In Cuba, more than 1000 persons were studied in 1983 during an outbreak of fascioliasis (95), 43 of whom were positive by stool examination; and the others were diagnosed by antibody detection assay. Another 300 persons were studied in 1994 during another outbreak, which occurred simultaneously in three endemic regions of the central province of Cuba. During this study 22 persons from seven family clusters were parasitologically confirmed (unpublished data). The most recent outbreak of fascioliasis occurred in that country in the winter of 1995, in which 64 patients were diagnosed early by antigen detection assay and some weeks later confirmed by parasitological examination (46). In addition, 14 chronic patients were diagnosed and 1 patient died as a consequence of severe chronic fascioliasis. During the last 10 years, at least 90 sporadic cases were diagnosed at the Institute of Tropical Medicine of Havana (63,72,79,80). In addition, a seroepidemiological study developed in an endemic community of Matanzas, Cuba, demonstrated a 9.1% of seroprevalence to *F. hepatica* antigens and four nonsymptomatic persons with *Fasciola* eggs in their stool (71).

The geographic distribution of *F. hepatica* is mainly determined by the distribution patterns of the snail intermediate host (96,97). Snails of the family Lymnaeidae exclusively transmit *Fasciola* (98). The amphibious snail *Lymnaea truncatula* in the palaeartic region, including Europe, transmits *F. hepatica*. In North America, *L. cubensis*, *L. columella*, and *L. bulimoides* are the transmitters, and in South America, *L. diaphana*, *L. viator*, and *L. cubensis* are the proven hosts. *L. columella* also occurs in the Caribbean, Central America, and South America (99), and it has been introduced to Australia, South Africa, and Europe (100). *L. truncatula* and *F. hepatica* have been reported in the cooler climates of Morocco (101). South Africa, and the tropical highlands of Ethiopia, Kenya, and Zimbabwe (102,103). In West Africa, *L. natalensis* is the only intermediate host found. It is common in regions with over 1000 mm rainfall and becomes more erratic in distribution northwards, where snails are only found in permanent waters adjacent to the river Niger. In the West African savannah, snail population density and animal infection increase at the end of the rainy season and peak in the early dry season (104).

Studies on habitats of the intermediate host of *F. hepatica* describe the presence of these snails in clean, shallow, slowly moving waters in open streams, ponds, and lakes with limited aquatic vegetation (102). *F. hepatica* eggs require a minimum of 10 days to form the miracidium, and this requires a minimum of 4–6 weeks to develop in snails. The climate conditions are important for

the survival of snails, eggs, and metacercariae. If elimination of metacercariae occurs during adverse climate or high temperatures, its infectivity is reduced (105). Metacercariae may survive 3 months in moist hay (103). Adult snails and eggs are unable to withstand desiccation well and have limited capability of surviving drought (107). Preston and Castelino reported in Kenya that *L. natalensis* population members increased progressively at a cattle farm between January and September during the decreasing temperatures of the wet season and peaked at the time of rising temperatures in the late rainy season (106). Other studies demonstrated that rediae do not produce cercariae at  $<16^{\circ}\text{C}$  but that cercariae develop when temperatures reach  $20^{\circ}\text{C}$  (107).

Habits of snails and their geographic distribution have the tendency to be stable from year to year. This behavior, together with the relative longevity of *Fasciola* in the mammalian host and the fact that livestock populations always follow the pattern of grazing in herds, makes it possible to use computerized geographic models, which provide the epidemiologist a systematic way to define the preferences and limits of tolerance of a parasite and to match these to the spatial and temporal suitability of the environment. In the classic work of Ollerenshaw and Rowlands (108), *F. hepatica* was the target of the first widely used climate forecast system for predicting acute outbreaks and appropriate control of a parasitic disease. The forecast was developed using prevalence data and climate values of five meteorological stations in the county of Anglesey from 1948 to 1957. Using this system and the formula  $Mt = N(R - PE + 5)$ , the authors calculated a monthly risk index based on N (the number of rain days) and the difference between R (precipitation) and PE (evapotranspiration calculated by the Penman method). They were able to estimate potential losses in sheep for both the major "summer infection" and a minor "winter infection" and the potential risk of infection in both seasons. Attempts have been made to adapt the Mt system to other areas of Europe, but it has not been routinely used due to the complex data needed for the Penman equation (109). Another climate-forecast system has also been developed for use in Northern Ireland (110), France (111), the United States, and Australia (112,113). However, they have not yet been extensively used outside the climate zone where they were developed.

Geographic information systems (GIS) and satellite sensor technology are emerging new tools for epidemiology studies on human and animal diseases. GIS allow computer-based analysis of multiple layers of mapped data in digital form, agroclimatic databases and maps of the host population and disease prevalence, which allows analysis of information by location and provides a dynamic updated mapping system that can be used by health authorities to plan and monitor control programs. GIS have recently been applied in fasciolosis (114,115) at Red River Valley ecosystems of Louisiana. Also, mathematical equations have been devised for the development of the intramolluscan stage of *F. hepatica* (116) and other stages of the life cycle (117), which have been useful to predict changes in the infection intensity of natural fluke populations in sheep after single and multiple doses of anthelmintic agents (118). More comprehensive reviews on various aspects of the epidemiology of fascioliasis have been published (105,119).

## V. CONTROL MEASURES

Three possible methods may be used for fasciolosis control: (a) diminishing to a minimum the intermediate host population, (b) applying chemotherapy systematically to the mammalian host, and (c) vaccination of the mammalian host for protection against infection.

Snail populations, the intermediate hosts, may be significantly reduced if their optimal habitat conditions are dramatically changed. This may be possible using chemical control methods. Snails are susceptible to chemical compounds such as copper sulfate. Applying such a chemical product on the soil, mainly in seasonable periods of higher snail proliferation, one may diminish the host population. However, this procedure not only affects the survival of snails but also changes the quality of the soil and therefore on a long-term basis would be dangerous for the surrounding ecosystem. Biological competitors such as snail species with a higher rate of growth than the snail intermediate host may also be used to diminish the *Lymnaea* population. In Cuba, *Marisa cornuarietis*

(121) and *Tarebia granifera* (122) have been suggested as biological controls against the *L. cubensis* and *L. columella* hosts (123). As part of this strategy the places where animals pasture should be continuously rotated and drained in order to avoid eggs excreted with fecal material reaching water, developing into miracidium, and finding appropriate intermediate hosts. At the same time animals should be adequately treated with anthelmintics.

Knowledge of the epidemiology of the disease, which basically depends on knowledge of seasonal rainfall, temperature, and suitable quality of soil, for different countries is essential for good strategic chemotherapy, as well as some basic knowledge of the biology of snails and flukes. The basic principles of chemoprophylaxis recommended by FAO (124) are as follows:

1. Apply the treatment towards the end of a period when larval development in the fluke eggs or in the snails is retarded, the reproduction rate of snails is low, or the activity of snails is impaired. At that time a prophylactic effect is expected by reducing pasture contamination with eggs before favorable climatic conditions occur for larval development of the fluke and for snail activity. Further treatments are required depending on seasonal conditions 2 or 3 months after known peak contamination of the pasture with metacercariae. Additional treatments may be necessary in areas where the contamination of the pasture is continuous due to favorable climatic conditions.
2. Rotate anthelmintics annually using those with different chemical groups and different modes of action in order to reduce the development of resistance in susceptible population is necessary.
3. Apply treatment of all animals from an endemic area within a short period of time in order to achieve a synchronized reduction of pasture contamination with eggs.
4. Use drugs that are effective against flukes aged 6 weeks and older. If the drugs used are only effective against adult flukes, additional treatments may be necessary.

Table 4 shows the comparative efficacy of nine of the most important compounds available for the treatment of fascioliasis in sheep and cattle. In humans, treatments with emetine and bithionol (125) at high and repeated doses have shown efficacy against adult flukes. However, both drugs are toxic and produce severe side effects. Praziquantel has been suggested for treatment of fascioliasis in many textbooks. However, no evidence of its efficacy against *Fasciola* spp. has yet been obtained. The drug of choice for treatment of human fascioliasis is triclabendazol. This compound is safe,

**TABLE 4** Comparative Efficacy Compounds Available for Treatment of Fasciolosis in Sheep and Cattle

Drug	Efficiency spectrum (>10%)	Route of application	Recommended dose rate (mg/kg)	
			Sheep	Cattle
Carbon tetrachloride	Flukes age >12 weeks	Oral	80	40
Hexachloroetano	Flukes age >12 weeks	Oral	250–300	300
Bithionol	Flukes age >12 weeks	Oral	75	30
Clixonamide	Flukes age >12 weeks	Oral	20	NR
Oxyclozanide	Flukes age >14 weeks	Oral	15	13–16
Niclofolan	Flukes age >12 weeks	Oral	4	3
Rafoxanide	Flukes age >6 weeks in sheep and >12 weeks in cattle	Oral	7.5	7.5
Albendazol	Flukes age >12 weeks	Oral	5	10
Triclabendazol	Flukes age >12 weeks	Oral	10	12



with no apparent side effects, and with the use of a single dose of 10 mg/kg, which may be repeated 24 hours later, has been very successful for the treatment of chronic or acute fascioliasis. Triclabendazol is the only drug that is effective against immature or mature flukes either in humans or in animals. Therefore, it is the ideal drug to develop a program control based on chemotherapy. Unfortunately the drug is not yet available for human use. For veterinary purpose, triclabendazol is commercialized under brand name Fasinex.

In any program control, chemotherapy success should be monitored using adequate diagnostic methods. Until now this has been performed using commercial kits based on evaluation of fecal egg counts (126), but as mentioned before, these are insensitive and time-consuming. Diagnostic methods to determine coproantigen prevalence (e.g., ES78 sandwich ELISA) may find use in more standardized infection monitoring programs in the future.

Until 1987, attempts to vaccinate animals against *F. hepatica* had been generally performed using a crude parasite extract, ES antigens, or radiation-attenuated metacercariae. The development of vaccines for the control of fascioliasis in ruminants has fortunately entered the molecular phase. Three different strategies have been used to date to identify candidate vaccine molecules with *F. hepatica*:

1. Cross-protective antigens (*F. hepatica* antigens capable of cross-protection against *S. mansoni*, i.e., Fh12)
2. Homologous antigens (molecules of *F. hepatica* homologous to an antigen known to protect animals against *S. mansoni* and *S. japonicum* infection, i.e., glutathione S-transferase)
3. Essential antigens (molecules used for the parasite or necessary for their survival during infection, i.e., cathepsin proteases)

Three candidate antigens from *F. hepatica* have shown efficacy in vaccine trials conducted in cattle or sheep.

The Fh12 antigen is a major component of a complex protein fraction isolated from adult *F. hepatica* with cross-reacting antibodies raised to *S. mansoni* adult worms. Fh12 is highly protective in mouse and cattle models when injected subcutaneously emulsified in Freund's complete adjuvant (127,128). Antiserum to Fh12 was used to screen a cDNA library, and the recombinant molecule identified had a molecular weight of 14.8 kDa and a 44% of identity with a family of fatty acid-binding protein including the Sm14 from *S. mansoni* (129). These results suggest that Fh12 is a fatty acid-binding protein that may play a role in the intracellular transport of long-chain fatty acid and their acyl-CoA esters.

Glutathione S-transferases (GST) are a family of housekeeping enzymes involved in the metabolism of xenobiotics, transport of anionic compounds, and detoxification of lipid peroxides. The GST or *F. hepatica* is a mixture of isoenzymes of size 23–26.5 kDa. GST induced a significant reduction in parasite burden in sheep when they were vaccinated with 10 doses of 100 µg of GST emulsified in Freund's complete adjuvant (130,131). Other studies showed that the vaccination of rats with two doses of 250 µg of GST did not induce protection (132). In cattle previously immunized twice with GST and challenged 2 weeks later, significant levels of fluke reduction (49%, 52%, and 69%) were obtained (133). Thus, GST is a relatively "novel antigen" in ruminants.

The cathepsin-L-like proteases are a family of proteases secreted by the adult parasite. At least eight different cathepsin-L-like proteases have been described (134). The identity between the different cathepsin-L-like proteases ranges from 80 to 99%. The cathepsin-L-like proteases in the adult fluke have been immunolocalized to granules in the parasite intestine epithelial and the intestinal lumen (135). This suggests that the cathepsin-L-like proteases in the secretory granules could be secreted into the intestinal lumen, where they presumably play an important role in extracellular degradation of host protein. Wijffels et al. (136) reported the first experiment testing the efficacy of fluke cathepsin-L as a vaccine in sheep. Vaccination induced high titers of antibody as determined by ELISA. It was found that vaccination did not affect the mean worm burden. However, fecal egg count was significantly reduced (by 70%). In addition, the eggs recovered from the vaccine sheep exhibit a mean of 80% reduction in viability (136).

One possible prospect for controlling infection with *F. hepatica* is the use of cocktail vaccines. The liver fluke undergoes extensive migration and development in its animal host, and successful parasitism will involve the expressions by the fluke of multiple gene products, which together must function to allow fluke survival. The juvenile flukes must invade through the gut and enter the liver, migrate through the liver and digestive tissue, evade the antibody and the inflammatory immune response, and finally enter the bile duct, where it degrades blood. These multiple steps will require the function of protein such as proteases for tissue penetration and immune evasion and detoxifying enzymes such as GST and superoxide dismutase for protection against damaging inflammatory responses. A vaccine that could simultaneously block protease function and detoxification may induce synergistic effects, which can severely debilitate the parasite and favor the host in its attempts to eliminate the invading parasite. A Fh12/GST combination would be anticipated to act at two different sites in the parasite: lipid uptake from the host via Fh12 and detoxification. A GST/cathepsin-L vaccine would be expected to reduce worm burdens by about 50% and simultaneously reduce egg release by the remaining 50% of parasites, resulting in a >80% reduction in egg release onto pasture. This also reduces the infection rate of the snails, and therefore further transmission of infection to cattle would also be reduced.

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## *Fasciolopsis buski*

**Bishnupada Roy\* and Veena Tandon**

*North-Eastern Hill University, Shillong, India*

### **I. BACKGROUND**

The disease caused by the intestinal giant fluke, *Fasciolopsis buski*, popularly known as fasciolopsiasis, is a distinct clinical entity with specific etiological agents. The parasite infects both humans and pigs. It can be considered one of the important deterrents to socioeconomic development in the impoverished areas of tropics and subtropics, where high rainfall and moderate water temperatures offer a suitable environmental condition for a high rate of snail reproduction together with conditions that allow the parasite to maintain its life cycle for most of the year (1,2).

In endemic zones, developmental activities, such as construction of dams for power and irrigation, associated with unhygienic food habits, particularly consumption of raw aquatic foodstuff and close proximity between human habitat and free-range pig farms, are found to be responsible for the occurrence and recurrence of the disease. Pigs serve as a reservoir of infection and help to maintain the parasite life cycle in nature throughout the year. As many as 152 flukes have been found in pig intestines, indicating the poor health status of the animals. Pork is a vital source of protein in the endemic areas.

### **II. CHARACTERISTICS**

#### **A. Habitat**

The adult fluke normally lives in the duodenum and jejunum of humans, but in heavy infections may be found in the stomach and most parts of the intestine. The eggs reach the outside through host excreta and develop further in water. Different stages of larval development occur inside the lymph spaces of planorbid snails belonging to the genus *Segmentina* or to the genus *Hippeutis* and finally encyst in stable aquatic vegetation (3,4).

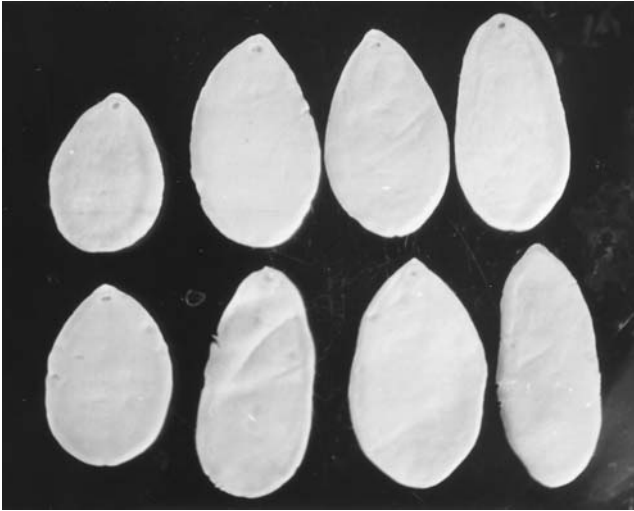
#### **B. Morphology**

The body of the parasite is thick, fleshy, pinkish, or dark brown in color when fresh, broadly ovate, elliptical with a rounded posterior end, 17–75 mm in length, 8–20 mm in width, and 0.5–3.5 mm in thickness (Figs. 1, 2). There is no cephalic cone or shoulders. The ventral sucker is large and muscular, lies close to the oral sucker, and is 1.22–2.48 mm in diameter. The prepharynx is very short, and the pharynx measures 0.36–0.72 mm in diameter. The esophagus is short, and the intestinal caeca are long, lateral, unbranched, and extend up to the subcaudal region of the body. The cirrus

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\* *Current affiliation:* Mizoram University, Aizawl, India.

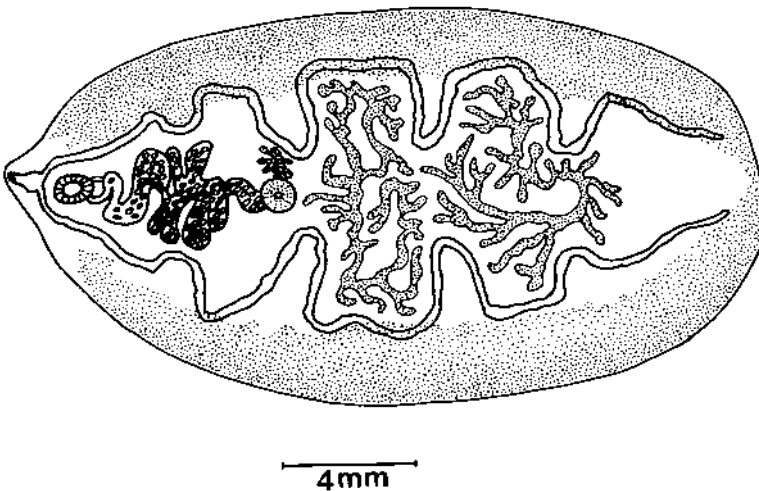




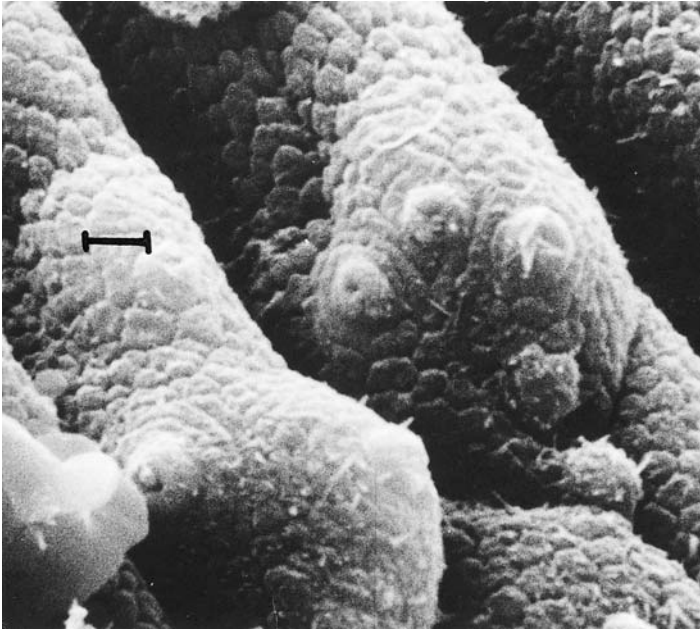
**FIGURE 1** *Fasciolopsis buski* flukes recovered from the intestine of pig.

sac is sinuous, the ovary is branched, and the uterus transversely coiled and confined to the preovarian region. The Mehlis' gland is median and postovarian. Eggs are brownish-yellow in color, operculate, ovoid in shape, 0.113–0.171 mm by 0.080–0.098 mm in size, and contain an unsegmented embryo in a mass of yolk cells.

The surface tegument of the buccal cavity is provided with longitudinally running ridges having fine tuberculation and domed aciliate papillae in groups of 2–4 (Fig. 3). The rim of the oval and ventral suckers is radially corrugated (Fig. 4). The dorsal surface of the fluke is provided with isolated bun-shaped round papillae with a smooth surface. At higher magnification, the general tegument on the dorsal surface including the lateral margins show a beaded texture formed by



**FIGURE 2** Diagrammatic representation of the parasite.



**FIGURE 3** *Fasciolopsis buski*, scanning electron micrograph showing grouped papillae on the surface of the buccal cavity (scale bar = 1  $\mu$ m). (From Ref. 6.)

compactly placed tuberculation (Fig. 5). The ventral surface of the fluke is provided with densely packed, posteriorly directed and broadly rounded scales (Fig. 6) (5).

Little deviation from this normal surface topography and anatomy of the fluke may be observed when collected from different geographical regions (6).

### C. Life Cycle

*F. buski* follows the typical trematode life pattern, involving an intermediate host, i.e., a fresh water snail of the genus *Segmentina* or the genus *Hippeutis* and a mammalian definitive host, i.e., human or pig. About 25,000 eggs escape out of the mammalian host every day through feces, and the shelled embryo develops in the water. In the course of 2–3 weeks, the miracidium develops inside the egg at an optimum temperature of 22–25°C. As it comes out of the egg through the operculum, the miracidium finds its way into a suitable snail host. Inside the lymph spaces of the snail, the miracidium changes into a sporocyst. The latter produces the first and second generations of rediae, the third larval stage. Finally, the daughter rediae produce tailed larva, the cercariae. The development from miracidium to cercaria takes about 2 months. Once out of the snail, the cercariae swim for a brief period and lose their tails and encyst on aquatic vegetation. Infection of the definitive host takes place through the ingestion of the encysted metacercariae along with infested vegetables. After entering the duodenum, the metacercariae excyst and liberates a juvenile worm, which develops into the adult fluke within about 3 months. The life span of the adult fluke seldom exceeds 6 months (7–10).

### III. DISEASE

Fasciolopsiasis causes obstructive, traumatic, and immunopathological changes in humans and is characterized by gastrointestinal pain, anorexia, vomiting, diarrhea, and mild fever. Infection in



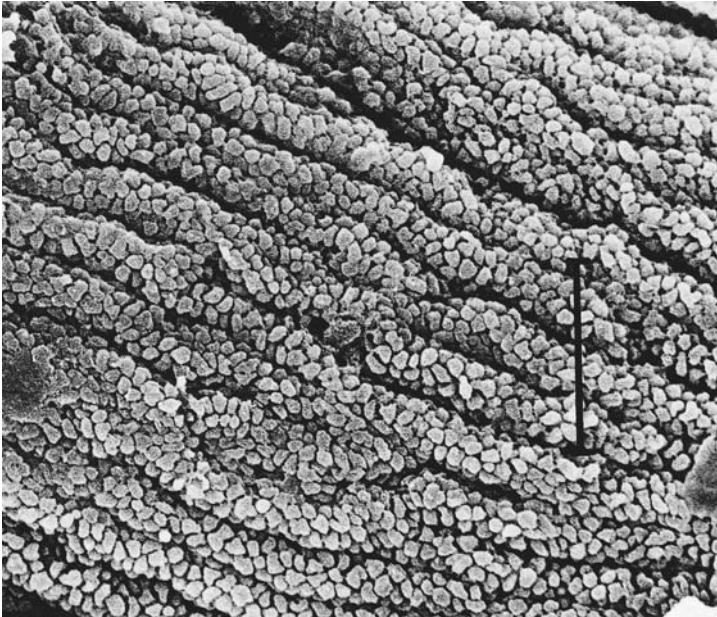
**FIGURE 4** *Fasciolopsis buski*, scanning electron micrograph showing the rim of the ventral sucker (scale bar = 10  $\mu\text{m}$ ).

other animals generally shows subclinical symptoms, but a heavy parasite load can mimic the human symptoms.

#### **IV. EPIDEMIOLOGY**

##### **A. Route of Infection**

Infection occurs by ingestion of infected raw foods like the bulb of lotus tuber (*Nelumbo* spp.), seed pods of water caltrop (*Trapa* spp.), the bulb of water chestnut (*Eliocharis tuberosa*), and other aquatic vegetation (*Salvinia*, *Valisneria*, *Eichornia* spp.) contaminated with encysted metacercaria (4,11–13). Peeling of these raw foods with the teeth makes entrance easy for the infective form.



**FIGURE 5** *Fasciolopsis buski*, scanning electron micrograph showing densely tuberculate dorsal surface of the body (scale bar = 10  $\mu\text{m}$ ).



**FIGURE 6** *Fasciolopsis buski*, scanning electron micrograph showing posteriorly directed scales on the ventral surface of the body (scale bar = 10  $\mu\text{m}$ ).

**Table 1** Prevalence of *Fasciolopsis buski* in Humans in Some Asian Countries

Country	% Infection	Ref.
China	0.8	24
India	22.4	1
Thailand	19.8	15
Taiwan	8.2	16
Indonesia	1 <sup>a</sup>	33

<sup>a</sup> A case report.

## B. Reservoir Hosts

Pigs, dogs, and rabbits act as reservoir hosts for the fluke (13,14).

## C. Geographical Distribution

*F. buski* is widely distributed in India, China, Pakistan, Bangladesh, Burma, Thailand, Malaysia, Indonesia, Taiwan, and Vietnam (Table 1) (10,13–17). In India, morbidity due to fasciolopsiasis has been recorded since 1843, when Busk first recovered *F. buski* from the duodenum of an Indian sailor (9). Subsequently, the parasite was reported from different provinces of India, namely Assam, Bengal, Bihar, Orissa, Maharashtra, Madhya Pradesh, and Uttar Pradesh, where poverty, unhygienic sociocultural practices, and food habits are associated with the existence of open types of large-scale pig farms (1,2,13,18–22). The planorbid snails, *Segmentina trochoideus* in Assam and *Hip-pertis coenosus* in Uttar Pradesh, act as intermediate hosts for the parasite (18,23).

In endemic areas of China mass chemotherapeutic practices failed to prevent the infection, particularly among children, because of the widespread practice of eating raw vegetables and ignorance about preventive measures for the disease (24–26).

*S. trochoideus* is the most common snail vector of *F. buski* in Bangladesh, followed by *H. umbilicalis* (3). In Vietnam *S. hemisphaerula* is the vector species (17).

## V. ISOLATION AND IDENTIFICATION

In endemic areas the history of the patient is taken and microscopic examination of feces is carried out to diagnose and identify the eggs of *F. buski*. The eggs are brownish in color, typically operculate, and large in size (0.113–0.17 × 0.080–0.098 mm). The adult *F. buski* can be distinguished and identified on the basis of the following characteristics:

1. Leaf-shaped unsegmented and fleshy flat worms, 17–75 mm in length and 8–20 mm in breadth
2. Cephalic cone absent
3. Long, unbranched intestinal blind ceca
4. The ventral surface of the body provided with posteriorly directed broadly rounded scales

Systematic classification of the fluke is as follows:

Phylum: Platyhelminthes  
Class: Trematoda  
Subclass: Digenea  
Order: Prosostomata

## VI. PATHOGENICITY

Young and adult *F. buski* remain attached to the duodenum and jejunum with the help of their ventral sucker and cause mechanical injury to the mucous membrane of the intestinal wall leading to local inflammation with hypersecretion of mucus, hemorrhage, and ulceration.

Heavy worm load causes intestinal bowel obstruction and interference with normal digestive juice secretion. The profuse stools become yellow-green in color and contain a considerable amount of undigested food (27). Absorption of toxic metabolites of the flukes leads to edema of the face, abdomen, and legs and eosinophilia (14). A long-standing heavy infection may cause patches of scar formation throughout the wall of the intestine and leakage of blood. There may be a high leukocyte count and depletion in red cell counts and vitamin B<sub>12</sub> levels in the serum of infected persons (15,28).

## VII. CONTROL MEASURES

The drug that has specific action on *F. buski* is tetrachloroethylene (C<sub>2</sub>Cl<sub>4</sub>) (12,29). For adults, the single dosage consists of 4 mL of drug in a hard gelatin capsule, and for children, 4 drops for every year up to the age of 15 years. Other drugs found effective against *F. buski* are oxyclozanide, praziquantal, niclosamide, dichlorophen, thiabendazole, and mebendazole (21,29–31).

In endemic rural areas where medical facilities are not accessible to the masses, indigenous plant medicines are used with success as traditional treatment to expel *F. buski* (5,32).

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# 34

## ***Echinostoma* Species**

**L. K. Eveland**

*California State University, Long Beach, California, U.S.A.*

**M. A. Haseeb**

*State University of New York, Brooklyn, New York, U.S.A.*

### **I. BACKGROUND**

Echinostomiasis is a parasitic disease of birds, animals, and humans resulting from intestinal infection with distomate, digenean trematodes of the family Echinostomatidae. Although echinostomiasis occurs worldwide, most human infections are reported from East and Southeast Asia. Despite being relatively rare, at least 19 species of echinostomes from 8 genera have been reported in humans from China, India, Indonesia, Japan, Korea, Malaysia, Philippines, Russia, Taiwan, and Thailand. Sporadic cases have also been reported from Brazil, Egypt, Italy, Mexico, and Romania (1).

### **II. CHARACTERISTICS**

Echinostomes are distomate, hermaphroditic flukes, whose most characteristic morphological feature is a horseshoe-shaped cephalic collar, which bears sensory papillae and spines. The number of spines is constant for a particular species and usually ranges from 27 to 51. The spines may be arranged in one or two circles around the collar. Three groups of echinostomes can be differentiated based on the arrangement of the collar spines: the Echinostomatinae species (*Artyfechinostomum*, *Echinoparyphium*, *Echinostoma*, *Paryphostomum*, *Hypoderaeum*) have collar spines arranged in two rows, interrupted ventrally; Himasthliinae have only one row of collar spines interrupted ventrally; and Echinochasmae have collar spines arranged in one row interrupted both dorsally and ventrally (2).

The worms are typically small, ranging from 4 to 20 mm in length and from 1 to 3 mm in width, although considerable intraspecific variation exists in size depending upon fixation, definitive host, and the worm burden (3). An oral sucker surrounds the mouth, and a muscular pharynx is located posterior to the oral sucker. The short esophagus bifurcates anterior to the acetabulum to give rise to intestinal ceca that run medial to the vitellaria and end blindly in the posterior aspect of the worm. The ventral sucker, which is larger than the oral sucker, is positioned in the anterior one third of the body. The rim of the suckers may contain papillae and spines. The tegument contains scale-like spines on both dorsal and ventral surfaces, although the number and size of tegumentary spines is reduced in the posterior half of the body (4). The spherical or ovoid ovary lies in front of the testes, which are in tandem in the posterior half of the body. The ootype lies between the testes and the ovary, and the uterus is between the ovary and ventral sucker. At the posterior tip of the body there is a terminal or subterminal excretory pore, which leads to an excretory bladder. The echinostomes lay yellow-brown eggs that are thin-shelled and have an inconspicuous operculum.



There is also a slight thickening of the shell at the abopercular end. They are unembryonated when passed in feces and usually measure 80–135  $\mu\text{m}$   $\times$  55–80  $\mu\text{m}$ , depending on the species.

### III. PATHOGENESIS AND DISEASE

Although pathogenesis in echinostomiasis is poorly documented, clinical symptoms are similar to those of other intestinal helminthiasis and are related to worm burden (5). Echinostomes cause inflammation and ulceration of the intestinal mucosa, with infiltration of polymorphs and eosinophils (6).

Light infections may remain asymptomatic. Moderate infections may produce headache, dizziness, anemia, gastric pain, and diarrhea. Heavy infections may be associated with anorexia, nausea, postprandial burning, and epigastric pain with hematemesis, vomiting, fever, flatulence, constipation, abdominal pain, profuse watery diarrhea, anemia, eosinophilia, edema (7–13), and hepatomegaly (14). Infection with *Artyfechinostomum mehrai* has been reported as a probable cause of bowel perforation (15). Different species parasitize humans for different lengths of time, so the duration of infection may also influence disease. *Echinostoma echinatum* reportedly can live in humans for as long as 10 months (16).

### IV. EPIDEMIOLOGY

Humans are infected with echinostomes through the consumption of raw or inadequately cooked freshwater mollusks, fish, or amphibians. Local customs and eating habits determine the exact mode and extent of transmission. Although snails are consumed in both Malaysia and Indonesia, the habit of cooking snails renders them harmless, and human infections with echinostomes are rare. By contrast, local food habits, which include eating uncooked snails and tadpoles, are responsible for the transmission of echinostomes in the Philippines and northeast Thailand.

The first human infection with *Echinostoma hortense* was reported in Japan in 1976 (17), and over the next few years more than 20 human cases were reported (18). Caused by eating raw or insufficiently cooked fish, echinostomiasis hortense was also shown to be highly endemic in humans in the southeastern part of Korea. In 1988, an infection rate of 22.4% was reported in Cheongsongun, an island area in the southeastern part of Korea (10).

*Echinostoma cinetorchis* was first described in humans in Japan (19), and four cases have since been reported in humans in Korea (8,10,20). Human infections are acquired from eating freshwater snails or freshwater fish.

*Echinochasmus japonicus* infects humans in Korea and Japan (17,21,22). Eating raw fish is believed to be the primary cause of infection. In Korea this echinostome has 18 species of freshwater fish as its secondary intermediate hosts (23). Cases of natural human infection have also been reported from China (24,25).

A highly endemic focus of *Echinostoma echinatum* (= *E. lindoense*) was discovered in Celebes, Indonesia (26,27), with reported infection rates in the Lindu Valley of Central Sulawesi ranging from 42.6–96% during 1937–1956. Infection is acquired primarily by the consumption of freshwater clams (16).

Predation by a newly introduced fish, *Tilapia mossambica*, resulted in almost complete disappearance of clams (*Corbi cola* spp.) that served as second intermediate hosts and were part of the local diet (5,16). The fish replaced clams in the local diet, and by the 1970s echinostomiasis in humans had been greatly reduced (28). Currently, it is encountered in 1% or less of the population (22,29). Other species reported from humans in Indonesia include *Echinostoma ilocanum*, *Echinostoma malayanum*, *Echinostoma revolutum*, and *Echinoparyphium recurvatum*. Human cases are attributed to eating freshwater snails and clams.

*Echinostoma revolutum* was reported in 4 of 507 males tested (0.8%) from northeast Thailand, but was not found in females (30). In Taiwan, 11–65% of persons surveyed had eggs of *Echinostoma revolutum*, *E. recurvatum*, or *E. melis* in their stools (31). The source of infection was considered

to be freshwater clams. Human infections with *Echinostoma revolutum* have also been reported from Indonesia (32).

Eggs of *Echinostoma* (= *Euparyphium*) *ilocanum* were first discovered in feces of prisoners at Bilibid Prison, Manila, the Philippines, in April 1907 (33). Although this fluke was redescribed and placed in the Genus *Euparyphium* in 1931, it is still called *Echinostoma ilocanum* or Garrison's fluke in the Philippines (34). In northern Luzon, the Philippines, *E. ilocanum* eggs were found in the feces of over 10% of persons examined (22), and the infection rate among ilocanos reached 44% in some areas (5). This high rate of infection in the north is probably owing to human consumption of the large rice field snail, *Pila luzonica*, which serves as a second intermediate host (22). In a survey in northeast Thailand, 8.1% of males were infected with *E. ilocanum* (30). It has also been reported from China (6) and Indonesia (32,35).

*Echinostoma* (= *Euparyphium*) *malayanum* has been reported from the Philippines (36) as well as in Malaysia and Singapore (37), with an overall prevalence rate of 20% in the municipality of Echague, Isabella (38). It also occurs in India (11), Indonesia (16), Malaysia and Singapore (37), and China (6). A survey in northeast Thailand found 8.3% of males and 2.9% of females infected with *E. malayanum* (30).

In China, *Echinochasmus* (= *Echinostoma*) *perfoliatus* has been reported from Anhui Province (39) and from 5% of the human populations of Fujian and Guangdong Provinces. It has also been reported from humans in Japan, Italy, Romania, and Russia (6).

In Taiwan, freshwater clams, eaten raw, half-boiled, or pickled overnight, are considered the main source of human cases of *E. recurvatum*, *E. revolutum*, and *Echinostoma melis*. Surveys throughout the country indicate infection rates ranging from 11 to 65% (22).

*E. malayanum*, *E. revolutum*, *E. ilocanum*, and *Hypoderaeum conoideum* have been recovered from humans in the northeast provinces of Thailand, who became infected from eating raw snails and tadpoles (40). Surveys have revealed that one half of the villages of a region reported human cases of echinostomiasis (41), mostly with *Hypoderaeum conoideum*. Raw snails and tadpoles were identified as the source of human cases. A Thai farmer from ROI-ET Province has also been reported infected with *Episthmium caninum*, presumably from the consumption of raw fish (42).

Several other echinostomes have been reported infrequently in humans. *Artyfechinostomum mehrai* was reported in humans in India where snails were the source of infection. *Echinochasmus angustitestis* and *Echinochasmus fujilanensis* were first reported in humans in China (43). *Echinochasmus liliputanus* infects humans in mainland China (43). A study showed that humans could be infected through drinking unboiled water containing cercariae or eating raw fish containing the metacercaria (44). Human infection with *Echinoparyphium paraulum* has been reported in Russia (37). *Echinoparyphium* (= *Euparyphium*) *recurvatum* infects humans in Taiwan (16,31) and Indonesia (32). Only one human infection has been reported with *Echinostoma macrorchis* (45). *Echinostoma melis* (= *Euparyphium jassyense* = *Echinostoma jassyense*) has been reported from humans in China (46) and Romania (37). *Episthmium caninum* was reported in a human as an accidental infection acquired from consuming raw fish in Thailand (42). A German in Hamburg infected with *Himasthla muehlensi* was believed to have acquired the parasite by eating the lamellibranch, *Venus mercenaria*, in North America (6). Thousands of *Paryphostomum sufrartyfex* were reportedly found in a 15-year-old Hindu boy who apparently died from the overwhelming infection (47).

## V. ISOLATION AND IDENTIFICATION

Most echinostomes have been identified in humans by recognizing their eggs in stools or by identifying adult worms in stools following treatment, even though the latter method, widely used, often causes alterations in the worms, which may result in difficulties in their identification (48). Their location within the human host, therefore, is usually not known. However, a case of echinostomiasis with ulcerative lesions in the duodenum, where the worms presumably resided, has been reported (49). In another reported case the patient vomited worms, suggesting that they had resided in the

upper small intestine or stomach (14). Perforated bowel has been documented in a child due to an infection of the ileum by *Artyfechinostomum mehrai* (15). These results suggest that echinostomes normally reside in the human small intestine.

*Echinostoma ilocanum* has been reported to range in size from 4 to 10 mm long, 0.5 to 1.5 mm wide, and 0.5 to 1.5 mm in thickness (6,14,34,37,40,50,51). The oral sucker is muscular, 174 (160–190)  $\mu\text{m}$   $\times$  200 (180–220)  $\mu\text{m}$ , and surrounded by a horseshoe-shaped collar, ventrally open, surmounted with a crown of 49–51 spines. There is a muscular pharynx 150  $\times$  175  $\mu\text{m}$ , which separates into two intestinal ceca that extend to the subcaudal region. The tegument is covered with small spines from the anterior to the middle half of the posterior portion of the body. The body is dorsoventrally flat and oval with rounded tapering ends, but the posterior end is more attenuated than the anterior. Two deeply lobed testes lie one behind the other in the third fourth of the body. The transversely ovoid ovary, 311  $\times$  287  $\mu\text{m}$  (40), lies just in front of the anterior testis. The vitellaria are medium-sized follicles situated in the lateral fields through the posterior three fourths of the body. The coiled uterus fills the intercecal field between the anterior testis and the ventral sucker. The eggs are straw-colored and operculate, measuring 83–116  $\mu\text{m}$   $\times$  58–69  $\mu\text{m}$ .

*Echinostoma lindoensis* worms are of a bright red color, which changes to a nondescript gray after death (27). They measure 13–15 mm in length and 2.5–3 mm in width. The head collar bears 37 spines including 5 corner spines (37), all of which are very uniform in size on any individual specimen (27). The size of the oral suckers ranges from 0.23 to 0.51 mm in diameter, and the pharynx ranges in size from 0.18 to 0.40 mm in diameter. The ventral sucker measures from 0.6 to 1.38 mm and is in the anterior quarter of the body. The tandem testes are in the posterior half of the worm and are definitely lobed, except in young worms, where they are spherical to oval (37). The anterior testis is usually slightly more voluminous in appearance than the posterior testis (27). The small and elliptical cirrus sac, which is not spinous, is situated just behind the bifurcation of the intestine. The ovary is oval to pyriform in shape with the largest axis from 0.3 to 0.5 mm and situated on the midline of the worm. The uterus forms 11 transverse loops. The vitellaria extend from the level of the posterior end of the ventral sucker. The operculate eggs measure 92–124  $\mu\text{m}$  in length by 65–76  $\mu\text{m}$  in width. They have a slight green tinge and a thick shell with a knoblike thickening at one pole and a faintly visible operculum at the opposite pole.

*Paryphostomum sufrartyfex* adults measure 8–9 mm in length, 2.5 mm in breadth, and 0.8–1.0 mm in thickness (37,47). The body is slightly curved ventrally at both ends. The ventral surface and part of the dorsal surface are spinous. There is usually a constriction at the level of the ventral sucker (47). The oral sucker is surrounded by head collar bearing 39–42 spines. All of the spines are similar in size except for one pair of larger ones at the outer ventral angle of the disk (37). The ventral sucker is approximately 1 mm in diameter and located in the anterior quarter of the body. Two deeply lobed testes lie in the third quarter of the body, one anterior and the other posterior. The cirrus sac is large. The single subglobose ovary lies to the right of the midline in front of the anterior testis. The genital pore is anterior to the ventral sucker. The vitellaria are situated in the lateral fields behind the ventral sucker to the posterior end, where they meet in the midline. The uterus is tightly packed between the ootype and the posterior margin of the ventral sucker. The operculate ova are ovoid and measure 90–140  $\mu\text{m}$   $\times$  60–80  $\mu\text{m}$  (37,47).

*Episthmium caninum* are elongated worms with rounded, tapered ends, but the anterior end is more attenuated than the posterior. They measure 0.931 (0.773–1.237) mm long and 0.376 (0.350–0.433) mm wide and are covered by small tegumental spines beginning at the posterior margin of the circumoral disc and extending to the mid-portion of the hind testis. The circumoral disc has 24 spines, which are prominent and interrupted dorsally by an oral sucker. Six of the spines exist on both dorsal sides arranged in one row, but the lateral and ventral spines are distinctly arranged in two continuous alternating rows (42).

The oval pharynx is 71 (48–84)  $\mu\text{m}$   $\times$  63 (54–77)  $\mu\text{m}$ . The ventral sucker measures 126 (19–151)  $\mu\text{m}$   $\times$  136 (105–154)  $\mu\text{m}$ . The anterior testis is transversely oval, measuring 108 (72–141)  $\mu\text{m}$   $\times$  164 (125–197)  $\mu\text{m}$ , and the posterior testis is 145 (113–179)  $\mu\text{m}$   $\times$  170 (143–195)  $\mu\text{m}$ . The cirrus pouch is anterior to the ventral sucker, as is the genital pore. The globose ovary is situated

to the left of midline between the ventral sucker and the anterior testis and measures 65 (59–69)  $\mu\text{m}$   $\times$  68 (56–77)  $\mu\text{m}$ . The uterus is short and contains 1–7 operculate eggs, which are thin-shelled, greenish-yellow in color, and measure 95 (84–102)  $\mu\text{m}$   $\times$  64 (56–69)  $\mu\text{m}$ .

*Echinoparyphium recurvatum* adults measure 2.8–4.0 mm in length and up to 0.7 mm in breadth (37). The anterior end is curved ventrally, and the cuticle on the anterior end of the body is covered with minute spines (52). The head collar has 43–45 spines arranged in two alternate rows. The spines are 0.021–0.051  $\mu\text{m}$  in length, and there are 4 larger corner spines on each side of the oral sucker. The circular ventral sucker is very muscular, measuring 0.240–0.330  $\times$  0.240–0.315 mm. The muscular pharynx is 0.087–0.111 mm long  $\times$  0.081–0.102 mm wide. Two oval testes, in tandem and almost equal in size (0.201–0.450  $\times$  0.147–0.258 mm), are not lobed and are in contact with each other. A large cirrus sac is situated at the anterior margin of the ventral sucker. The ovary is small (0.099–0.174  $\times$  0.114–0.117 mm) and transversely oval. The short uterus contains 3–7 operculate eggs, which are widely ovoid to oval, measuring 108–110  $\mu\text{m}$   $\times$  81–84  $\mu\text{m}$  (37).

*Echinostoma revolutum* adults are 8–22 mm in length and 1–2.5 mm in width (37,52,53). The body is elongated, with an attenuated anterior portion, and fresh specimens are pinkish in color. The body is widest at the region of the ventral sucker, then attenuates again towards the posterior end. They have a distinct collar armed with a row of 37 large (0.042–0.069 mm in length) spines [2 corner groups (each of 5 spines), 2 lateral groups (6 spines), and 1 dorsal group (15 spines)]. The cuticle spines cover the anterior half of the worm, being especially noticeable from the head to the ventral sucker. The oral sucker is subterminal, circular or oval, and 144–371  $\times$  171–400  $\mu\text{m}$  in diameter. The muscular cup-shaped ventral sucker, 171–1028  $\times$  371–1028  $\mu\text{m}$  in size, is nearly circular and is located in the posterior part of the first quarter of the body. A muscular pyriform or oval pharynx 150–204  $\mu\text{m}$  long  $\times$  110–163  $\mu\text{m}$  wide follows a short prepharynx. The esophagus (240–258  $\mu\text{m}$ ) divides in front of the ventral sucker into two small simple ceca, which run through the lateral sides to nearly the end of the body. The testes are tandem, almost equal in size, and have an irregular shape. They are in the posterior half of the worm, situated in the space between the cecal branches. The elliptical cirrus sac is small (258–272  $\mu\text{m}$   $\times$  150–245  $\mu\text{m}$ ) and situated just behind the bifurcation of the intestine. The lateral vitellaria extend from the level of the second half of the ventral sucker to the end of the body. The ovary is spherical or oval and is located at a short distance above the testes. The operculate eggs measure 90–126  $\mu\text{m}$   $\times$  59–71  $\mu\text{m}$  and are immature when passed in the feces.

*Artyfechinostomum mehrai* are pinkish, freely moving, approximately 8 mm  $\times$  3 mm, flat worms with conspicuous collar and about 43 spines (15).

*E. cinetorchis* adults are elongated and leaf-like and measure 5.6–6.6 mm in length and up to 1.4 mm in breadth (37,54). Their head crown spines vary from 36 to 38 (usually 37) without dorsal interruption and include 6 corner spines and dorsal and lateral spines in two alternating rows. A characteristic feature of this species is that the number (0, 1, or 2) and location of the testes are variable, as suggested by the species name (55). The uterus lies in the intercecal field between the ventral sucker and ovary, and the vitellaria are extending from the posterior border of ventral sucker. The genital pore opens at the front of the ventral sucker. The eggs average 105  $\times$  65  $\mu\text{m}$  and have an indistinct opercular ridge.

*E. macrorchis* adult worms measure 4.6–7.8 mm in length and up to 1.3 mm in breadth (37). The body is moderately elongated, maintaining the same width all the way except for the preacetabular and posttesticular regions (45). The body is spinous both ventrally and dorsally only in the preacetabular region; there are about 50 transverse rows of spines on the postacetabular ventral surface only (45). The head collar is reniform with 43–47, usually 45, spines: there are 6 corner spines on each lappet, 3 lateral spines on each side, and 27 dorsal spines. The dorsal collar spines are arranged in 2 alternating rows, 14 anteriorly (oral) and 13 posteriorly (aboral). The corner spines are significantly longer than the others (45). The oral sucker is subterminal, and the acetabulum is in the anterior fifth of the body.

Both the bifurcation of the intestine and the genital pore are anterior to the acetabulum. The oval-shaped testes are tandem, intercecal, and posterior to the mid-body. The more posterior testis

is longer than the other and has a more pointed posterior end. The cirrus sac is elliptical and is situated between the intestinal bifurcation and the ventral sucker. The ovary at mid-body is spherical to slightly oval. The uterus winds throughout the intercecal field between the ventral sucker and ovary, and the vitellaria extend from the posterior to ventral sucker. The operculate eggs measure 93–110  $\mu\text{m} \times 55\text{--}65 \mu\text{m}$ .

*E. hortense* adult worms are elongated, dorsoventrally flattened, attenuated anteriorly, and measure 6.7–9.2 mm in length and up to 1.3 mm in breadth (37). The reniform collar around the oral sucker has 27–28 spines including corner spines of 4 each arranged in two rows. Two large testes, which are usually spirally lobed with three or four folds, or rarely tandem, are located near the center of the body (24,55). The anterior testis is usually longer than it is wide (37). The spheroid ovary is just anterior to the anterior testis and morphologically distinguished from other echinostomes because it is located toward the right of the median line of the body. Well-developed vitellaria occupy the posterior end of the body meeting behind the posterior testis. The coiled uterus lies in the intercecal space between the ovary and the ventral sucker. The large, yellowish-brown eggs measure 109–148  $\times$  43–83  $\mu\text{m}$  (mean 120  $\times$  82  $\mu\text{m}$  (13,37,55).

*Echinoparyphium paraulum* adults measure 6–10.5 mm in length and 0.8–1.4 mm in breadth, and tegumental spines cover the cuticle almost to the posterior extremity (37). The oral sucker is surrounded by a head collar, which is continuous across the ventral surface, bearing 37 spines: 27 in a double dorso-lateral row and 5 corner spines at either end. The tandem testes lie in the third quarter of the body; the anterior testis frequently has three, and the posterior four lobes. The cirrus sac may extend back to the middle of the ventral sucker. The ovary lies just anterior to the testes. The eggs measure 105–108  $\mu\text{m} \times 632 \mu\text{m}$ .

*Echinochasmus perfoliatus* adults vary in size from 0.5 to 12 mm in length and 0.1 to 2 mm in breadth (37). Preserved specimens are usually curved ventrally. The tegument contains spines on both dorsal and ventral surfaces. The head collar bears 24 spines, which are interrupted mid-dorsally as well as mid-ventrally. Two large, subglobose testes lie one behind the other in tandem in the third fourth of the body. The retort-shaped cirrus pouch lies anterior to the ventral sucker on the right of the midline. The small globose ovary is somewhat in front of the anterior testis, and the seminal receptacle lies to the right of the midline. The vitellaria extend from the levels of the genital pore to the posterior end of the body. The uterus is a relatively short, slightly coiled tubule, containing only a few eggs at one time. These eggs are ellipsoidal, operculate, thin-shelled, hyaline to greenish-yellow color, and measure 90–135  $\mu\text{m} \times 55\text{--}95 \mu\text{m}$ .

*Echinochasmus japonicus* are characteristically small and plump adult worms, measuring 0.45–0.95 mm in length and up to 0.3 mm in breadth (24,37). The body is elongate and leaf-shaped, with lateral edges of anterior body curved ventrally. The head collar is prominent, bearing 24 collar spines, interrupted dorsally with a row of 12 spines on each side; the ventral spines are in two alternating rows, and the dorsal spines are in a single row. The tegument is spinous. The testes are tandem, and the ellipsoidal cirrus sac lies just behind the bifurcation of the intestine. The ovoid or elliptical ovary is behind the acetabulum, and the vitellaria extend from in front of the acetabulum to the posterior extremity. The short uterus contains elliptical eggs averaging 85  $\times$  56  $\mu\text{m}$ , with an indistinct opercular ridge. The abopercular pole is thickened into a spine or flat protuberance.

*Echinostoma melis* (= *Euparyphium jassyense* = *Echinostoma jassyense*) adult worms are elongate, measuring 5.5–7.5 mm in length and up to 1.2 mm in breadth. The head collar bears 27 spines dorsally, of which 4 large ones are situated on each side at the ventral angle, and the remaining 19 small ones are disposed in an uninterrupted double row on the dorsal border of the disk. The testes are median, tandem, and are situated at the posterior part of anterior half of the body. The cirrus sac lies somewhat behind midline of the ventral sucker. The ovary is spherical and lies anterior to the testes to the right of the midline. The vitellaria extend backward from the level of the midline of the ovary. The uterus is short and the eggs measure 132–154  $\mu\text{m} \times 79\text{--}85 \mu\text{m}$  (37).

Adult *E. malayanum* worms are elongate and pinkish in color in fresh specimens. The body is curved ventrally. The cuticle is covered with small spines extending almost to the posterior end (48). They measure 6.5–9.3 mm in length and 2–2.3 mm in breadth (37,53). They have a well-developed head collar with 2 rows of 43–45 spines. There are usually 3 oral and 2 aboral corner

spines in each ventral flap (48). On each lateral side 11 spines are arranged in a single row. Of 11 alternating dorsal spines, 6 are oral and 5 aboral. The prepharynx is short; the muscular pharynx is nearly the same size as the oral sucker. The esophagus divides laterally into two ceca in front of the ventral sucker, which extend nearly to the end of the body. The cirrus sac is ovoid, extending to the posterior border of the ventral sucker. The deeply lobulated tandem testes lie one behind the other in the third quarter of the body. The ovoid ovary is globular or slightly flattened and lies in front of the testes. The cirrus pouch extends beyond the posterior margin of the acetabulum. The vitellaria extend into lateral fields, beginning behind the ventral sucker. The tubular, coiled uterus lies between the anterior testis and the ventral sucker. It contains yellowish-brown operculate eggs that measure  $106\text{--}131\ \mu\text{m} \times 69\text{--}80\ \mu\text{m}$ .

*Himasthla muehlensi* adult worms measure 11–17.7 mm in length by 0.41–0.67 mm in breadth (37). The head collar bears 32 spines, 2 of which are situated on each side of the ventral angle and the remaining 28 are arranged in an uninterrupted horseshoe pattern around the sides and dorsum of the disk. The two testes are situated in the posterior part of the body and the transversely compressed ovary lies just in front of the anterior testis. The cirrus sac consists of a very long seminal muscular cirrus organ armed with rose thorns. In its proximal portion the uterus is broadly coiled; more distally it extends as a slightly coiled tubule up to the genital pore. The numerous, irregular, ovoid eggs are indistinctly operculate and measure  $114\text{--}149\ \mu\text{m} \times 62\text{--}85\ \mu\text{m}$ .

*Hypoderaeum conoideum* adults are elongate, tapering posteriorly, and pinkish in color when fresh (53). They are approximately 8 mm in length and 1.5 mm in breadth. Cuticular spines cover the tegument from the head to the ventral sucker. The head collar is poorly developed with two rows of 45–53 small spines. The oral sucker is  $136\ \mu\text{m} \times 171\ \mu\text{m}$ . The cup-shaped ventral sucker is located in the first fourth of the body. The prepharynx is very short, and the pharynx is the same size as the oral sucker. The short esophagus divides in front of the ventral sucker into two lateral ceca, which extend to nearly the end of the body. The vitellaria consist of small follicles in the lateral fields, commencing behind the ventral sucker through nearly the posterior end of the body. There are two sausage-shaped testes in tandem in the third fourth of the body; the ovoid ovary lies in front of the anterior testes. The uterus is tubular and coiled.

## VI. PREVENTION AND CONTROL

Echinostomes cause infection in many animals and birds and more rarely in humans. Human infections are acquired through the ingestion of raw or insufficiently cooked fish, mollusks, or tadpoles. Therefore, the control of echinostomiasis and the prevention of human infections depends upon understanding the bionomics of snail vectors and other intermediate and definitive hosts and human behavior (56,57).

Human traditions and customary food habits are not the only factors that must be taken into account in the prevention of echinostomiasis. In some endemic areas fuel is expensive and/or cooking facilities are not available, and other methods of preparing foods such as fermenting, pickling, and smoking are used. Alternatives to cooking may not kill metacercariae. A recent increase in the export and import of foods, along with increased travel into and from endemic areas, is also important in the spread of infection (58). Other foodborne trematodes exist in echinostome-endemic areas, and control and prevention methods frequently overlap (57).

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## *Clonorchis sinensis*

Sung-Tae Hong

*Seoul National University College of Medicine, Seoul, Korea*

### I. BACKGROUND

*Clonorchis sinensis* was first recovered from a Chinese man by McConnell during an autopsy (1). It was named as *Distoma sinense* by Cobbold in 1875, changed to *Opisthorchis sinensis* by Blanchard in 1895, and finally to *Clonorchis sinensis* by Looss in 1907. Its common name is the “Chinese liver fluke,” and it belongs to the family Opisthorchidae, which is a trematode family that, like Dicrocoeliidae and Fasciolidae, infects human biliary trees (2).

Although the oldest substantiation of infection by this organism was the discovery of *C. sinensis* eggs from viscera of an ancient corpse buried in 278 B.C. (3), this organism must have been prevalent among humans since the prehistoric era, like many other human helminths. Most of the live metacercariae introduced into humans have a successful parasitic life, and most infected humans show few symptoms; both host and parasite may have been familiar to each other for a long time. Since fishing was a major means of livelihood for humans in that era, there were probably numerous opportunities for infection.

Nowadays, clonorchiasis is limited to East Asia, including China, Korea, Far East Russia, Taiwan, and Vietnam. Distribution of its intermediate hosts and the fish-eating customs among residents determine its distribution. In the endemic areas, it is one of the major foodborne pathogens.

After the development of chemotherapy using praziquantel in the 1980s, successful control of clonorchiasis was expected. However, it is still prevalent except in Japan, where clonorchiasis was controlled before the introduction of praziquantel. Since chronic clonorchiasis of high intensity induces serious medical complications, it is a major public health concern and must be controlled in the endemic areas as soon as possible. Thorough cooking of fish, proper diagnosis, and praziquantel treatment are major requirements for its control.

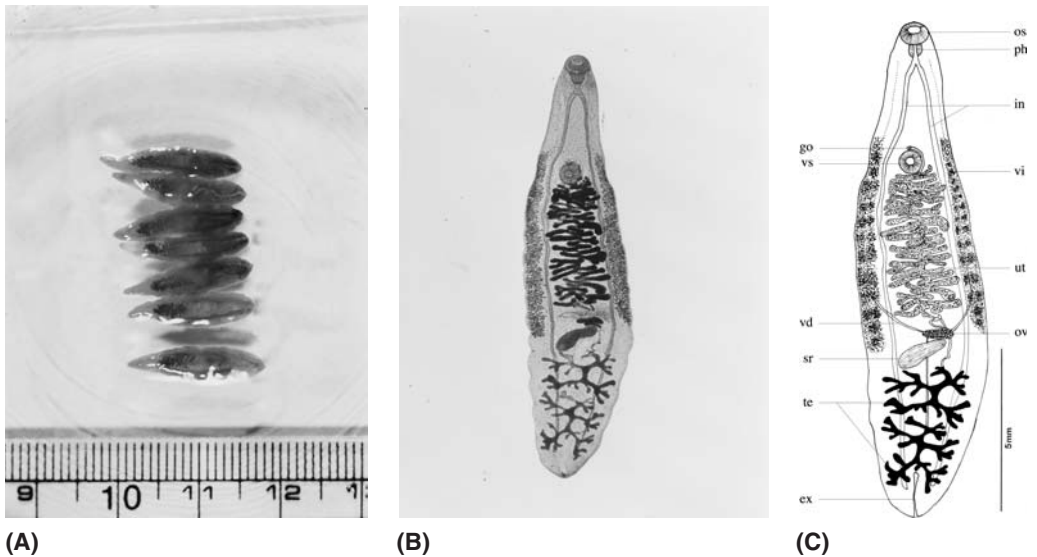
### II. LIFE HISTORY AND MORPHOLOGICAL CHARACTERISTICS

*Clonorchis sinensis* has the characteristic life history of digenetic trematodes. It multiplies by both polygametic asexual division during its larval stages in the snail host and by sexual reproduction by formation of eggs from adult worms in definitive hosts.

#### A. Adult

One to two hours after ingestion of a live metacercaria, the larva excysts out of the wall in the duodenum of infected humans. The excysted young worm migrates against the bile flow through the biliary tree within 4–7 hours and reaches the intrahepatic bile duct around 24 hours after ingestion (4). It takes 4 weeks for a metacercaria to mature and be able to produce eggs after infection to humans (2).

An adult worm looks like a small slender leaf, 10–20 mm long, 2–4 mm wide, and 1 mm thick. It tends to be a little bigger according to age and size of its host. The color of the adult worm,



**FIGURE 1** Morphology of an adult *C. sinensis*: (A) Gross view of unstained mature worms from a human; (B) light microscopic view of an acetocarmine-stained worm; (C) drawing of a worm indicating the organs. os, oral sucker; ph, pharynx; in, intestine; go, genital opening; vs, ventral sucker; vi, vitelline gland; ut, uterus; vd, vitelline duct; ov, ovary; sr, seminal receptacle; te, testes; ex, excretory bladder.

especially the anterior half of the worm, is pinkish due to the presence of fresh eggs in the uterus. The aged worms become grossly dark because of pigmentation with lipofuscin (5). An adult worm is equipped with two suckers, oral and ventral, two intestinal ceca, two branched testes, oval ovary, oval seminal receptacle, vitelline follicles, and coiled uterine loops (Fig. 1). The testes produce spermatozoa that are used for either self-fertilization or cross-fertilization. The seminal receptacle is full of spermatozoa, and the spermatozoa fertilize the mature oval cell when it is released from the ovary into the oviduct. The fertilized oval cell is then supplemented with 5–7 yolk cells and surrounded by the shell in the ootype. The shelled egg moves into the uterus, where the egg matures (1).

## B. Eggs and Larvae in the Snail Host

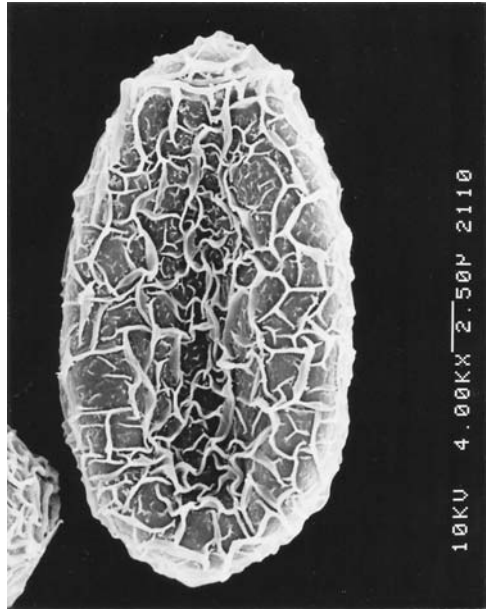
The egg is operculated and measures  $26\text{--}30 \times 14\text{--}15 \mu\text{m}$ . Its shell surface is wrinkled like that of a muskmelon. In the egg, a mature miracidium is enclosed (Fig. 2). After the egg is passed from the human host and flows into rivers, the snail intermediate host ingests it. Its first intermediate hosts are in four families: Hydrobidae, Melaniidae, Assimineidae, and Thiaridae. *Parafossarulus manchouricus* (Fig. 3), the most widely spread species, *P. anomalospiralis*, *Alocinma longicornis*, *Bithynia fushsiana*, and *B. misella* are in the family Hydrobidae. *Melanoides tuberculata* and *Semislucospora libertina* are in the family Melaniidae, *Assimineia lutea* is in Assimineidae, and *Thiara granifera* is in the family Thiaridae (3).

In the snail host, the miracidium hatches out in the rectum and penetrates the wall to reach the perirectal tissue. The miracidium is pyriform and measures  $32 \times 17 \mu\text{m}$ . The ciliated membrane covers its surface. A penetrating stylet is on the anterior end with a penetrating gland connected to it. At the posterior end, 8–25 germinal cells grow. After maturation at the perirectal tissue, sporocysts are liberated from the germinal cells (Fig. 4) (2).

The sporocyst is a sac-like larva, which measures  $90 \times 65 \mu\text{m}$ . It contains several germ cells, which will be rediae (Fig. 4). After liberation from the sporocyst, most of the rediae migrate into

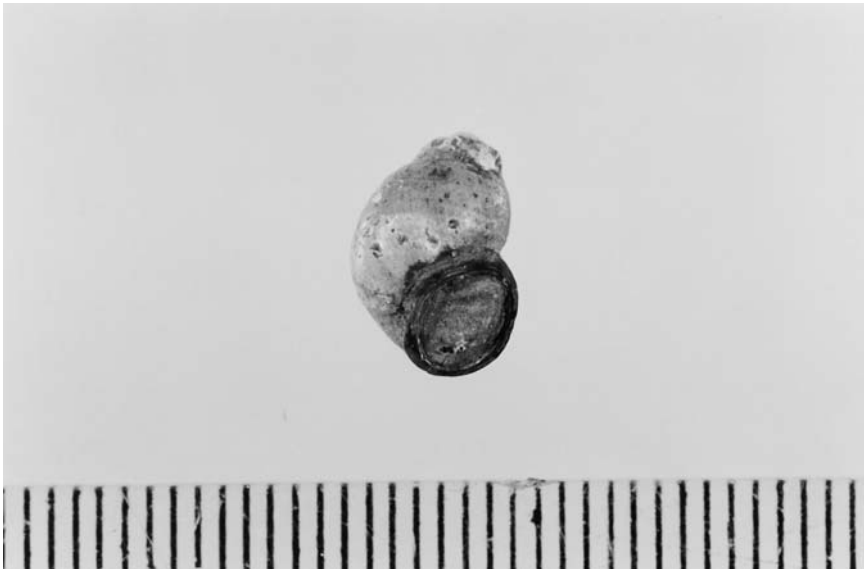


(A)

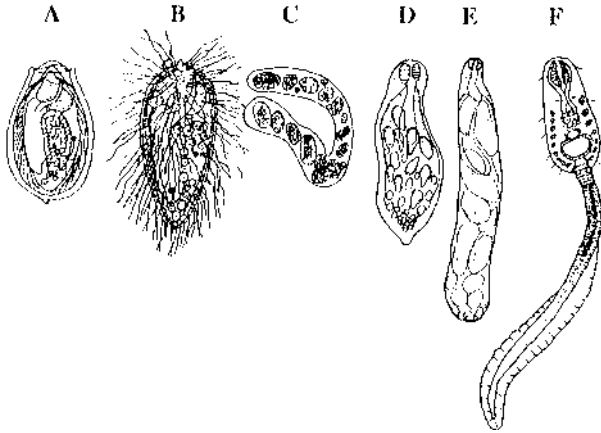


(B)

**FIGURE 2** An egg of *C. sinensis*: (A) light microscopic observation, original magnification  $\times 1500$ ; (B) scanning electron microscopic observation, original magnification  $\times 3500$ . Operculum, shoulder, and surface wrinkling are distinctive.



**FIGURE 3** Gross view of the first intermediate host, *Parafossarulus manchouricus*.



**FIGURE 4** Larval stages of *C. sinensis* in the snail host: (A) mature miracidium in the egg shell; (B) liberated miracidium; (C) mature sporocyst; (D) developing redia; (E) mature redia including cercariae; (F) cercaria. (Adapted from Ref. 2).

the lymph system of the liver, and some of them migrate into other near viscera. Liberation of the redia takes 17 days after ingestion of an egg by the snail (4,6).

A mature redia measures  $1.7 \times 0.13$  mm and has a pharynx, intestine, and about 50 embryos, which will be cercariae. As rediae mature, the cercariae escape from the rediae into the hepatic lymphatic tissue of the snail host. After maturation in the snail's lymphatic tissue, mature cercariae freely escape into the water through the snail's anus. It takes about 100 days from ingestion of the egg by the snail host before the mature cercariae are released (6). By counting the number of germ cells in each larval stage, it is estimated that 3200–10,000 cercariae are produced from one egg. One infected snail is known to release as many as 5840 cercariae daily under optimal conditions (3).

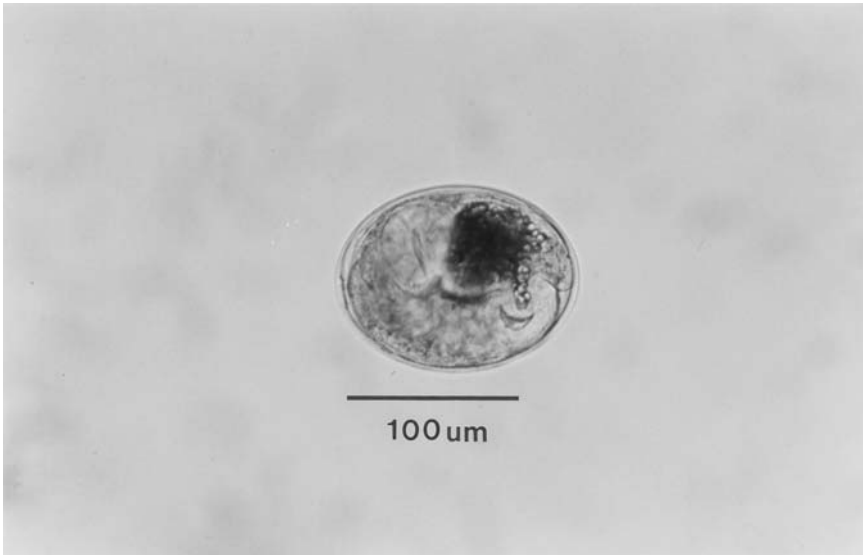
The cercaria is comprised of body and tail; the body 0.216–0.238 mm long and 0.062–0.092 mm wide, and the cylindrical tail 0.374–0.488 mm long and 0.045–0.053 mm in diameter. The body possesses the oral sucker, ventral sucker, pharynx, two eyespots, 7 pairs of penetration glands, and an excretory bladder (Fig. 4). A membranous keel is found on the dorsal and ventral surfaces of the tail. The cercariae swim actively for the first 12 hours but lose their activity between 24 and 72 hours in water unless they penetrate the skin of the second intermediate host (7).

### C. Metacercariae and the Second Intermediate Host

When a cercaria comes into contact with a fish, the body attaches to the skin of the fish and its anterior end begins to penetrate the skin. To facilitate the penetration, a histolytic substance is secreted from its penetration gland and the tail moves vigorously. The penetration process takes 5–10 min (7).

After successful penetration into the subcutaneous tissue or the muscle, the cercarial body secretes cystogenic material, becomes encysted, and turns into a metacercaria. Maturation of a metacercaria usually takes 23 days (7). The metacercaria is oval to round measuring 0.135–0.145 mm by 0.09–0.10 mm. The larva in the cyst wall loses eyespots and makes a big excretory bladder and has oral and ventral suckers of equal size. Its bladder is filled with many dark excretory granules (Fig. 5). The metacercaria is expected to maintain its infectivity for about one year. When the final host, which includes man, ingests the metacercaria, the larva excysts and then migrates into the biliary tree.

The second intermediate host includes predominantly all species of freshwater fish in family Cyprinidae, and several species of many other families (1–3). However, since each species of the



**FIGURE 5** A mature metacercaria of *C. sinensis* recovered from the fish host, original magnification  $\times 300$ .

fish shows different susceptibility, the infection rate and intensity of illness by the metacercariae vary greatly between species. Some species, such as *Pseudorasbora parva* (Fig. 6), *Sarcocheilichthys sinensis*, and *Hemibarbus labeo*, are more susceptible than others to the infection rate and intensity even though they live in the same place. The favorite fish for raw consumption are *Cyprinus carpio* and *Carassius carassius*, which harbor few metacercariae. Experimental challenges have shown that the surface mucus of these fish is hostile to the invading larva. The varying susceptibility



**FIGURE 6** *Pseudorasbora parva*, the most suitable second intermediate host of *C. sinensis*.

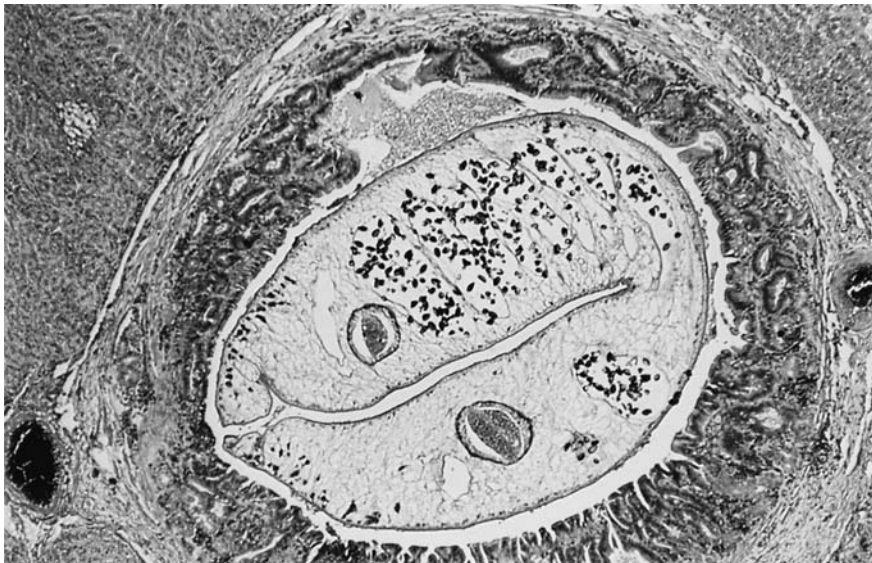
between fish species has been explained as being due to different amounts of linoleic acid in the mucus on the skin surface of the fish (8). The linoleic acid in the mucus was regarded as a cercaricidal substance, which kills most of the invading cercariae.

### III. PATHOGENESIS AND DISEASE

Infection of *C. sinensis* induces pathological changes of the infected bile duct and presents different clinical symptoms. Severity of the pathology and disease depends upon intensity and chronicity of the infection. The host responses vary; they can include inflammation in the bile duct and periductal tissue, hyperplasia of the bile duct mucosa, metaplasia of mucus-secreting cells, neoplastic changes of the mucosal cells, and immunological responses.

Inflammation in the ductal wall and periductal tissue is similar to other parasitic infections, such as infiltration of inflammatory cells of neutrophils, eosinophils, and lymphocytes and edema. Like many other helminthiases, eosinophilia is common in clonorchiasis. In addition to these, proliferation of fibroblasts and fibrosis around the duct and metaplasia of mucus-secreting cells follow the inflammatory reaction. Under normal conditions, mucus-secreting cells are not found in the biliary mucosa. However, in clonorchiasis, these cells do appear in the biliary mucosa. The acidic mucus-secreting cells are mainly located at the base, and neutral mucus-secreting cells are mainly found in the upper part of the mucosa (9,10).

The hyperplastic response of the biliary mucosa is characteristic in clonorchiasis (Fig. 7). The stimulation caused by the worm and desquamation of the epithelial layer induce much more active division of epithelial cells than required (11). The actively dividing cells result in an increase in cell density of 6.3% one week after infection, 9.1% after 2 weeks, 9.4% after 5 weeks, and 11.2% after 15 weeks, but 0.6% in the control. The overproduction of these cells forms a hyperplastic mucosa in a glandular arrangement at the base and in a tubular arrangement at the top (12). The



**FIGURE 7** Histopathological findings of the intrahepatic bile duct in clonorchiasis, showing dilatation of the duct, glandular hyperplasia of the mucosa, infiltration of inflammatory cell in the ductal wall, and periductal fibrosis. Original magnification  $\times 60$ . (From Ref. 13.)

hyperplasia of the mucosa, dilatation of the duct, and periductal fibrosis are progressive during the infection and make the bile duct very thick. These reactions are only partially reversible after treatment (13).

Neoplastic response is also associated with clonorchiasis. Cholangiocarcinoma was found significantly high in prevalence at endemic areas (14–17). In hamsters, cholangiocarcinoma was experimentally induced by simultaneous inoculation of dimethylnitrosamine and *C. sinensis* (18). In this study, *C. sinensis* was confirmed to be a promoter instead of an initiator. The neoplastic change of the biliary epithelial cells can be explained as an outcome of the increased dividing cells. During the cell division, some carcinogens of ineffectively low concentration, endogenous or exogenous, may be incorporated into the dividing nuclei and the cells become neoplastic. The carcinogen associated with clonorchiasis is a subject of further study.

The pathology of clonorchiasis is basically cholangitis. The infected humans usually feel no symptoms because most of them have only mild localized cholangitis. Even though some symptoms are apparent, they are nonspecific, such as general malaise, anorexia, nausea, vomiting, abdominal discomfort, epigastric tenderness, diarrhea, and mild fever. Some patients may complain of dizziness, headache, and back pain. However, when the infection is high in intensity with many worms and becomes chronic, the symptoms are more serious. The presence of worms in the bile duct and fibrotic changes of the duct may obstruct the bile flow, inducing obstructive jaundice and abdominal cramps. As the fibrosis progresses, the hepatic parenchyme may become compressed and shrunken. In chronic infection, the liver may become cirrhotic. In such cases, serious complications of liver cirrhosis may occur (2–4).

Many complications are known to occur with clonorchiasis. Secondary infection by enteric bacteria or pyogenic cholangitis is commonly associated. Formation of calculi in the biliary lumen or in the gall bladder is also frequent. In heavily infected cases, the worm mass may block bile flow in the intrahepatic ducts, a symptom that is often clinically confused with intrahepatic stones. Cholecystitis is also common, and pancreatitis may occur. Cholangiocarcinoma may be the most serious complication, though its occurrence is rare (2–4).

Immunologically, humoral response to this organism is well known. Serum IgG and IgE antibodies to *C. sinensis* antigen were recognized (19,20). Among many proteins, bands of 43, 34, 28–26, 17, and 10–8 kDa have been identified as major antigenic molecules (19–21).

## **IV. EPIDEMIOLOGY**

### **A. Geographical Distribution**

Clonorchiasis is prevalent in East Asia, including East Russia, Korea, China, Taiwan, and northern Vietnam. It was previously prevalent in Japan but has been completely controlled since 1960s. Presently, at least 20 million people are thought to carry this worm (2,3). If there is an average of 5 worms per infected case, it is estimated that 100 million worms exist in the world.

### **B. Intermediate Hosts and Ecology**

Since freshwater fish mediate *C. sinensis*, its endemic foci are scattered along rivers and lakes in the countries mentioned above. These foci vary among the individual villages. Even within the same river, the egg positive rate ranges from 5% to over 60%. Population density of optimum intermediate hosts and habits of consumption of raw freshwater fish determine the endemicity. It requires the first intermediate snail host, mainly *Parafossarulus* spp. and *Bithynia* spp. These snails dwell in rivers or reservoirs where water flows slowly and many water plants live on the mud floor. Because of the environmental requirements, it is prevalent along the middle or lower reaches of a big river but not along upper reaches, where water flows rapidly and the floor is covered by pebbles instead of mud (2–4).

As for the second intermediate host, most freshwater fish are implicated. However, only a few serve as a major source of human infection because not only are they the favorite species for raw consumption, they are also heavily infected by the metacercariae. The number of metacercariae in the muscle of a susceptible fish may be up to the thousands in endemic areas (2,3). Some species of freshwater shrimp were recorded in China as the second intermediate host (3,4), but the role of shrimp is not fully established.

Recently it has been reported that in certain zones in China and Korea, the metacercarial density is rapidly decreasing mainly due to rapid destruction of natural ecology by water pollution. Inappropriately treated industrial waste and pesticides for agriculture are polluting the water of major rivers. The pollution is definitely hazardous for maintaining the life cycle of the trematode by directly killing *C. sinensis* larvae and also by reducing the population of the intermediate hosts. The most significant ramification of water pollution is that local inhabitants avoid consuming fish caught in the polluted rivers, thus reducing the number of new infections due to *C. sinensis*.

### C. Definitive Hosts and Reservoirs

Most mammals other than humans are definitive hosts of *C. sinensis*. In nature, dogs, cats, pigs, rats, rabbits, buffaloes, camels, and yellow weasels are hosts for *C. sinensis*. Hamsters, guinea pigs, mice, gerbils, nutrias, and monkeys are known hosts by experiments (2). In the field, major reservoirs are pigs, dogs, cats, and house rats. Chicks and ducks are questionable as reservoirs (3).

### D. Characteristics of Human Infection Pattern

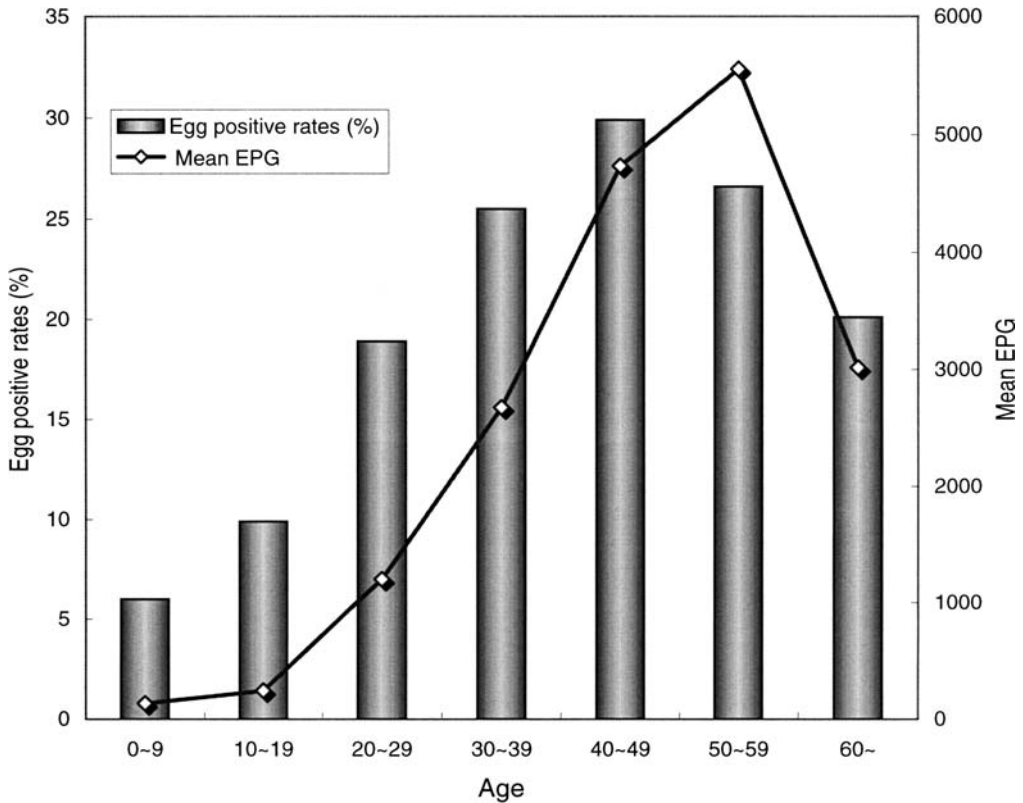
The major cause of human infection is eating raw fish, which is very customary in endemic areas. This habit has lasted over thousands of years. Due to the characteristic mode of infection, the infection is directly related to fishing, and thus more males are infected than females (2–4,22). Also, the infection rate in the elderly is higher than that in young people, and the finding of higher infection rate in older males is regarded as an accumulation effect. The accumulatory infection suggests that host immunity to *C. sinensis* may be of little effect for prevention of reinfection. Since the life span of *C. sinensis* in humans is known to be relatively long, more than 10 years, the worms may accumulate in the biliary tree for life.

Epidemiological data have repeatedly shown that the prevalence rate increases by age until the 50s by the accumulation effect, but it decreases over the age of 60 years (2,3,22). In a study conducted in 1983 in Kimhae, the most endemic area in Korea, the prevalence rate by age was 4.5% under 10 years old, 23.1% between 10 and 20, 54.5% in the 20s, 60.9% in the 30s, 64.5% in the 40s, 76.2% in the 50s, and 66.7% in those over 60 years of age (Fig. 8) (2). The decrease in the prevalence rate among groups over 60 years could be interpreted as a phenomenon caused by more death of infected people than uninfected people over 60 in the endemic areas.

The accumulation effect represents an increase of the infection intensity (or worm burden) in the older age groups as well as an increase in the infection rate. The worm burden is much higher in people within the 50s age group than that in the other age groups. The study at Kimhae demonstrated mean counts of EPG (number of eggs per gram of feces) as 133 under 10 years old, 1000 in the 10–20 year age group, 3963 in the 20s, 6738 in the 30s, 4884 in the 40s, and 7363 in the 50s, but it dropped to 2456 in those over the 60s (Fig. 8) (2,22). The sudden drop of EPG in those over 60 represents the serious impact of clonorchiasis with heavy worm burden in the endemic areas. Clonorchiasis had been one of the most significant health problems in the prepraziquantel era.

The worm burden is important for development of clinical problems. Lightly infected cases usually do not exhibit any symptoms of clonorchiasis, but heavily infected cases suffer from various symptoms and complications. The worm burden is roughly estimated by counting the EPG. The EPG is an additional index for evaluation of the endemicity of clonorchiasis to the egg positive rate in certain areas. As a whole, the EPG counts are more in areas where the prevalence rate is higher (2–4,22). When an intervention measure is introduced to an endemic area, the EPG decreases much more rapidly than does the prevalence.





**FIGURE 8** Egg positive rates and mean counts of EPG of clonorchiasis by age. (From Refs. 2 and 22.)

Clonorchiasis is known to show a pattern of familial aggregation. Even in the same endemic area, all members of some families are infected, but no members of other families are infected (2,4). This familial aggregation pattern is common in infection of foodborne pathogens.

## V. IDENTIFICATION AND DIAGNOSIS

Diagnosis of human clonorchiasis is primarily made by detection of the eggs on fecal examination. The eggs are oval in shape, likened to sesame seeds by their slender opercular end and blunt abopercular end with a terminal knob (Fig. 2). The operculum is well demarcated by the surrounding shoulder. The outer surface of the eggshell is wrinkled, which differentiates it from heterophyid eggs (Fig. 2), but it is hard to differentiate its eggs from those of *Opisthorchis* spp., which also have a wrinkled surface.

In addition to the qualitative fecal examination, quantitative fecal examination is necessary. For the quantitative examination, Stoll's egg counting technique and the Kato-Katz method are widely used by counting the EPG. The EPG is extrapolated to give the EPD (number of eggs per day) by weighing one-day-old feces. The worm number is roughly estimated by dividing the EPD by the mean number of eggs produced by a worm in a day. For example, when the EPG of an infected case is 100 and a one-day-old feces sample weighs 150 g, the EPD is 15,000. Since one *C. sinensis* worm is known to produce 2500–4000 eggs in a day, it is estimated that there are 4–6 worms present in that particular case. The majority of the clonorchiasis cases are lightly infected

with an EPG of 100 or less, but some of them are heavily infected with EPG levels of 30,000, where the worm burden may be about 1200–1800 (22).

It is hard to define diagnostic sensitivity of fecal examination because setting golden standards is impossible in human clonorchiasis. Any kind of fecal examination method shows good diagnostic sensitivity in heavily infected cases, but the sensitivity is generally low in lightly infected cases with an EPG of 100 or lower. In Korea and China, a cellophane-thick smear (Kato's method or Kato-Katz method) is widely used for mass screening in the field, and the formalin-ether sedimentation technique is the standard method of fecal examination in the hospital. One study compared the egg detectability of the examination methods by four repeated examinations of both methods (23). For clonorchiasis, the diagnostic sensitivity of the cellophane smear was 44.7% and that of the sedimentation smear was 67.4%.

Diagnostic measures other than fecal examination include the intradermal test, serology, sonography, and recovery of worms at surgery. The intradermal test is performed by intradermal injection of the antigen, and the immediate skin induration by Arthus reaction is measured. This test is known to be sensitive but not specific. Its specificity is very low, 21.6% in Korea (24). This low specificity is due to cross-reaction with other trematode infections and to long-lasting residual reaction after praziquantel treatment. Therefore, the intradermal test is inappropriate for diagnosis of individual cases but can be used as a mass screening test in the field.

Many serodiagnostic techniques have been developed, but the ELISA is the most common (2,3,25). Recently multiantigen screening has been commonly applied for serodiagnosis of parasitic diseases, including clonorchiasis. Crude extract of the adult *Clonorchis* worms is usually used for the serodiagnostic antigen. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting techniques have been used to analyze the antigen (19,20). Several researches are investigating the use of other antigens to improve the sensitivity and specificity of serodiagnosis, which has become a hot topic of present research on clonorchiasis.

Radiological techniques are also helpful for diagnosis of clonorchiasis. The most popular technique, however, is abdominal sonography, which visualizes dilated and thickened intrahepatic bile ducts due to infection with *C. sinensis* (26,27). However, diagnostic sensitivity and specificity of the sonography are also too low for a definite diagnosis. One field study evaluated the sensitivity as 52.2% and the specificity as 51% (28). Other techniques are cholangiography, computed tomography (CT) scan, and nuclear magnetic resonance (NMR) scan, but these are seldom used for diagnosis.

In cases of complicated heavy infection, biliary surgery is occasionally necessary so that the worms can migrate out through the opened duct or T-tube after surgery. Also, observation of adult worms during or after the surgery confirms the diagnosis.

## VI. PATHOGENICITY

The pathogenicity of clonorchiasis closely depends upon infection intensity, duration of infection, and secondary bacterial infection. A mild infection is usually asymptomatic. The *C. sinensis* worm can irritate the host tissue in three ways: mechanical, chemical, and immunological. Mechanical stimulation is the primary mechanism of destruction of the mucosal epithelial layer (2,3). The worm attaches to the epithelial mucosa by its oral sucker and ingests desquamated epithelial cells and blood. It also moves continuously in the duct. This direct contact of the worms and mechanical stimulation of the epithelial cells may induce inflammation and fibrosis in the bile duct wall.

Mechanical obstruction of the bile flow also occurs when the bile duct lumen is filled with worms. The worm mass may clinically mimic sandy stones in the liver. The mechanical stimulation of the bile duct mucosa and obstruction of the duct and bile flow are the primary points of the pathogenicity.

Chemical stimulation has been suspected but not clearly identified as yet, although some proteases and excretory secretory proteins have been recognized (29). The infected mucosa secretes neutral and acidic mucin by mucin-secreting cell metaplasia (10). The mucin in the bile is suspected of playing a role in the protection of the mucosal tissue, but this has not been clearly determined, and

any other biochemical action of the mucin in the bile is still unknown. Immunological stimulation is well known to induce humoral and cellular responses by several excretory secretory molecules (19–21). However, it is still uncertain whether the immunological responses serve to protect the host or to aggravate the disease. Concomitant infection is usually superimposed by bacterial contamination causing pyogenic cholangitis or abscesses. The infection can spread along the biliary tree to the pancreatic duct and can also raise the rate of complications.

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

There may be some genetic factors of the host that contribute to pathogenicity, but no data are available for clonorchiasis.

## VIII. Control Measures

Control of clonorchiasis is an important health issue in endemic areas. One of the most powerful control measures is health education, which mainly targets prevention of the infection. Since eating raw freshwater fish is the major source of infection, cooking of fish is extremely important for its prevention and is the primary subject of health education. However, its efficacy is quite limited because eating raw fish is a traditional culture in the endemic areas. Some of them prefer eating raw fish, even though they know the risk of clonorchiasis. In the endemic area of Korea, some people eat raw fish and then take praziquantel.

Praziquantel is the drug of choice for treatment of trematode or cestode infections and is also a potent anthelmintic for clonorchiasis (2). Three doses of praziquantel to give a total dose of 75 mg/kg is recommended for treatment of clonorchiasis, with 85.7% cure rate and 99.5% egg reduction rate (30). A single dose of 40 mg/kg praziquantel has been applied for mass control of clonorchiasis in Korea with an excellent egg reduction rate of 95.5%, although the cure rate was as low as 25.0% (30). The excellent effect on egg reduction by a 40 mg/kg single dose of praziquantel was regarded effective in interrupting the transmission cycle of the worm, and thus the regimen has been applied for mass control in the field in Korea (31). Moreover, a reduced dose of praziquantel is less expensive and a single dose is more convenient. However, mass control with a 40 mg/kg single dose of praziquantel has now been replaced with regular dose medication because curing the individual case has become more important than mass control.

Praziquantel has some adverse effects; the neurological symptoms include headache, dizziness, and sleepiness; the gastrointestinal symptoms include diarrhea, nausea, vomiting, abdominal pain or cramp; the allergic symptoms include rash and itching (30,31). These symptoms are dose dependent, and the abdominal cramp is dependent on worm burden. However, all these symptoms are transient and need no special care for relief.

A trial of clonorchiasis control in the field by repeated praziquantel treatment of egg positive cases every 6 months demonstrated that the positive egg rate in the village was 22.7% at the beginning but decreased to 6.3% after seven medication applications for 3½ years (27). The data suggest that control of clonorchiasis by praziquantel treatment requires much longer than expected. Such low control efficiency is partly because of treatment failure and partly reinfection after cure.

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## *Opisthorchis viverrini*

Yupaporn Wattanagoon and Swangjai Pungpak

Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

### I. BACKGROUND

*Opisthorchis viverrini* is one of a number of flukes that infect the human biliary system. It is closely related to *O. felineus* and *Clonorchis sinensis*. This parasite was first found at autopsies of two prisoners from northern Thailand in 1912 (1). It was thought to be *O. felineus* until an epidemiological survey was conducted in Thailand by Sadun, who recognized the difference between *O. felineus* and the liver fluke found in Thailand and reported this in 1955 (2). *O. viverrini* infects millions of people in the northeastern and northern parts of Thailand (3), and most of the clinical studies of this disease were performed during the 1980s and 1990s.

### II. CHARACTERISTICS

#### A. The Adult Worm

The adult worm resides in the biliary tracts of infected humans and animals, e.g., dogs, cats, and other fish-eating mammals. It is flat and lancet-shaped,  $7-12 \times 2-3$  mm in size, and has two suckers: an oral sucker at the anterior end and a ventral sucker at the anterior one third. The coiled uterus fills the midportion of the body between the ventral sucker and the lobed ovary. There are two lobed testes, which lie behind the ovary in the posterior portion. The vitelline glands are situated along the lateral margin in the midportion of the fluke (Fig. 1).

#### B. The Egg

The egg of *O. viverrini* is oval in shape,  $28 \times 15$   $\mu\text{m}$  in size, and yellowish-brown in color. It has an operculum that seats on a shoulder and a tubercle-like knob at the abopercular end. It contains a miracidium when laid (Fig. 2).

#### C. The Metacercaria

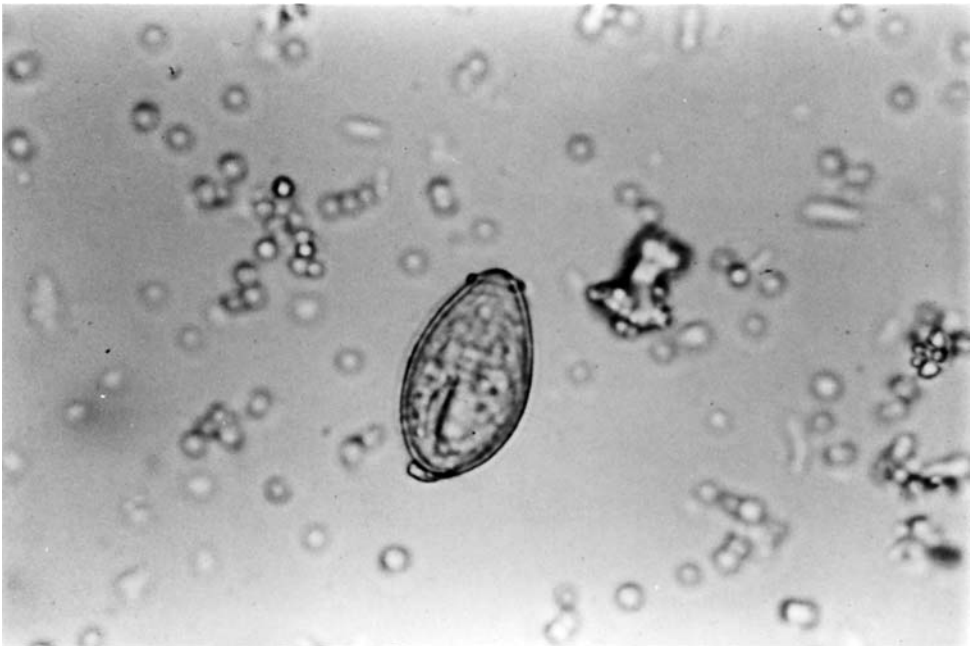
The *O. viverrini* metacercaria, the infective stage in cyprinoid fish, is oval in shape,  $18.5 \times 17.5$   $\mu\text{m}$  in size, and yellowish-brown in color with a smooth cyst wall. The oral and ventral suckers are clearly visible within the metacercaria, which is actively moving inside the cyst. A large black, ovoid-shaped bladder occupies one third of the cyst (Fig. 3).

#### D. Life Cycle

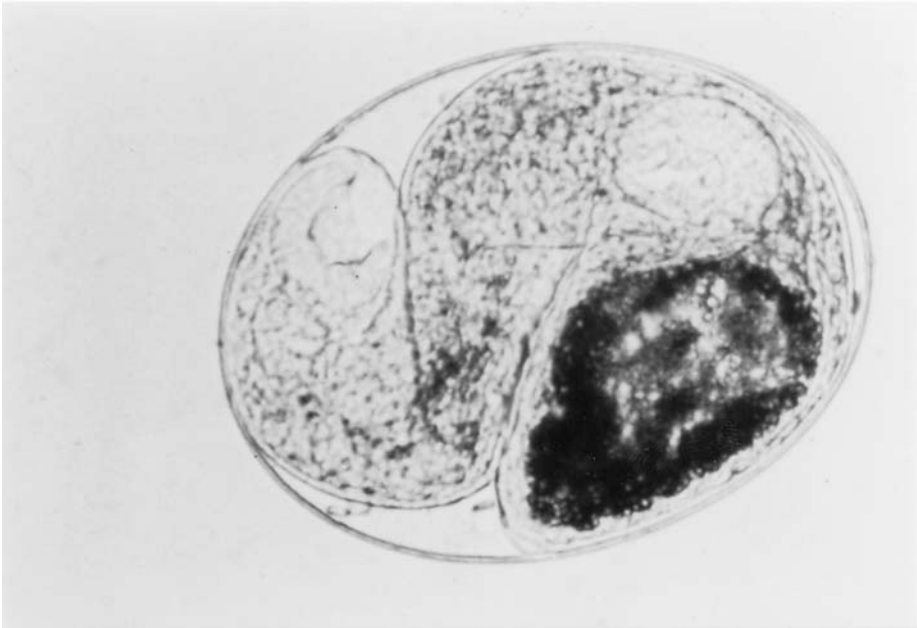
Embryonated ova are excreted in the feces of an infected patient. Once they reach the water, they are ingested by the freshwater snail (*Bithynia* spp.), which is the first intermediate host; they then develop into the cercarial stage within 2–3 months. The cercariae emerge from the snail and penetrate the cyprinoid fish (Fig. 4), the second intermediate host, where they become encysted in the



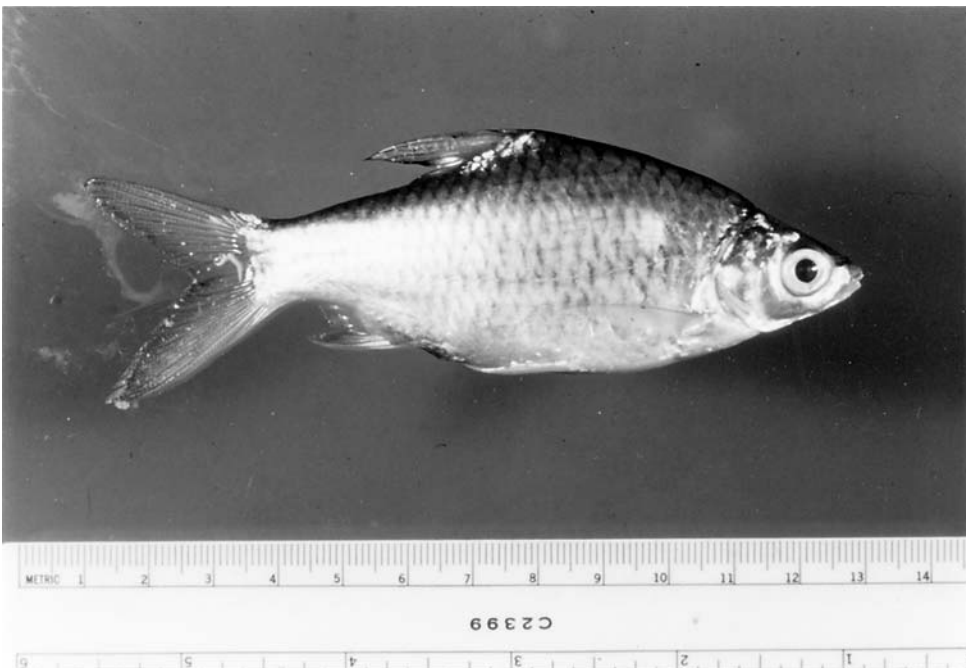
**FIGURE 1** *Opisthorchis viverrini* adult worm.



**FIGURE 2** *Opisthorchis viverrini* egg.



**FIGURE 3** *Opisthorchis viverrini* metacercariae.



**FIGURE 4** *Cyprinoid* spp. fish. (Courtesy of J. Waikagul, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand.)

flesh under the scales or skin. Cysts further develop into metacercariae, which infect humans who consume the raw fish preparation. The metacercariae excyst in the duodenum and migrate through the common bile duct to the distal bile passages in the liver, where they mature in 3–4 weeks (4). The life span of adult worms may be as long as 20–25 years. Cats, dogs, and other fish-eating mammals are reservoirs for human infection.

### III. DISEASES

The disease caused by *O. viverrini* varies in severity. It ranges from asymptomatic to mildly symptomatic to the most severe form, causing obstruction of bile ducts, ascending cholangitis, and cholangiocarcinoma. There has been no definite association between clinical signs and intensity of infection (5), but the severe form is believed to be associated with longstanding infection.

#### A. Clinical Manifestation

Symptoms of infection include dull pain and discomfort in the right hypochondrium, sometimes spreading to the epigastrium. A peculiar hot sensation is commonly felt in the abdominal region; lassitude, anorexia, and flatulence are common complaints. Patients may present with low-grade fever, hepatomegaly, and an enlarged gall bladder. The severely ill patients present with obstructive jaundice with signs of cholangitis and/or evidence of cholangiocarcinoma (5–8).

#### B. Diagnosis

##### 1. Parasitological Examination

Diagnosis of opisthorchiasis is based on finding ova of the parasite in feces from infected patients. The diagnosis is more sensitive if a concentration technique is used instead of a simple smear. *O. viverrini* ova may also be found in duodenal fluids. Patients present with obstructive jaundice. *O. viverrini* ova may not be found in feces but can be recovered from bile contents after surgery. The ova can also be confused with heterophyid intestinal fluke ova.

##### 2. Serological Diagnosis

Many serological tests have been studied, but the diagnostic value is not good, especially in endemic areas where the reinfection rate may be high due to persistent presence of antibody after effective chemotherapy. Cross-reactivity can occur with various other parasitic infections (9–11). However, a recent study using the monoclonal antibody–based enzyme-linked immunosorbent assay (Mab-ELISA) to detect coproantigen in the feces of infected patients produced good results. The assay detects current infection, and therefore could be used for a large epidemiological survey (12).

##### 3. Radiological Examination

Ultrasonography of liver and gallbladder of opisthorchiasis patients reveals changes varying from normal to hepatomegaly to an enlarged dilated gallbladder with sludge with or without irregular bladder wall and enhanced portal vein radical echoes. The abnormalities are significantly associated with intensity of infection, and gallbladder abnormalities are reversible after elimination of infection (8,13–15).

#### C. Treatment

Treatment consists of the following:

Praziquantel at 40 mg/kg body weight, given as a single dose at bedtime, or 25 mg/kg, given three times in one day, is an effective treatment for opisthorchiasis with a cure rate of 90–97% (16–18).



Albendazole at 400 mg, given twice daily for 7 days, has a low cure rate of 63% but can reduce the number of eggs in 92% of patients (19).

Mebendazole at 30 mg/kg body weight/day, given for 3–4 weeks, has a cure rate of 94% (20).

Cholangiocarcinoma is usually detected very late due to the slow-growing nature of this tumor. Patients with resectable mass have a better prognosis with a more than 40% 2-year survival rate. However, palliative surgery for patients with unresectable tumor does not prolong survival, and the median survival time is 8 months (21).

#### IV. EPIDEMIOLOGY

*O. viverrini* is endemic in Thailand, Laos, Cambodia, and Vietnam. The northeastern and northern parts of Thailand have a higher incidence than other parts of the country due to the habit of consuming raw freshwater fish preparation of “Koi Pla” or “Pla Som.”

Epidemiological surveys reported 7 million infected patients in the northeast of Thailand in 1984. Despite effective chemotherapy and health education, a recent survey demonstrated an infection rate of 21.5% of the Thai population, mainly in northeast and northern Thailand (3). Sporadic cases have been reported from other countries due to migration of Thai laborers into the area.

The infection can start very early in life, in infants of less than one year, if they are fed raw freshwater fish preparation. Disease symptoms manifest in people in endemic areas usually in the fourth decade of life.

#### V. ISOLATION AND IDENTIFICATION

*O. viverrini* ova can be detected in feces of infected patients by a simple smear or concentration technique. It may be difficult to differentiate *O. viverrini* ova from those of other small intestinal flukes, especially those of heterophyid species, by light microscopy.

Adult worms are usually expelled from the biliary tract into the intestine and then excreted in the feces after effective chemotherapy. The morphology is as described earlier. It differs from *C. sinensis*, which has branched testes, and is similar to *O. felineus* except for the size.

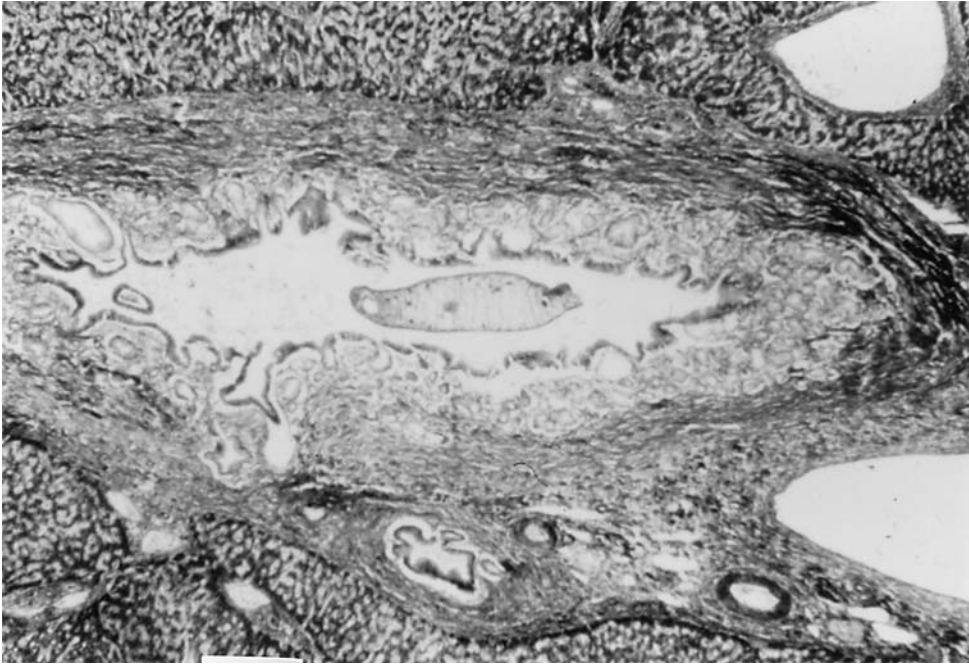
The ova may also be detected in the duodenal fluid obtained from aspiration.

#### VI. PATHOGENICITY

The pathological changes caused by *O. viverrini* infection result from mechanical irritation by the worm and its metabolites, the host immune response, and secondary bacterial infections. The pathology is related to intensity and duration of infection. The lesions are confined to the biliary system, where the flukes reside. Histological examination has revealed epithelial cell hyperplasia, proliferation and desquamation, glandular proliferation, and periductal infiltration with eosinophils and round cells. The intrahepatic bile ducts are dilated, with distal clubbing or formation of cysts, which in the late stage are enlarged (Fig. 5) (22). The gall bladder may be enlarged and contain white bile with hypertrophic glandular epithelial linings. Gallstones may be present in some cases with parasite debris and ova found in the center of the stones.

#### VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

So far there is no evidence suggesting that genetic factors determine the disease virulence in opisthorchiasis. Cholangiocarcinoma is found as a late consequence of the disease in several species, including *O. viverrini*, *C. sinensis*, and *O. felineus*. Host factors may also play a role because the incidence



**FIGURE 5** Pathology of liver and bile ducts in opisthorchiasis. (Courtesy of M. Riganti, Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Thailand.)

is higher in males than females. Other factors may be more important, i.e., nutritional status, alcohol consumption, or other underlying hepatic diseases.

## VIII. CONTROL MEASURES

Consumption of raw freshwater fish preparation should be discouraged. Improvement of sanitation and mass treatment of an infected population in an endemic area with praziquantel has effectively reduced the infection rate in northeastern Thailand. However, continuous health education should be implemented due to the observation of reinfection in previously treated individuals.

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# 37

## *Paragonimus* Species

**Wanchai Maleewong**

*Khon Kaen University, Khon Kaen, Thailand*

### I. BACKGROUND

The lung fluke *Paragonimus*, one of the most harmful parasites, causes paragonimiasis in humans and animals, mainly in Asia, some parts of West Africa, and South and Central America. It is estimated that the number of people infected is over 20 million (1). Up to the present time, at least 40 species have been reported throughout the world (2), and approximately 15 species of *Paragonimus* are known to infect humans. From the medical point of view, while *P. heterotremus* is the etiological agent of human paragonimiasis in China, Laos, and Thailand (3–5), *P. westermani* is the most common species elsewhere in the world. The species of *Paragonimus* reported to infect humans and their distribution are listed in [Table 1](#).

### II. CHARACTERISTICS

All species of *Paragonimus* require two intermediate hosts, molluscs and crustaceans, for the completion of their life cycle. Adult flukes are found in the lungs of the final hosts, humans and mammals, especially members of the families Felidae and Canidae, in whom they lay their eggs. The eggs are then either expectorated with the sputum or swallowed and passed in the feces. The immature eggs develop into miracidia in the water. Hatched miracidia swim freely in the water and then invade the freshwater snails. In the case of crustaceans, such as freshwater crabs ([Fig. 1](#)), crayfish, and, on rare occasions, shrimp, they probably acquire the infection by consuming free-living cercaria or eating infected snails containing the fully developed cercaria (6–8). Penetration by the cercaria through the skin takes place at the joints of the appendages or at any point on the soft exoskeleton after ecdysis (9). The cercaria then develops into metacercaria, the infective stage. When the crustacean host is eaten raw or undercooked by the final host, the metacercaria excysts in the small intestine and penetrates through the intestinal wall into the abdominal cavity, prior to migration through the subperitoneal tissues, the muscle, the liver, and the diaphragm. It finally enters the lung, where maturation occurs (10). The life cycle of the fluke is shown in [Fig. 2](#). In addition, the final host can also acquire an infection by eating the raw flesh of some paratenic hosts, such as wild boars, pigs, rodents, and small mammals (11–13). The time of development, maturity, and the kind of hosts are different, according to the species of *Paragonimus*. Sometimes the wandering larvae deviate from the normal pathway to an ectopic site.

The living worm, reddish-brown when removed from the host, has an indefinite shape owing to its constant expansion and contraction ([Fig. 3](#)), but the preserved parasite is a fleshy, plump, oval fluke, resembling a coffee bean. The integument is covered with spines. The oral sucker is located at the anterior end, and the ventral sucker is situated in the midline just anterior to the equatorial plane of the body. The digestive tract consists of a globose pharynx, a short esophagus, and two ceca that extend in a zigzag manner to the caudal portion of the body. The excretory bladder is a

**Table 1** Species of *Paragonimus* Known to Infect Humans

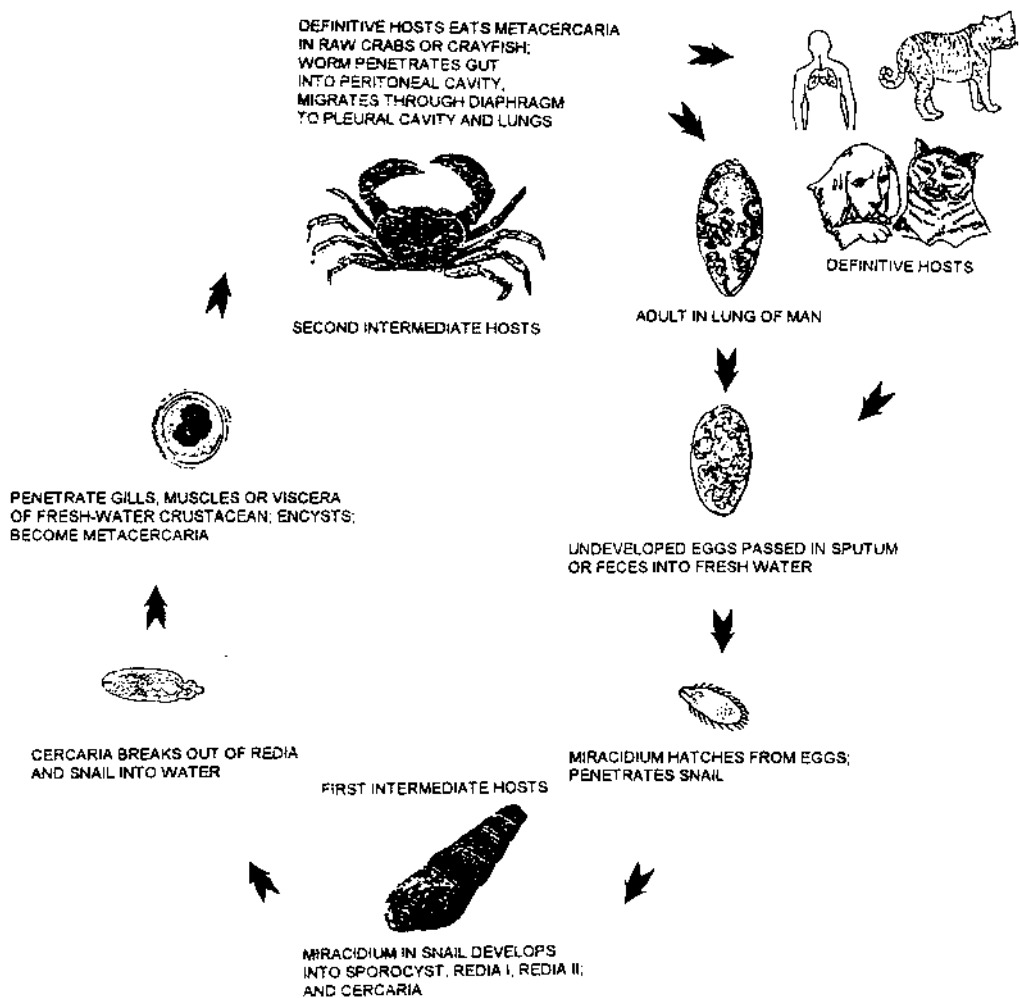
<i>Paragonimus</i> species	Geographical distribution
<i>P. westermani</i> (Kerbert, 1878)	India, Ceylon, Thailand, China, Taiwan, Japan, etc.
<i>P. ringeri</i> (Cobbold, 1880 <sup>a</sup> )	Taiwan
<i>P. pulmonis</i> (Nakahama, 1883 <sup>a</sup> )	Japan
<i>P. pulmonalis</i> (Baelz, 1880 <sup>a</sup> )	Japan, Korea, Taiwan
<i>P. kellicotti</i> (Ward, 1908)	USA, Canada
<i>P. skrjabini</i> (Chen, 1959)	China
<i>P. miyazakii</i> (Kamo, Nishida, Hatsushika, and Tomimura, 1961)	Japan
<i>P. heterotremus</i> (Chen and Hsia, 1964)	China, Thailand, Laos
<i>P. tuanshanensis</i> (Chung, Ho, Cheng, Tsao, 1964 <sup>b</sup> )	China
<i>P. africanus</i> (Voelker and Vogel, 1964)	Cameroon, Nigeria
<i>P. uterobilateralis</i> (Voelker and Vogel, 1965)	Cameroon, Liberia, Nigeria, Guinea
<i>P. mexicanus</i> (Miyazaki and Ishii, 1968)	Mexico
<i>P. philippinensis</i> (Ito, Yokogawa, Araki and Kobayashi, 1978 <sup>a</sup> )	Philippines
<i>P. ecuadoriensis</i> (Voelker and Arzube, 1979)	Ecuador
<i>P. heitungensis</i> (Chung, Hsu, Ho, Kao, Lan and Chiu, 1977)	China

<sup>a</sup> Appears to be identical to *P. westermani*.

<sup>b</sup> Appears to be identical to *P. heterotremus*.

long, slightly coiled pouch, which is located from the posterior extremity to the level of the pharynx (10). The testes are situated side by side in the posterior third of the body. The vasa efferentia join together in a common vas deferens, which forms the seminal vesicle. The worm has no cirrus sac, but the terminal part of the seminal vesicle develops into a prostatic portion and an ejaculatory duct, which empties through a common opening with the metraterm into the genital atrium. The genital pore is located close to the posterior margin of the ventral sucker. The ovary lies to the left or the right of the midline, somewhat posterior to the ventral sucker. On the way to the ootype, the oviduct is joined by the small seminal receptacle and Laurer's canal. Mehlis' gland is also present. The vitellaria consists of numerous branch follicles in the lateral fields and extensively overlies the ceca

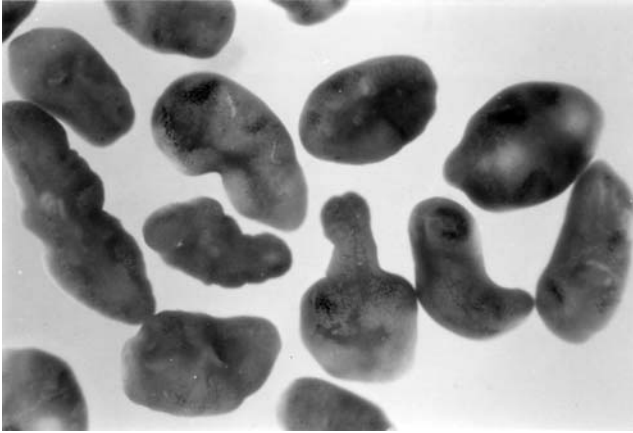
**FIGURE 1** Mountain stream crabs (*Potamon* spp.), the second intermediate host of *Paragonimus heterotremus* in Thailand.



**FIGURE 2** Life cycle of *Paragonimus* spp.

from the region of the pharynx to the caudal extremity. The uterus forms a series of knotted coils on the opposite side and is slightly anterior to the plane of the ovary. It opens externally through the metraterm and joins the ejaculatory duct to enter the genital atrium (14,15).

For species differentiation of the genus *Paragonimus*, many criteria are used, but the most common and important method used is to examine the morphology of the adults and metacercaria. The following items are important criteria for differentiating adult worms: shape of the whole body, arrangement of the cuticular spines, shape and size of the ovary and testes, comparison of the transverse diameter between oral and ventral suckers, shape and size of the uterine eggs, and features of the egg shell (10). For the metacercaria, significant differentiating criteria include the number of cyst walls, shape and size of the inner cyst (the outer cyst is unsuitable because of its changing ability and fragility), thickness of the inner cyst wall, characteristics of the excysted larva such as body size, oral and ventral sucker ratio, intestines, excretory bladder, oral stylet, and the existence of pinkish granules within the larval body. Species differentiation is usually more easily performed using features of sufficiently flattened adult worms and living metacercaria. The characteristics of



**FIGURE 3** Living adult of *Paragonimus heterotremus*.

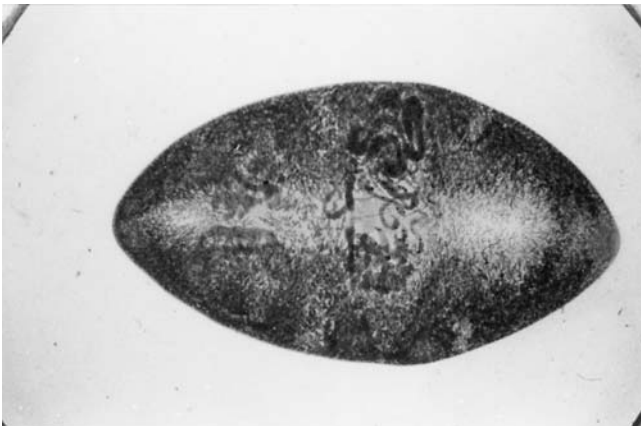
two species of *Paragonimus* that have been found to be important in human infection are described below.

**A. *P. westermani***

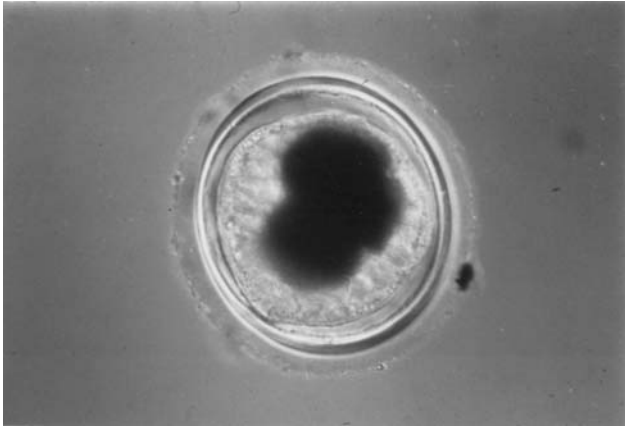
The adult worms are 7.5–12 mm in length and 4–6 mm in width. The body is covered with single-spaced cuticular spines. The size of oral sucker is about subequal to the ventral sucker. The ovary divides into six lobes. The testes are about the same size as the ovary. The metacercaria cyst is spherical, with an average size of about 430–500  $\mu\text{m}$ . The larval body contracts and envelopes within two layers of the cyst wall. The inner cyst wall is thick. The average size of the operculated egg is  $48 \times 90 \mu\text{m}$ . The egg shell is uneven in thickness. Some eggs show the thickening of an egg shell at the posterior pole.

**B. *P. heterotremus***

The *P. heterotremus* adult worm (Fig. 4) is characterized by having a very large oral sucker, almost twice as large as the ventral sucker, singly spaced cuticular spines, profusely branched ovary, and



**FIGURE 4** *Paragonimus heterotremus* adult.



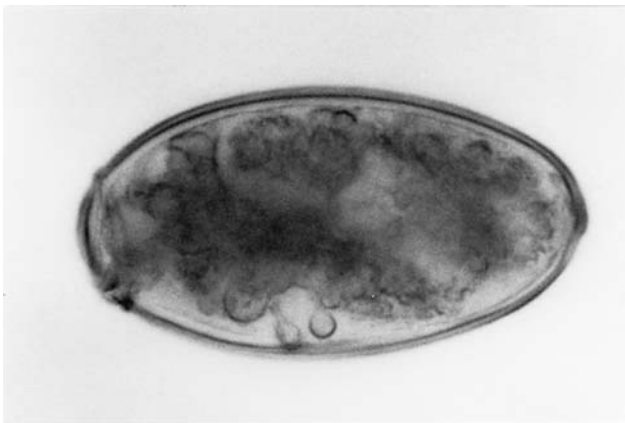
**FIGURE 5** Living metecercaria of *Paragonimus heterotremus*.

a small uterine mass (16). The metacercaria is oval in shape and provided with a thin outer cyst wall and a thicker inner cyst wall (Fig. 5). The average size is  $210 \times 240 \mu\text{m}$  in diameter. The cyst wall is thickened at both poles. The eggs vary in size and features, but are relatively even in thickness of the egg shells (Fig. 6). The average size is  $47 \times 86 \mu\text{m}$ .

### III. DISEASES

The symptoms and signs associated with paragonimiasis make it possible to characterize the infection into acute and chronic stages. The acute stage of human paragonimiasis is usually asymptomatic. Sometimes, however, the migration of the immature worms causes an allergic reaction manifested by an allergic skin reaction, such as rashes, urticaria, and by diarrhea and abdominal pain caused by the penetration of the worms (1,2). Fever, coughing, chest pain, and dyspnea might be presented in acute lung symptoms (2). The chronic stage is divided into pulmonary and extrapulmonary types.

In extrapulmonary paragonimiasis, the clinical features vary from asymptomatic to severe forms. The most remarkable clinical symptom of an infected individual is a chronic cough with a



**FIGURE 6** Egg of *Paragonimus heterotremus*.



gelatinous, rusty brown, pneumonia-like, blood-streaked sputum (17–19). Hemoptysis is usually induced by heavy work. Pneumothorax, empyema is caused by secondary bacterial infection, and pleural effusion might be present. When only a chronic cough is present, illness may be misinterpreted as being either chronic bronchitis, bronchiectasis, or bronchial asthma. Pulmonary paragonimiasis is frequently confused with pulmonary tuberculosis (1,20).

In extrapulmonary paragonimiasis, the symptoms of extrapulmonary lesions vary depending on the location of the fluke. Cerebral paragonimiasis is the most serious complication (20–22). Worm migration through the brain can cause hemorrhage, edema, or meningitis. A severe headache, confusion, epilepsy, seizure, hemiparesis, hypoesthesia, blurred vision, diplopia, homonymous hemianopsia and meningismus may be present. Death is common among patients with intracranial hypertension (1).

Symptoms and signs of abdominal paragonimiasis include abdominal masses associated with abdominal pain, tenderness, diarrhea, nausea, and vomiting. Abscess formation can occur in the intestinal wall, mesentery, omentum, liver, and spleen. In paragonimiasis caused by *P. heterotremus* and *P. skrjabini*, subcutaneous swelling occurs more often than in *P. westermani* infection (1,4).

#### IV. EPIDEMIOLOGY

The distribution of human paragonimiasis is highly focal in three endemic areas: Asia, including China, Japan, Korea, Laos, Philippines, Taiwan, and Thailand; Africa, including Cameroon, Gambia, and Nigeria; and South and Central America, including Ecuador, Peru, Costa Rica, and Columbia. A very crude estimate of the global number of infections is in the order of 20 million (1). These areas can be identified as those in which the inhabitants eat raw crabs or crayfish, and control programs could therefore be targeted towards educating the people.

In China, at least four species are of medical importance: *P. westermani*, *P. skrjabini*, *P. heterotremus* (synonym: *P. tuanshanensis*), and *P. hueitungensis*. Human paragonimiasis has been reported from a total of 21 provinces. The popular dishes are drunken crab (immersion of live crab in wine), raw crab sauce, crab jam, raw crayfish, and crayfish curd. At the present time, the prevalence has decreased due to control measures such as health education and chemotherapy (1).

In Korea, approximately 6 million people in four provinces are currently at risk of paragonimiasis due to *P. westermani* and *P. iloksuenensis* (1). Freshwater crabs soaked in soybean sauce (kejang) has been a favorite dish. Survey data in the 1990s show that the prevalence of human paragonimiasis has lowered to about one 1/100 of that in the early 1970s. This may be due to ecological damage and the change in attitude of the people and/or the long-term effect of mass chemotherapy (23).

In Japan, the raw juice from crayfish or crabs is used as an antipyretic for measles, urticaria, and diarrhea (2). Paragonimiasis has been reported in many parts of the country. Between 1954 and 1968, the average positive skin test rate was 3.5%. However, with treatment and control measures according to the Parasitosis Prevention Laws of the Ministry of Health, the prevalence has decreased since 1981 (1). In 1991, about 1000 people were estimated to be infected, or less than 1 per 100,000 population.

In Thailand, *Kung Plah*, *Kung Ten* (raw crayfish salad), and *Nam Prik Poo* (crab sauce) are popular dishes. Nowadays, human paragonimiasis has been reported in a total, of 10 provinces, and there are many important evidences to support that *P. heterotremus* is the major etiological agent of human paragonimiasis (19). The prevalence and intensity of infection among inhabitants are slightly lower among males than among females (24). However, when combining both sexes, the prevalence is highest in the 25- to 29-year-old group.

In the Philippines, *Kinagang*, which is a popular dish, consists of insufficiently cooked freshwater mountain crabs. Human diseases are known to be caused by two species: *P. westermani* and *P. philippinensis* (25).

In Ecuador, approximately one fifth of the total population is at risk. In Peru, paragonimiasis is also suspected to occur in the Amazon region, but no epidemiological study has been undertaken (1).

In Cameroon, both *P. africanus* and *P. uterobilateralis* appear to be present. Prevalences between 5 and 10% have been reported (1). Persons 10–19 years of age and women are most frequently affected.

## V. ISOLATION AND IDENTIFICATION

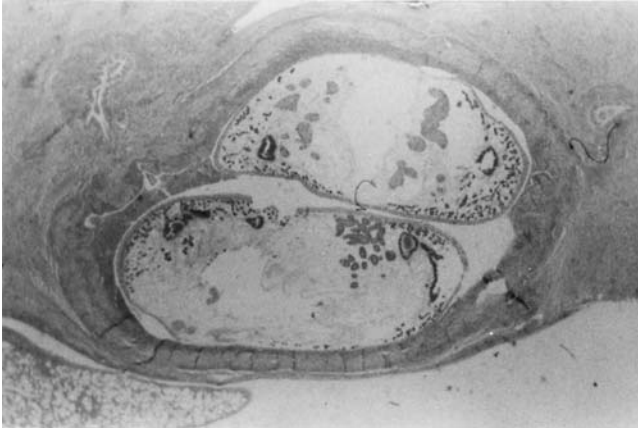
Diagnosis of human paragonimiasis is based on clinical features as well as laboratory tests. The current method is based on the demonstration of *Paragonimus* eggs in the feces and/or sputa by microscopic examination (20). The eggs of *Paragonimus* can be found in blood-stained sputum of patients with paragonimiasis by the direct smear method. However, when there are only a few eggs, the sample should be concentrated and the pellet examined with 1–2% of sodium hydroxide. In a light infection, however, the sputum is not always produced, and in those patients who have a habit of swallowing it, the eggs can be found only by the examination of the stools. X-ray examination of the lungs has demonstrated that 10–20% samples are normal. Those with positive changes show a thin-walled single cyst lesion, patchy infiltration, cavitation, fibrosis, pleural thickening, and multiple cystic appearance. However, the shadow appeared similar to pulmonary tuberculosis. Nowadays, two approaches comprising a genetic probe and immunological test have been developed, which claims to be as good as or even better than microscopic examination. The serological tests are also supplementary methods in the diagnosis of the extrapulmonary type or during the incubation period of the disease, when the worm is still sexually immature. Several serological tests have been applied for immunodiagnosis of paragonimiasis, such as intradermal test (26,27) complement fixation test (28,29), enzyme-linked immunosorbent assay (ELISA) (29–39), and immunoblotting technique (40–46).

For the immunodiagnosis of *P. westermani*, the immunoblotting technique, using an approximately 8 kDa protein from a complex *P. westermani* chaffee antigen, is highly sensitive (96%) and specific (99%) (40). The 94, 76, and 66 kDa tegument proteins of the *P. westermani* adult are also specific for *P. westermani* infection (47). There is no significant difference between the ELISA values of the same sera reacted against crude antigens prepared from *P. westermani*, *P. miyazakii*, *P. heterotremus*, and *P. siamensis* (32). In addition, the specific local IgE and IgG antibodies, found in the lung and the pleural effusions from patients with paragonimiasis, are more suitable than serum for immunodiagnosis (35). On the other hand, the ELISA inhibition test, using a *P. westermani* specific monoclonal antibody, shows a higher specificity in comparison with the micro-ELISA (36). The diagnostic potential of antigen detection assay by dot ELISA has also been developed, and it has been suggested that it might constitute a sensitive and specific test for active infection (48). Recently, ELISA, using fluke cysteine proteinase as an antigen, has been used to improve the specificity (49).

In the diagnosis of *P. heterotremus* infection, the antigenic polypeptide band at approximately 31.5 kDa is eligible for diagnostic use by the immunoblotting technique (41–43). In addition, the monoclonal antibody–purified metabolic antigens give a high sensitivity and specificity for detecting specific antibodies in the indirect ELISA (37). Another model is the development of a sensitive and specific DNA probe, namely pPH-13, and is used in a DNA hybridization assay for the detection of the parasite DNA in feces. The DNA hybridization might be used as the taxonomic aid in parasite identification for diagnosis and epidemiological surveys (39).

## VI. PATHOGENICITY

From the time of metacercarial ingestion until the settling down of the larvae, there is no indication of any significant pathological process in the infected individual. The pathogenesis results from the migration caused by the flukes, and toxic substances might be produced by the parasites. Worms in the lung causes hemorrhage, inflammatory reaction with leukocytic infiltration, and necrosis of



**FIGURE 7** *Paragonimus heterotremus*. Section of lung showing a worm surrounded by leukocytic infiltration and fibrous encapsulation.

lung parenchyma that gradually proceeds to the development of fibrotic encapsulation (Fig. 7), except for the opening to the respiratory tract (20). The cysts are chocolate brown in color and are 1–2 cm in diameter. A long-standing cyst wall is thick, fibrosclerotic, and calcified. Granuloma, due to *Paragonimus* eggs, is frequently seen in the vicinity of the cyst. The cysts might be single, multiple, or in clusters. The lesions having worms in unusual locations can occur in the brain, liver, abdominal wall, peritoneal cavity, cervical lymph nodes, adipose tissues around the kidney, pericardium, pancreas, omentum, mesentery, stomach wall, scrotum, epididymis, spinal cord, eye, orbit, eyelids, bone marrow, subcutaneous tissue, urinary bladder, ovary, and uterus (7,20).

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

C-banding techniques and zymodeme analysis have been widely used to study the genetic difference in *Paragonimus* spp. (50). A diploid type ( $2n = 2x = 22$ ), a triploid type ( $2n = 3x = 33$ ), and a tetraploid type ( $2n = 4x = 44$ ) of *P. westermani* have been reviewed (51). Several studies have determined that the triploid form should be called *P. pulmonalis* but the diploid form should remain as *P. westermani*. There is evidence that the origin of tetraploid flukes come from triploid and diploid parents. Both diploid and triploid types are found in Japan. Triploids are reported in Korea, Taiwan, and Japan. Diploids, triploids, and tetraploids are found in Liaoning, China. The pathology caused by the diploids and triploids is different, i.e., the triploid fluke mainly forms cysts in the lung, but the diploid fluke causes lesions in the pleural cavity and pleura (50). Reproduction of the diploid form requires the exchange of sperm between the adults cohabiting within a cyst in the lung. If a worm does not meet a partner, it migrates around in the pleural cavity, and there is no development of the eggs, which have been laid in the lung cyst. Thus, the migration tends to be prolonged and accordingly causes severe damage to the pleura. In contrast, reproduction in the triploids occurs by parthenogenesis. Thus, coupling with a partner is not a requirement.

Recently, however the phylogenetic classification of *P. westermani* has been divided into two groups as determined by comparison of nucleotide sequences from the second internal transcribed spacer of ribosomal gene repeat unit and a part of the mitochondrial cytochrome *c* oxidase subunit I gene (52). One is from China, Japan, Korea, and Taiwan, which is relatively uniform and includes both diploid and triploid forms. Another one is from Malaysia, Thailand, and the Philippines, which is genetically distinct from the former.

## VIII. CONTROL MEASURES

The strategy for control and eradication of these infections requires the interruption of the life cycle of the parasite. Eradication of an intermediate host, such as snails, is difficult to accomplish, since they are a part of the environment and widespread. Molluscicides might destroy fish, which is a dietary protein source. Improvement in environmental sanitation can be carried out successfully by massive health education and the complete use of latrines in the endemic areas. Changing the custom of eating raw crabs and crayfish can prevent infections with metacercaria among humans. Cobalt-60 irradiation at a dose of 2.5 kGy can destroy the infectivity of *P. westermani* metacercaria (53). Effective treatment is one of the most important measures of control. Praziquantel given in doses of 25 mg/kg of body weight for 1–3 days produces a cure rate of 70–100%. Finally, collaboration between the sectors of public health, agriculture, aquaculture, education, and politics plays an important role in any effort to control infections (1).

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*Anisakis simplex* and *Pseudoterranova decipiens***M. T. Audicana and M. D. del Pozo***Hospital Santiago Apóstol, Vitoria-Gasteiz, Spain***R. Iglesias and F. M. Ubeira***Santiago de Compostela University, Santiago de Compostela, Spain***I. BACKGROUND**

The consumption of raw or undercooked fish may lead to infection with any of several helminths, the most important being species of the cestode genus *Diphyllobothrium*, the digenean families Heterophyidae (*Heterophyes* spp. and *Metagonimus yokogawai*) and Opisthorchiidae (*Clonorchis sinensis* and *Opisthorchis* spp.), and the nematode genera *Anisakis* and *Pseudoterranova* of the family Anisakidae (1). As will be reviewed in Sec IV, the anisakid species most commonly involved in human infections are *Anisakis simplex* and less frequently *Pseudoterranova decipiens*. Infection with *A. physeteris* and *Contracaecum* spp. has only been reported in a very few cases (2–5). At one time, the anisakid species *Hysterothylacium aduncum* was considered a possible human parasite (6,7), but this hypothesis was later discarded in view of experimental evidence indicating that it is unable to penetrate the gastric mucosa of laboratory animals and it cannot survive at temperatures close to 37°C (8,9). However, it has recently been demonstrated that larvae of some species of this genus are capable of invading the gastrointestinal tract of primates (10), and therefore the possibility of human infection should not be ruled out completely.

The first case of human infection by a species of the Anisakidae family was reported in the Netherlands by Van Thiel in 1960 (11). This author described the presence of a marine nematode in the center of an eosinophilic intestinal phlegmon from a patient suffering from acute abdominal pain, as a “very unusual finding.” Later the nematode was identified as *Anisakis* spp., a common parasite of marine fishes and mammals, and the human parasitosis was named anisakiasis (12). This infection was associated with the consumption of smoked fish and obliged the Dutch health authorities to introduce legislation requiring the freezing of all fish destined to be eaten raw (13). Since then, the majority of anisakiasis cases have been described by Japanese authors (5), reflecting the frequent consumption of raw fish in that country. However, over the last few years there has been an increase in the number of cases reported in other countries, including Korea (14), the United States (15), France (16), and Spain (17).

Since the 1960s, the term anisakiasis has been used to designate not only the human disease caused by the third-stage larvae (L3) of *Anisakis* spp., but also the human disease caused by L3 of other members of the family Anisakidae. In 1998, a group of experts (SNOAPAD) (18) recommended the use of three different terms: (a) anisakidosis for disease caused by any member of the family Anisakidae, (b) anisakiosis for disease caused by members of the genus *Anisakis*, and (c) pseudoterranovosis for disease caused by members of the genus *Pseudoterranova*. In this review we use this terminology.

## II. CHARACTERISTICS

### A. Morphological Characteristics

In sea fishes, the third larval stage of anisakids is frequently found encysted in the viscera (Fig. 1) and/or the musculature or moving actively in the body cavity. To the naked eye, L3 of *Anisakis simplex* are pinkish-white and cylindrical in form, tapering at either end, with a small whitish elongated spot (the ventricle) on the anterior third of the body. Body length at this larval stage ranges from 20 to 30 mm.

The mouth consists of a small oral opening surrounded by one dorsal and two ventrolateral lip bulges (Fig. 2A). Between the latter, there is a triangular boring tooth, which inserts just behind the excretory pore. The end of the tail bears a mucron (tail spine) about 0.015–0.030 mm long (Fig. 2B).

Light microscopy clearly reveals a thin muscular esophagus followed by a ventricle (1 mm in length) joined obliquely to the proximal portion of the intestine (Fig. 3). Ventral to the alimentary canal, the excretory cell runs along the anterior third of the body, opening to the outside via the excretory pore. A dorsal gland arising in the ventricle and opening into the esophagus lumen (at the level of the nerve ring) occupies the dorsal part of the esophagus. The subventral gland surrounding the dorsal gland in the ventricle opens into the distal region of the esophagus. It appears that the ventral gland is involved in intraluminal digestion, whereas the dorsal gland is involved in extracorporeal digestion (19).

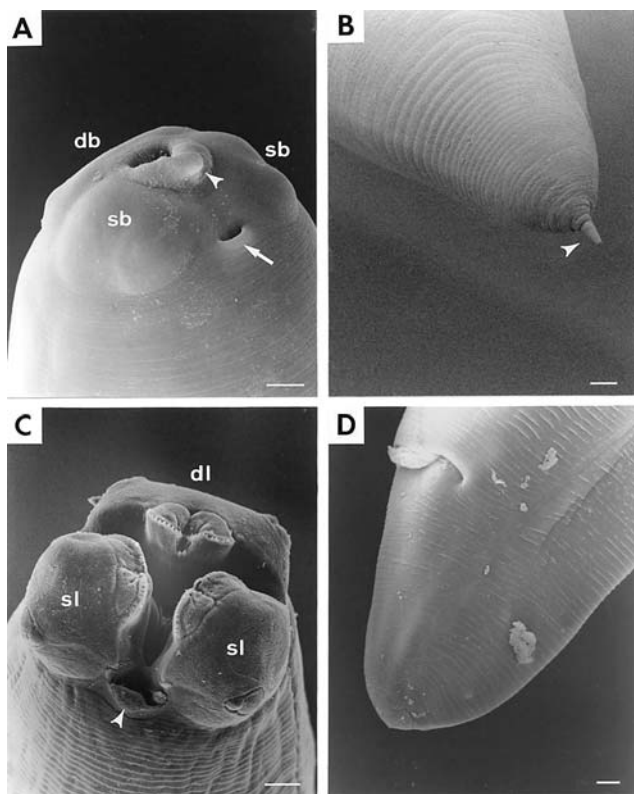
Most of the excretory cell is occupied by a single nucleus, while the rest comprises of a granular cytoplasm with drainage tubules that flow into the main duct. It is thought that this cell operates as both an excretory and a secretory system.

L3 of *Pseudoterranova decipiens* are yellowish-brown and larger than *Anisakis* larvae (30–40 mm in length). External structures include three more prominent lip bulges, a boring tooth, an excretory pore, and a longer and more slender terminal mucron. The ventricle is shorter and joins the intestine via a horizontal junction. A characteristic intestinal cecum projects from the intestine to the anterior end in the ventricular region (Fig. 4). The remaining internal structures are similar to those observed in *Anisakis*.



**FIGURE 1** Multiple third-stage *Anisakis simplex* larvae encysted in the viscera of a blue whiting (*Micromesistius poutassou*). Larvae are especially abundant in the liver. (From Ref. 17.)

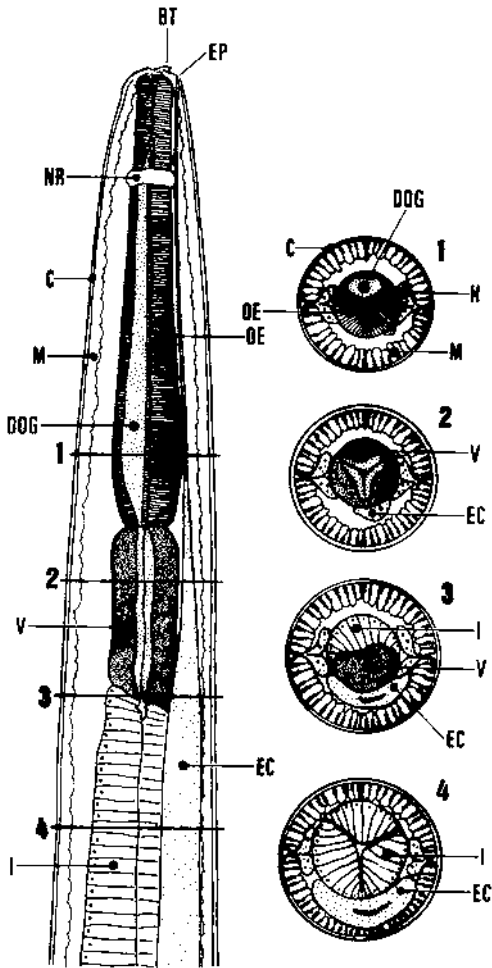




**FIGURE 2** Scanning electron micrographs of third-stage (A, B) and fourth-stage (C, D) *Anisakis simplex* larvae. (A) Anterior end of L3, showing the dorsal (db) and subventral (sb) lip bulges surrounding the oral opening, the boring tooth (arrowhead) and the excretory pore (arrow). (B) Caudal region of L3, showing the mucron at the end of the tail. (C) Anterior end of L4, showing the dorsal (dl) and subventral (sl) lips and the excretory pore (arrowhead) surrounding the oral opening. (D) Caudal region of L4, showing that the terminal mucron disappears after the molting process. (Scale bars=10  $\mu$ m)(By courtesy of Prof. M.L. Sanmartín.)

## B. Life Cycle

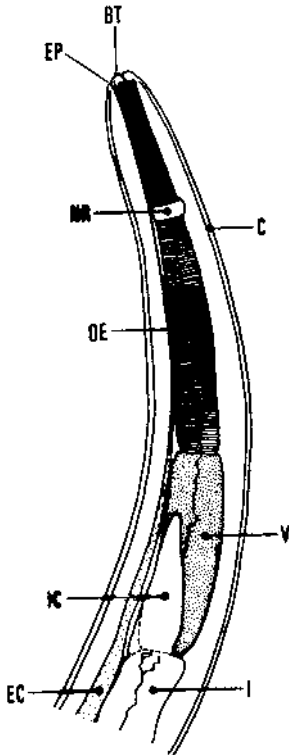
*Anisakis simplex* has an almost worldwide distribution, although it is especially abundant in polar and moderately cold marine waters. It reaches sexual maturity primarily in the stomach of cetaceans (dolphins, porpoises, whales, etc.) and, less frequently, in pinnipeds (seals, sea lions, walruses) (Fig. 5). Unembryonated eggs are passed in the feces of the final host and embryonate in seawater, the speed of hatching being controlled by the temperature of the water (range 4–8 days at 13–18°C to 57–82 days below 5°C) (20,21). Recent evidence shows that two molts occur within the egg before hatching, the emerging ensheathed free-living larva being the third stage, not the second stage as was previously believed (22). These larvae may be ingested by euphausiid crustaceans (the intermediate hosts), and indeed this step is probably necessary for completion of the life cycle. It has been suggested that L3 may also be transferred to euphausiid hosts as a result of euphausiid predation of copepods that have ingested parasite larvae (21).



**FIGURE 3** Schematic drawing of the anterior end of a third-stage *Anisakis simplex* larva, showing the morphological structures observed in sections at different levels (1–4) and the intestine obliquely joined to the ventricle. BT: boring tooth, EP: excretory pore, NR: nerve ring, C: cuticle, OE: esophagus, M: musculature, DOG: dorsal esophageal gland, H: hypodermis, V: ventricle, EC: excretory cell, I: intestine. (From Ref. 17.)

Fish (mainly teleosts) and cephalopods (mainly squids) may act as paratenic hosts, contributing to dissemination of the parasite by ingestion of L3 as a result of feeding on euphausiids or contaminated fishes or cephalopods. Commercially important species that may act as paratenic hosts include herring, cod, rockfish, salmon, mackerel, hake, sardine, anchovy, and common squid.

Marine mammals are normally infected as a result of predation of contaminated teleosts and cephalopods, although direct infection by feeding on infected euphausiids (krill) also seems possible, since infection has been observed in whale species that feed only on these planktonic crustaceans (23). After ingestion, the L3 penetrate the gastric mucosa of the final hosts, molting twice before



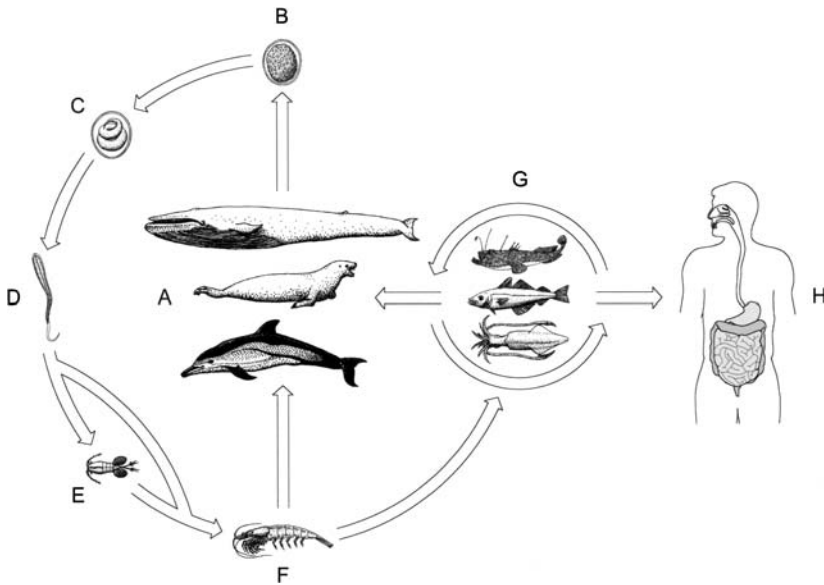
**FIGURE 4** Schematic drawing of the anterior end of a third-stage *Pseudoterranova decipiens* larva, showing the horizontal ventricle-intestine junction and the characteristic intestinal cecum. BT: boring tooth, EP: excretory pore, NR: nerve ring, C: cuticle, OE: esophagus, V: ventricle, IC: intestinal cecum, EC: excretory cell, I: intestine.

reaching sexual maturity. Typically, 50–100 individuals of the third, fourth, and adult stages are grouped in clusters in the center of gastric ulcers measuring 1–6 cm in diameter (24) (Fig. 6).

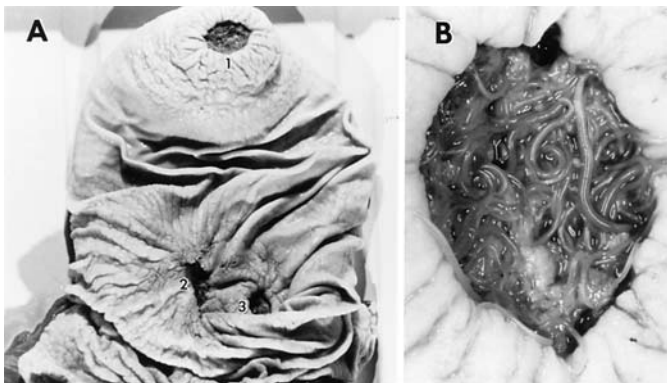
In general, the life cycle of *P. decipiens* is similar to that of *A. simplex*, although the final hosts of the former are almost exclusively pinnipeds (25,26). As in the case of *A. simplex*, the hatching speed of the eggs and the survival rate of emerging L3 (22) are strongly influenced by water temperature (27). The tail of the cuticular sheath that surrounds the larva has adherent properties that facilitate attachment to the sea bottom substrate until ingestion by its intermediate hosts, which appear to be primarily mysid crustaceans and to a lesser extent gammaridean amphipods (28–32). The transmission of the free-living larva to these benthic hosts, and more especially to mysids, may also occur via harpacticoid and cyclopoid copepods (33,34).

In the North Atlantic, L3 of *P. decipiens* have been detected in more than 60 different fish species, including cod, haddock, halibut, long rough dab, and sculpin (35,36). Squid is not usually infected by this species (38). Like *A. simplex*, *P. decipiens* can be transmitted from fish to fish, as has been demonstrated experimentally (37). The capacity of *P. decipiens* L3 to reach fish muscle appears to be greater than that of *A. simplex* (25).

Humans may be accidentally infected when they eat raw or undercooked fish contaminated with L3 of these anisakid nematodes. Once in the human gastrointestinal tract, L3 of *Anisakis*, and



**FIGURE 5** Summary of the life cycle proposed for *Anisakis simplex*. Adult parasites are found in the stomach of a broad range of marine mammals including cetaceans and less frequently pinnipeds (A) Unembryonated eggs (B) are passed out into seawater with the feces of the final hosts. Once the eggs are completely embryonated (C) they hatch, releasing an ensheathed free-living third-stage larva (D) that is then ingested by euphausiid krill (F). The emerging larvae may also reach these intermediate hosts via copepods (E). The infective third-stage larvae are transmitted to the final hosts (A) or to fishes and squids (G; paratenic hosts), which feed on contaminated euphausiids or other infected fishes and squids. Humans (H) become accidentally infected when they eat raw or undercooked seafood. The life cycle proposed for *Pseudoterranova decipiens* is similar, except that the final hosts are almost exclusively pinnipeds, the intermediate hosts are mainly mysid crustaceans, and squids are not usually infected.



**FIGURE 6** (A) Luminal face of the stomach of a common porpoise (*Phocoena phocoena*) with three deep ulcers (1–3) resulting from the presence of *Anisakis simplex*. (B) Detail of ulcer 1 containing multiple parasites at different developmental stages. (From Ref. 17.)

especially *P. decipiens*, may progress to L4 (39–44). In exceptional cases, the immature adult stage may be reached (45,46).

### III. DISEASES

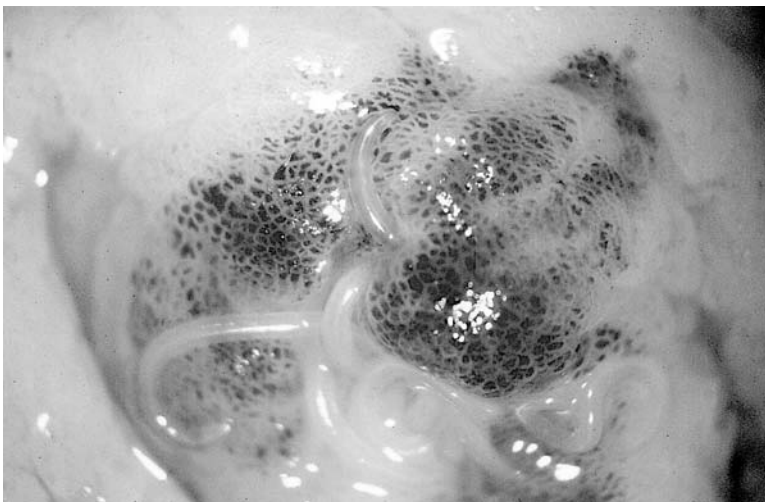
Human anisakid infections frequently cause gastrointestinal symptoms, which may be associated with mild to severe allergic reactions. In addition, some patients show allergic reactions without any associated digestive disorders. We therefore consider that anisakid larvae may be responsible for four clinical forms of illness in humans. The first three are characterized by the location of the lesions, and the fourth by allergic manifestations alone.

#### A. Gastric Form (Gastric Anisakidosis)

This form arises when anisakid larvae penetrate the gastric wall (frequently just the mucosa or submucosa). The acute clinical course is generally characterized by acute epigastric pain, nausea, and vomiting, all appearing a few hours after ingestion of the contaminated fish (47). Less frequently, a bloated abdomen, diarrhea, pyrexia, hematemesis and other nonspecific symptoms may also appear. Hematology often reveals leukocytosis and, to a lesser extent, eosinophilia. Allergic symptoms (i.e., urticaria/angioedema and even anaphylaxis) are reported in about 10% of cases, and the combination of infection and allergic symptoms has recently been named “gastroallergic” anisakiosis (48).

Endoscopic studies have revealed that the lesions and the associated larvae are usually distributed in the body of the stomach, along the greater curvature (49,50). Ooiwa et al. (51) described three types of lesions normally observed at the penetration site in humans: (a) tumor-like (also called “vanishing tumor”), characterized by the presence of the larvae at the center of an elevation, (b) swollen-fold, when the larva is found penetrating an edematous mucosal fold, and (c) flat, if there is no change in the penetrated mucosa. Mild spotty hemorrhage, petechiae, or erosion at the penetration site are frequently found, together with diffuse edematous changes in the gastric mucosa. Damage to the gastric mucosa may also be observed in experimental infections (Fig. 7).

Histopathological studies of the acute form are rare, because these cases are frequently diagnosed and treated endoscopically (see below) without taking biopsies. Typical findings include the



**FIGURE 7** Multiple *Anisakis simplex* larvae penetrating the gastric mucosa of an experimentally infected rabbit. Petechiae are evident at the surface of the edematous mucosa surrounding the penetration site.

presence of intact larva embedded in the edematous submucosa and surrounded by neutrophils and eosinophils (52). The gastric wall shows marked thickening, with edema and massive eosinophilic infiltration.

Some patients may develop a subacute or chronic form, characterized by clinical manifestations such as relatively severe epigastric pain that later becomes dull, and dyspepsia, vomiting, and anorexia, which can persist for months or even years (53). Endoscopic examinations in chronic cases may show erosion or ulcer with edema at the surface of a tumor-type induration or nodule mimicking stomach cancer or ulcer (5). Kikuchi et al. (54) described three types of histopathological processes normally detected in these cases: (a) abscess formation (a marked abscess normally located in the submucosa, with massive eosinophilic infiltration around the degenerating larvae), (b) abscess-granuloma formation (the degenerating larva is located in the center of a reduced abscess surrounded by granulation tissue with collagenization, and lymphocytes as predominant cell type), and (c) granuloma formation (the most advanced histopathological stage of chronic anisakidosis: larval debris, often practically undetectable, is embedded in the center of granulomatous tissue with collagenization, foreign-body giant cells, lymphocytes, and, to a lesser extent, eosinophils).

## **B. Intestinal Form (Intestinal Anisakidosis)**

In acute or fulminant cases, acute abdominal pain (usually appearing 24–48 hours following ingestion of the larvae) may be accompanied by nausea, vomiting, abdominal bulging, induration, and deviation from established bowel rhythm with either constipation or diarrhea (55,56). When fever over 38°C appears, this may indicate secondary infection with bacteria. Laboratory findings often show left-shifted leukocytosis, but eosinophilia is not generally observed. Both obstructive syndromes (57) and arthralgias (58,59) have also been described.

Topographically, the phlegmonous lesions usually appear in the terminal ileum, within 50 cm preceding the Bahuin valve (56). In most acute cases, abdominal ascitic fluid with a high eosinophil content (more than 30% of infiltrating cells) is found (60). Unlike peritonitis, the ascitic fluid has a serum-like transparent or yellowish translucent appearance. As in the gastric form, inflammatory changes (including severe local edema that may lead to obstruction and proximal dilatation) are strictly localized. In addition to edema, petechiae, hyperemia, and cloudy swelling of the serosa and mesenterium may also be observed. Microscopic examination usually shows the intact larva embedded in the submucosa, with intense edema and marked cellular infiltration throughout the serosal layer. These changes lead to a three- to five-fold thickening of the intestinal wall. The phlegmonous lesion is histopathologically similar to that occurring in the acute gastric form, but more severe.

In the subacute or chronic forms, the granulomatous changes (often misdiagnosed as tumors) cause a thickening of the wall, luminal stenosis, and thus chronic abdominal symptoms. In these cases, the macroscopic and microscopic findings are again similar to those observed in chronic gastric anisakidosis (60).

## **C. Extragastrintestinal or Ectopic Form (Ectopic Anisakidosis)**

On certain occasions larvae may perforate the gastrointestinal wall completely, reaching the abdominal cavity and migrating to organs and tissues such as the greater omentum, mesenterium (61,62), lung (63,64), pancreas (65), or liver (66). In the majority of these cases, the clinical manifestations are slight, but intestinal symptoms of greater severity may occur as a result of the response elicited by the larva during its penetration of the intestinal wall (5,62).

In other cases the larva may migrate back along the alimentary tract from the stomach, to settle in the esophagus or in the oropharynx, in the latter location provoking the so-called “tingling throat syndrome,” which normally leads to the expulsion of the larva by coughing up. These types of symptoms have been very common among the cases of pseudoterranovosis described in the United States (67).

## D. Allergic Form

Urticaria has been reported in gastrointestinal infections with *Anisakis simplex* (47,68). In 1990, Kasuya et al. suggested that this parasite should be considered as a possible etiological factor in the differential diagnosis of allergic reactions induced by fish (69). However, it is only recently that *Anisakis simplex* has been identified as an important cause of allergic reactions mediated by IgE antibodies (70).

Allergic reactions to *Anisakis simplex* with neither gastric nor intestinal symptoms have been observed in sensitized patients, following the ingestion of contaminated seafood (fish or cephalopods). The first symptoms appear very early, within the first few hours, and very frequently within even the first 60 minutes. The clinical course follows the general pattern for type I allergic reactions, and its severity varies from simple urticaria or angioedema to anaphylactic shock. Clinical manifestations are severe in 20–60% of cases and may affect several organs, including the skin, respiratory apparatus, digestive system, and cardiovascular system (71,72). Rheumatic symptoms may appear in the course of anaphylaxis but are extremely rare (73). Recently, two episodes of anaphylaxis have been reported in direct association with skin prick testing using an *A. simplex* extract (74), and it has been reported that *A. simplex* can be an airborne antigen (75,76).

An unexpected characteristic of *Anisakis simplex* allergy patients is their high average age of about 50 years (71,77,78), despite the fact that food-related allergies are usually more common in children and young adults. This may be because adults more frequently eat raw or undercooked seafood. In the Basque country (northern Spain), *Anisakis simplex* is a frequent cause of allergic reactions, being responsible for about 8% of cases of acute urticaria/angioedema and/or anaphylaxis (78); this prevalence is similar to or higher than that for other major allergenic foods and should therefore be borne in mind when diagnosing food allergy. Indeed, in our clinical experience, *Anisakis simplex* is the most frequent cause of allergy among adult patients who experience an acute episode of urticaria/angioedema and/or anaphylaxis after eating seafood (79).

Although our research has focused mainly on *A. simplex* allergy, it seems very likely that *P. decipiens* will cause similar problems because of the antigenic homology between the two species (80).

## E. Diagnosis

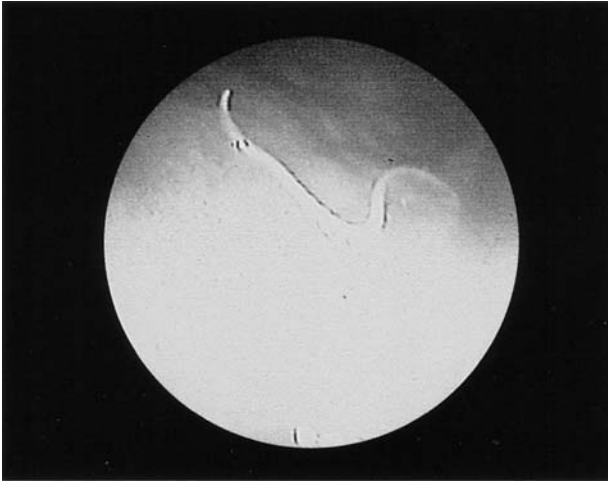
Human anisakidosis is peculiar in that (a) about 90–95% of the cases described are caused by a single larva (81), (b) although the most frequent site for larval settling is the gastrointestinal tract, larvae may also penetrate the gastrointestinal wall and migrate to ectopic localizations, and (c) humans are not adequate hosts for the parasite. These peculiarities mean that diagnosis by coprologic techniques is useless. In fact, endoscopy is the most common routine aid for diagnosis when the parasite is settled in accessible sites.

Anisakid infection should be suspected in patients with gastrointestinal symptoms and a history of consumption of raw or undercooked fish or cephalods. If carried out soon after the onset of symptoms, endoscopic examination of the stomach (Fig. 8), duodenum, or colon usually reveals the larvae penetrating the mucosa, thus confirming the diagnosis (82–84). At more advanced stages, when the larvae have already penetrated the wall, and especially in the intestinal form, a histopathological study of the resected area may prove useful. In the most advanced chronic cases, only a granulomatous lesion with larval cuticular debris is observed (54).

Alternative techniques that may aid diagnosis include radiographic studies with barium (in which larvae can be identified as thread-like filling defects) (55,85,86), ultrasonography (87,88), and endoscopic ultrasonography (89,90).

As a complement to direct visualization methods, serodiagnostic tests are useful in suspicious cases in which the larva cannot be located because it has migrated out of the gastrointestinal tract, is inaccessible to gastroscopy or colonoscopy, or has been partially or totally destroyed.

Serodiagnostic tests available for anisakiosis have included procedures based on latex agglutina-



**FIGURE 8** Gastroendoscopic image obtained during diagnosis of human gastric anisakiosis showing an *Anisakis simplex* larva penetrating the gastric mucosa.

tion (91), Ouchterlony tests and immunoelectrophoresis (92,93), immunofluorescence (94,95) indirect hemagglutination (96), complement fixation (97), immunoblotting (93,98), and ELISA (99–102). All these methods use whole or partially purified antigens and thus show poor specificity, since *Anisakis* antigens show strong cross-reactivity with antigens from many other parasites (103,104). This is especially relevant to determinations of specific IgG or IgM antibodies, which are frequently induced by immunodominant carbohydrates (105–107) that may be present in parasite glycoproteins.

For patients showing allergy symptoms only, until recently the initial diagnosis was done on the basis of three criteria: (a) compatible clinical history (urticaria/angioedema after the ingestion of seafood), (b) presence of IgE antibodies against whole antigens of the parasite, using *in vivo* tests (prick test) or *in vitro* tests such as IgE-immunoblotting (108,109) and/or ImmunoCAP (78,108), and (c) noninvolvement of fish proteins. Nevertheless, there have been reports of sensitization to *A. simplex* (defined only as presence of anti-*Anisakis* IgE antibodies using whole parasite antigens) in patients with no relevant clinical history (110,111).

Recently, however, important progress has been made with regard to the sensitivity and specificity of serodiagnostic tests for *Anisakis*. Of particular significance was the development of *Anisakis*-specific monoclonal antibodies (17,112–114). In this connection, it was recently demonstrated that ELISA using the mAb UA3 (UA3-ELISA), which recognizes two major *Anisakis* antigens of 139 and 154 kDa, allows detection of specific IgG and IgE antibodies in sensitized patients showing gastrointestinal or allergic symptoms with 100% sensitivity and 100% specificity (115,116). By contrast, ImmunoCAP showed 100% sensitivity but only about 50% specificity, while IgE-immunoblotting showed 83% sensitivity and 93% specificity (116). False positives are thus fairly common in ImmunoCAP and IgE-immunoblotting, as expected given that both methods use complex parasite antigen mixtures containing carbohydrates that can cross-react with similar epitopes occurring in antigens from other parasites, crustaceans, insects, mites, bacteria, and plants (117–120). Other sources of cross-reactivity in whole antigens preparations may include the so-called “panallergens,” such as tropomyosin, found in crustaceans, insects, mites (121), and probably also *A. simplex* (122), as well as highly conserved molecules such as biotinyl-enzymes (123–126), which stimulate the production of IgE antibodies in some patients (127).

Finally, with all serological tests it should be borne in mind that in populations highly exposed to *Anisakis* antigens (such as the Japanese population), in which a large proportion of subjects can



be expected to have these anti-*Anisakis* antibodies in serum, the tests may be less useful than in populations in which consumption of raw or undercooked sea fish is infrequent.

## F. Treatment

The most effective treatment for gastric, duodenal, and colonic anisakiosis is extraction of the larvae during the endoscopy itself, after which symptoms typically disappear within a few hours (84,128). In many cases of acute intestinal anisakiosis, in which the larvae are not accessible, laparotomy and resection of the affected fragment must be carried out. However, when suspicion of anisakiosis is firm, a conservative treatment with serotherapy and antibiotics may be sufficient (55,88,129,130). *Anisakis simplex* larvae has been shown to be highly resistant to anthelmintics (131), though larvae of *P. decipiens* appear to be sensitive to ivermectin, at least in vitro (132).

*Anisakis*-induced allergy should be treated in the same way as any other anaphylactic reaction.

## IV. EPIDEMIOLOGY

At the moment, about 97% of reported anisakidosis cases are due to anisakiosis, the other 3% being cases of pseudoterranovosis. Anisakiosis is particularly common among the Japanese population, due to widespread consumption of raw fish. Of the total of about 14,000 cases reported worldwide to date, about 95% are from Japan, where about 2000 cases are reported per year (66). In the United States and Europe, the disease is much less frequent: to date, about 50 cases have reported in the United States (67), and about 500 cases in Europe. Within Europe, more than 95% of cases have been from Holland, Germany, France, and Spain. In Spain, the cumulative total number of reported cases (more than 80 cases confirmed by endoscopic techniques) has increased rapidly during the last few years because of increased awareness among physicians.

Pseudoterranovosis, normally affecting the stomach, is especially frequent in northern Japan (5) and the United States (15). In Japan, gastric anisakidosis is apparently far more common than intestinal anisakidosis (95% of cases), while in Europe and the United States the opposite is true. These differences have been attributed to the more widespread use of endoscopic techniques by Japanese doctors and to their greater awareness of the disease (5).

The transmission of these foodborne pathogens is clearly related to traditions of consumption of raw or improperly cooked fish. A number of fish dishes are considered to be high-risk for the contraction of anisakidosis, including Japanese sushi and sashimi, Dutch salted or smoke herring, Scandinavian gravlax, Hawaiian lomi-lomi, South American cebiche, and Spanish boquerones en vinagre (pickled anchovies) (127).

Epidemiological studies in Japan have found that anisakiosis is more frequent in coastal populations (and in particular in people involved in the fish industry) and in males aged 20–50 years (133). The main transmitter species is the spotted chub mackerel (*Scomber japonicus*) and Japanese flying squid (*Todarodes pacificus*) (38,134). In the United States, the majority of reported cases were due to ingestion of Pacific salmon (*Oncorhynchus* spp.) (135), and one study has shown that up to 10% of the salmon consumed in the sushi bars of the Seattle region contained *Anisakis* larvae (136). In western Europe, herring (*Clupea arengus*) is the main species involved (13,130,137), although cases with other species that were insufficiently cooked (microwaved, grilled, or shallow-fried) have also been reported. In Spain, most cases have been related to the consumption of pickled anchovies (*Engraulis encrasiclorus*) and raw sardines (*Sardina pilchardus*).

The principal transmitters of pseudoterranovosis are possibly Pacific cod (*Gadus macrocephala*) and the Pacific halibut (*Hippoglossus stenolepis*) in Japan and the red snapper (*Sebastes* spp.) in the United States (138).

The recent increase in the number of cases worldwide may be associated with the following factors: (a) a better knowledge of the disease and its diagnostic techniques, (b) proliferation of Japanese restaurants in the western world, (c) recent tendencies to cook food for a very short time

in order to preserve its nutritional value, and (d) the increase in marine mammal populations as a result of protection movements, as can be found off the western coast of the United States, where the higher incidence of anisakidosis cases has coincided with an increase in the numbers of some cetacean species (67).

*Anisakis simplex* allergy is also an important and frequent disorder. Since the authors of this chapter started to work with this parasite, it has become clear that it is the most important food allergen in the Basque country in northern Spain (78). Japanese authors have reported IgE to excretory/secretory antigens from *A. simplex* in 87.5% of endoscopically diagnosed gastric anisakidosis patients and in 75% of patients with urticaria induced by spotted chub mackerel, but only in 8.3% patients with urticaria of unknown origin and 19% of normal controls (139). The studies in northern Spain (78) demonstrated that the prevalence of *A. simplex* allergy alone (12%) was similar to that of Rosaceae pollen, nuts, and shellfish combined. This illustrates the importance of *A. simplex* in the etiology of acute urticaria/anaphylaxis. Furthermore, the prevalence of allergy and sensitization to *A. simplex* (ImmunoCAP values > 0.7 kU/L) is higher than the prevalence of allergy and sensitization to fish (12% vs. 1.3% and 26% vs. 3%, respectively)(78). The identification of *A. simplex* as a foodborne allergy is of particular importance since these cases would otherwise be considered as idiopathic. However, it should be borne in mind that ImmunoCAP overestimates the number of sensitized subjects (116). There is a need for further studies of this aspect using UA3-ELISA.

## V. ISOLATION AND IDENTIFICATION

Third-stage larvae causing anisakidosis can be isolated from the human gastrointestinal tract by endoscopy and identified by light microscopy after fixation (Berland's fluid) and mounting and clearing (in lactophenol) on the basis of morphological characteristics including the boring tooth and excretory pore in the cephalic region, the ventricle and the plane of its junction with the intestine (horizontal in *P. decipiens*, oblique in *A. simplex*), the intestinal cecum (only present in *P. decipiens*), and finally the tail and the terminal mucron. However, it should be pointed out that the larvae of these nematodes can survive for approximately 7–14 days in experimental hosts as well as in humans (17,140) and may molt to the fourth larval stage 3–4 days postinfection (41,141). In such cases neither the boring tooth nor the terminal mucron are present (Fig. 2C and D), because they disappear during the molt. It should also be borne in mind that the three lip bulges become three intended lips at this stage (Fig. 2C).

Identification is difficult when the larva cannot be isolated by endoscopy and only histological biopsies are available. In such cases, identification would be easier if the structure of the larva is conserved, allowing observation in histological sections of internal structures such as the excretory cell, the ventricle, the hypodermal lateral cords, and the intestine (Fig. 3).

## VI. PATHOGENICITY

Over the last few years, studies have indicated that, as with other helminthoses, the pathological changes occurring within the human gastrointestinal tract during infection with *A. simplex* are the combined result of (a) the direct action of the larva during tissue invasion and (b) the complex interaction between the host immune system and the substances released by or contained within the parasite.

### A. Invasive Mechanisms

In order to invade the gastrointestinal mucosa, L3 of *A. simplex* probably used both the mechanical tooth, present in the cephalic region, and their potent proteolytic enzymes, which are capable of degrading the extracellular matrix. These enzymes are probably produced by the dorsal esophageal

gland and the excretory cell of the larva, to be secreted through the excretory pore and the oral opening, respectively (19,142,143). Proteolytic molecules of 54.3 kDa (144), 23.4 and 46 kDa (103), and 25–26 kDa (145,146) have been isolated from excretory-secretory (ES) products released by *A. simplex* L3 in vitro. The latter is probably the same as the 23.4 kDa protein described by Kennedy and colleagues (103) and is similar to mammalian trypsin in structure and function. Recently, a 40 kDa enzyme that degrades chondroitin sulfate-A and hyaluronic acid (147), and also a 30 kDa serine proteinase similar to that present in the bacterium *Dichelobacter nodosus* (148), have been characterized. The great invasive capacity of the larvae, together with the presence of anticoagulant substances in the ES products (149), explains the existence of the multiple well-defined and erosive and/or hemorrhagic lesions usually detected near the main lesion within the gastric mucosa of patients suffering from anisakidosis (58).

## B. Immunopathological Mechanisms

The involvement of immunological mechanisms in the pathogenesis of acute anisakiosis was first proposed in 1964 by Kuipers with the “double hit” hypothesis, which explained why more severe pathological changes occurred after reinfection in rabbits (150). Subsequent experimental studies confirmed that previous sensitization caused lesions of greater severity, and also indicated that type I, type III, and type IV hypersensitivity reactions may be involved in the immunopathology of anisakiosis (151–154).

To date there have been insufficient studies to provide a detailed understanding of host-parasite interactions in the various clinical and histopathological forms of human anisakiosis. Type I and/or type IV hypersensitivity responses are certainly involved. The presence of type I responses in acute anisakiosis is indicated by the fact that serum anti-*Anisakis* IgE levels increase rapidly during the first few days and remain high for months (114,155,156). Eosinophils are not capable of destroying *Anisakis* larvae in vitro (157); however, eosinophil infiltration of the tissue surrounding the parasite is one of the most distinctive features of the local inflammatory lesions observed in anisakiosis. The presence of these cells may reflect the late stage of the type I response, following release of eosinophilic chemotactic factors during the acute stage of the response. In addition, it is possible that some parasite-derived substances also attract eosinophils to the damaged tissue. For example, Iwasaki and Torisu (158) have observed that *A. simplex* extracts applied to the ileum of nonsensitized rabbits induce lesions characterized by local eosinophilia and scant presence of mast cells and neutrophils. Although blood eosinophilia is a common feature of nematode infections (159), it appears to occur in less than 30% of cases of anisakiosis (47,86,160).

The granulomatous lesions observed in the chronic gastrointestinal and ectopic forms of anisakiosis are typical of type IV hypersensitivity reactions. No granulomatous lesions were observed in a recent study of an immunosuppressed patient suffering from both AIDS and intestinal anisakidosis (161), arguing in favor of the involvement of cellular immunity in these lesions.

In the allergic form of anisakidosis, the most relevant immunopathological finding is the increase in specific and total IgE levels in patients’ serum (71,108,109). This response, a hallmark of helminth infections, is typical of Th2 immune responses (162) and dependent on IL-4 (163). IgE levels can persist in these patients for years even if marine fish are eliminated from the diet.

Western blot studies showed that the human IgE and IgG antibody response to *Anisakis simplex* antigens is highly heterogeneous and varies dramatically between individuals (108). This may be explained by genetic differences between individuals, by differences in the nature and number of immunizations, and/or by the nature of the immunogen to which the patient had been exposed. In human anisakidosis, the patient can be exposed to *Anisakis* antigens from three sources: (a) ES and somatic (including cuticular) antigens, as a result of tissue penetration and subsequent degeneration of the larvae, (b) ES antigens only, in cases which there is a rapid expulsion of the parasite through the anus, and (c) ES and somatic antigens from dead larvae contained in food. In experimental studies of mice, differences in antibody response have been found depending on whether animals are exposed to living *A. simplex* or to antigen preparations only: infection elicited a strong Th2

response, while exposure to antigens alone did not (M. W. Kennedy, personal communication). This may suggest that freezing or cooking of fish could in the long term reduce the incidence of allergic reactions, though it would not prevent them in already sensitized individuals. What is more, it is possible that other related nematodes present in marine fish (e.g., *Hysterothylacium*) may contribute to the stimulation of anti-*Anisakis* IgE antibodies as a result of cross-reactivity.

### C. Other Related Pathologies

In addition to the aforementioned mechanisms, there are some aspects of the pathogenesis of diseases caused by *Anisakis* and *Pseudoterranova* that need to be studied in more depth. In some cases, for example, secondary infections with bacteria have been observed, which may cause more severe inflammatory changes (56,152). What remains unclear is the link between *A. simplex* and rheumatological symptoms (arthralgias/arthritis)(58,59,73). These symptoms could be caused by immunocomplexes, as has been described for other parasitoses such as trypanosomiasis, malaria, Chagas' disease, and schistosomiasis. Finally, it has been suggested that infection with *Anisakis simplex* may be related to the gastric carcinogenic lesions observed in some patients (82,164).

## VII. GENETIC FACTORS INVOLVED IN VIRULENCE

Virulence may be influenced by reproduction rate, avoidance of host defenses (anatomical seclusion, antigenic disguise, antigenic variation, etc.), and production of enzymes, antigens, and toxins (159,165), all of which can be considered to be under genetic control. Presumably these strategies are aimed primarily at the natural hosts, not at accidental hosts (like humans for *Anisakis*). Genetic studies of *A. simplex* based on multilocus electrophoresis have confirmed the existence of three reproductively isolated sibling species with no morphological differences at the L3 stage (*A. pegreffii* or *A. simplex* A, *A. simplex sensu stricto* or *A. simplex* B, and *A. simplex* C) (166). A similar pattern is observed in *P. decipiens* complexes (*P. decipiens* A, B, and C) (167). There have been no studies of possible among-genotype differences in virulence for animals and humans. However, some authors have suggested that *P. decipiens* larvae, especially in the United States, are less invasive and less pathogenic than *A. simplex* larvae (25,67,138).

Virulence may also be determined by the susceptibility of the host. Due to the high incidence of infection of fish by anisakids, it is striking that only a few individuals appear to be susceptible to both anisakiosis and *Anisakis* allergy. The disproportionately large number of cases occurring in the Basque country, compared to other regions with similar diets within Spain, might be due to a genetic predisposition to this allergy or to different culinary practices in this region. This should be further investigated.

## VIII. CONTROL MEASURES

One of the most effective approaches for controlling infection is to inform the public about the possible health hazards of eating raw or undercooked fish. Despite attempts to develop alternative strategies, deep-freezing to a core temperature below  $-20^{\circ}\text{C}$  and/or heating when cooking to a core temperature higher than  $60^{\circ}\text{C}$  remain the most effective measures to prevent human infection. In Holland, the prevalence of anisakidosis dropped dramatically after 1967, when legislation was introduced obliging deep-freezing of herring before consumption (13). Note, however, that efficient freezing has certain requirements, which mainly depend on the size of the fish and the type of freezer used. Larvae in a whole fish (2–4 kg) take 5 days to die at  $-20^{\circ}\text{C}$  in a home freezer (168), 2–3 hours at  $-20^{\circ}\text{C}$  in an industrial freezer (with a faster temperature ramp) (169), and one hour at  $-40^{\circ}\text{C}$  in commercial blast-freezing procedures (170). Fish-smoking techniques using moderate temperatures (below  $40^{\circ}\text{C}$ ) are reported to be ineffective for killing all the larvae embedded in the

musculature of fish (171). However, the “English method,” performed at 80°C, kills all larvae (172). Baking in a microwave oven for a short time is not effective (173–175), and other culinary practices such as shallow-frying or barbecuing may also be unsafe in view of the case described with fried fish (4).

The nematodes present in fish muscle can survive several traditional food-preserving methods for different periods of time. *Anisakis* larvae can survive up to 25 days in the salt and vinegar mixtures used to marinate herring in Holland (176), 21 days of salting (161), and 35 or 42 days in marinating mixtures, prepared according to German and Danish recipes, respectively (177). The mortality rate in marinated fish appears to be a matter of time, dependent mainly on the salt concentration in the aqueous phase of the fish tissue: approximately halving the salt concentration while keeping the acetic acid concentration constant leads to a threefold increase in survival time (177).

In addition to these procedures, the fish industry and researchers have over the last three decades searched for other methods to detect and eliminate or kill the anisakid larvae present in the fish musculature before marketing and consumption. Unfortunately, none of the candidate methods are effective. Some of these procedures involve:

1. Examination of fish fillets by transillumination (“candling”). This method, widely used in the cod industry, is more effective for *P. decipiens* than for *Anisakis*, because of the former’s larger size and darker color. Bratney has observed that this technique may overlook about 30% of the larvae present in the musculature (178).
2. Artificial peptic digestion. This is laborious and expensive, making it unsuitable for the industry, although it is useful for experimental studies (179).
3. Observation of tissue homogenates under ultraviolet light. This is more effective when the larvae are dead and therefore does not solve the fish industry problem (180).
4. Visual examination. This is easy to perform, but exhibits errors of 17–55% in some small species (181).
5. Gamma irradiation. Unfortunately, doses sufficient to kill larvae damage the flesh (182).

Current European Community (EC) legislation (183,184) concerning the production and commercialization of fish and fish products requires measures including (a) visual examination of the fish and extraction of all visible parasites, (b) withdrawal from the market of heavily infected specimens or pieces, and (c) freezing at temperatures lower than  $-20^{\circ}\text{C}$  for at least 24 hours in the case of fish (e.g., salmon, herring, mackerel) destined for smoking at less than  $60^{\circ}\text{C}$ , marinating, pickling, and/or salting. Similarly, since 1987 the U.S. Food and Drug Administration (FDA) requires that all fish products that will not be cooked or processed at temperature higher than  $60^{\circ}\text{C}$  should be previously deep-frozen at  $-35^{\circ}\text{C}$  for at least 15 hours or frozen at  $-23^{\circ}\text{C}$  for at least one week (156).

As regards allergy, it is worth highlighting that while anisakiosis can only be contracted by the ingestion of raw or undercooked seafood, allergic reactions cannot be prevented by cooking, since the allergens involved are thermostable (185,186). In recent studies, however, oral challenge tests have indicated good tolerance of frozen or lyophilized larvae in some patients with allergy after eating marinated fish (187,188). In addition, serological analysis of a Spanish population using UA3-ELISA suggests that IgE sensitization is only conveyed with the consumption of undercooked fish (189). These results are in contrast with the view that allergy to *Anisakis simplex* can be caused by ingestion of nematode allergens alone. However, and as has been noted for other allergic disorders, we cannot completely rule out the possibility that some individuals with increased susceptibility or with alterations of the gastrointestinal mucosa due to pathological processes or drugs (e.g., nonsteroidal anti-inflammatory drugs) may show responses to the allergens of the nematode alone. For this reason, some allergologists have proposed fish-free diets for patients in risk groups of this type or who have a history of severe allergic disorders.

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# 39

## *Trichinella*

**Wanda Kocięcka\* and Krystyna Boczoń‡**

*University of Medical Sciences, Poznań, Poland*

**Edoardo Pozio§**

*Istituto Superiore di Sanità, Rome, Italy*

**Franz van Knapen¶**

*Utrecht University, Utrecht, The Netherlands*

### I. BACKGROUND

Trichinellosis represents a parasitic zoonosis induced by the nematodes of the genus *Trichinella*. This parasitic invasion follows consumption of raw or semiraw meat of animals infected with *Trichinella* larvae and is cosmopolitan in distribution.

The knowledge about *Trichinella* and trichinellosis has developed in stages. Increasingly frequent detection of *Trichinella* invasions has paralleled progress in investigative techniques. In the second half of the twentieth century, knowledge of the parasite and its taxonomy as well as the disease induced by the parasite and the techniques of detecting the invasion increased rapidly. This has helped to define the geographic range of *Trichinella*, its reservoirs, and its circulation among domesticated and wild animals. This progress has permitted identification of new *Trichinella* species. Molecular techniques have demonstrated that *Trichinella* is heterogeneous and contains eight species: *T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli*, *T. nelsoni*, *T. papuae*, and *T. zimbabwensis*. *Trichinella* antigens have been characterized that allow specific evaluation using immunodiagnostic techniques.

*Trichinella* and trichinellosis continue to attract the attention of a wide range of specialists: medical doctors, veterinarians, biologists, immunopathologist, biochemists, epidemiologists, and geneticists. Their common aims involves definition of the invasion reservoirs among animals, characterization of *Trichinella* genetic types, and rational treatment of patients. In several research center investigations are in progress on mechanisms of the immune response in the host, employing various experimental models and including studies on patients. Detection of *Trichinella* invasion is aimed both at animals from wild and synantropic habitats and at humans from various groups, including ethnic groups exposed to the invasion risk. Recently it has been recognized that infections develop not only in epidemic foci but also as sporadic individual cases. Frequently these cases are unusual, demonstrating a severe course, asymptomatic, or with untypical disease pattern.

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\* Sections I, II, and III.

‡ Section VI.

§ Sections IV, V, and VII.

¶ Section VIII.

The clinical pattern of trichinellosis has been increasingly enriched by new observations and comparative analyses, as related to selected traits of the identified *Trichinella* species and the available new therapeutic potential. Control strategies represent an important aspect of trichinellosis, the foodborne zoonosis. They include enforcement of cooked waste of raw meat regulations, animal inspection, and procedures of pork irradiation to destroy *Trichinella* larvae in meat. Special attention should be paid to the education of consumers, who should be aware that raw meat of pigs, wild pigs, or horses may be the source of *Trichinella* infection.

There exists no better example of successful (veterinary) public health measures to prevent human disease than the abattoir control of *Trichinella* infection. After the recognition in 1895 (Raiillet) of *T. spiralis* as a nematode infection of humans caused by the consumption of infected pork, effective microscopic tools were introduced to identify infected pigs directly after slaughter.

This led to the first meat inspection measures regulated by legislation in many European countries by the end of the nineteenth century. Inspections were carried out initially by veterinarians using a trichinoscope (1). Since then there has been international debate as to the accuracy of the control methods employed and the economic importance of free trade versus sanitary measures to prevent human disease (2).

Because expensive large-scale inspection systems are needed and private home slaughtering often is not reported those are main reasons why trichinellosis is still a worldwide problem. Moreover, there are a variety of other reasons why trichinellosis is still not under control in many parts of the world. These include: socio-economic changes, changing of food habits, immigrant food habits, unexpected sources for infection other than from pork, international trade and tourism (3). Economic drawback and educational failure in large parts of the world prohibit proper control measures at the farm level, the abattoir control system, and information of consumers and awareness of the medical profession.

In this chapter control measures will be discussed against the background of available laboratory methods, policy decisions about objectives in control of trichinellosis and endemicity of trichinellosis in man and animals in given geographical areas.

## II. CHARACTERISTICS

*Trichinella* (formerly named *Trichina*), represents a dioecious nematode belonging to the phylum Nematoda, class Aphasmidia, and family Trichinellidae. It is the smallest nematode parasite in humans and inhabits tissues both in its mature form, inhabiting walls of the small intestine, and in its larval form, developing in muscles. It is a polyxenic parasite, i.e., it is capable of developing and of inhabiting a wide range of hosts. All mammalian species are susceptible to experimental infection with *Trichinella*. Naturally infected species include humans and carnivorous animals (cats, dogs, wolves, foxes, bears, walruses, seals), omnivores (pigs, wild pigs), rodents (rats, mice, marmots), and herbivores (horses, sheep, coypu, rabbits). These organisms may all represent the source of invasion for another host, including humans.

### A. Morphological Characteristics of Developmental Forms

The developmental cycle of *Trichinella* spp. includes mature (female and male) forms and larval forms. Depending upon the stage of development, newborn larvae, migrating larvae, and encapsulated larvae are distinguished morphologically.

The anterior portion of the *Trichinella* body contains the esophagus, the nervous system, and the excretory system. In the alimentary tract, the esophagus reaches the midpoint of the body length. It consists of the proximal part, with strongly developed muscles, and the glandular part. The intestine forms a straight tube terminated by a cloaca at the end of the body.

The stichosome represents a characteristic organ consisting of a single layer of 50–55 semicircular cells (stichocytes), which surround the esophagus. The stichosome is present both in mature forms and in larvae. The number of stichocytes changes depending upon developmental stage, sex, and species of *Trichinella*. The stichosome represents an important morphological and functional



element in biological development of *Trichinella* (4–6). The stichocyte contains the cell nucleus and granules, which are the source of antigen secretion. Two types of granules have been distinguished, best recognized in invasive larvae. Beta granules are smaller and are located in the frontal part of a stichocyte, while the lighter and more coarse alpha granules are positioned in the rear portion of the cell. During ecdysis, stichocytes release their content to the fine lateral canaliculi, which drain to a larger duct. The ducts open to the esophageal lumen. Antigenic (excretory-secretory) material released by stichocytes is particularly effective in inducing an immune response in the host.

The female of *Trichinella* spp. is 3.0–4.8 mm in length, 60–72  $\mu\text{m}$  in diameter, and contains a broad, single ovary located in the posterior fragment of the body. The latter extends into a short oviduct and then into an elongated uterus. The seminal receptacle that accumulates sperm and fertilized eggs is located between the ovary and the uterus. The uterus contains multiple egg cells of oval shape,  $30 \times 40 \mu\text{m}$  in size. The eggs develop in the uterus. By 36 hours postfertilization the young larvae are formed and freed from their delicate egg envelopes and arranged in a row. At 5 days postfertilization the larvae are expelled through the short vagina, which opens in the anterior 20% of the female's body.

The male of *Trichinella* is smaller, 1.5–1.6 mm in length and 33–40  $\mu\text{m}$  in diameter. It contains a single testis and a single vas deferens, which opens up to the seminal vesicle. This is terminated at the ejaculatory duct, releasing its content to cloacae. The region is equipped with two appendages, which serve as holding organs during copulation. The appendages represent an important morphological element in distinguishing the sex of *Trichinella*. Between the appendages, four tubercles are located: two ventral conical papillae and two dorsal papillae, believed to play a role during fertilization. Sperm production takes place just before or immediately after the last ecdysis.

The newborn larva is 80–120  $\mu\text{m}$  in length and has a diameter of around 6  $\mu\text{m}$ , so that it can pass through capillaries. The inner organs of the newborn larva are poorly developed and primitive. The larva contains an intestine, preceded by an esophagus in the form of a thin tube. Newborn larvae have not been noted to possess a stichosome. Fragments of stichosome, consisting of a few stichocytes, do not appear in the course of stepwise development until the seventh or eighth day. Despommier (7) noted in his morphological description that the frontal part of the body in newborn larva, just before the esophagus, contains a small dagger, the stict, which probably facilitates larval penetration from tissues to the circulation. This structure has been observed in migrating larvae but not in invasive larvae or adult forms.

The encapsulated larva represents the fully developed larva in the striated cell of a skeletal muscle, the so-called nurse cell, which forms an intracellular niche for the larva (7). The larvae in muscle cells encapsulate only in five species (*T. spiralis*, *T. nativa*, *T. britovi*, *T. nelsoni*, and *T. murrelli*). The collagen capsule of the larva is oval in shape,  $200 \times 600 \mu\text{m}$ . The capsule shape varies with the *Trichinella* species and the host. In the human host, the capsule has an oval shape; in the pig or wild pig, the capsule is more spherical and the dimensions are somewhat smaller. The encapsulated larva represents the invasive form, hazardous for the subsequent host.

## B. Life Cycle

Life cycle is strictly linked to parasite morphogenesis. Following ingestion of the muscle larva, the nurse cell is digested by gastric juice and, in subsequent segments of alimentary tract, by bile acids. The larva released in the small intestine of the host grows and reaches sexual maturity in 3 days. In the developmental cycle of *Trichinella*, two basic biotopes can be distinguished: the multi-intracellular enteral niche and the multi-intracellular parenteral niche (7).

After fertilization, which may take place in the third day of invasion, embryogenesis commences in the uteri of the females, and on the fifth day newborn larvae are delivered directly to the lamina propria of the intestine. From there, the larvae penetrate to the capillary lymphatic vessels, flow to the thoracic duct, and with the venous blood enter the right heart and the lungs. Due to their small size they may pass through the network of capillaries that entwine the pulmonary alveoli and, then, through the left heart enter the greater circulation. In this manner the migrating larvae may penetrate all organs and tissues, although they permanently settle only in selected tissues. The latter include

skeletal striated muscles, which represent the principal and final biotope of the second generation of *Trichinella*. Thus, the migrating larvae appear in the circulation on the fifth or sixth day; in the course of their migration they manifest a 10-fold increase in size and exhibit development of internal organs and of the stichosome. By the twelfth or thirteenth day of infection, the larva is capable of invading the muscle cell. In the cells, the larva orients itself longitudinally and then coils up and encapsulates, inducing, in parallel, basophilic transformation of the muscle cell. After penetrating the muscle cell, larval organogenesis progresses to terminate on the seventeenth day. At that stage the larva is already fully invasive. According to Despommier (7), from the twentieth day of invasion the encapsulated larva and the sarcoplasm of the invaded muscle cell form the so-called nurse-cell-infective L<sub>1</sub> larva complex. The complex is stable and may exist until the end of host's life or until it degenerates or becomes calcified.

### **C. Characteristics of *Trichinella* Antigen**

Studies on the detection and identification of *Trichinella* antigen concern the surface antigen, present in the cuticle or in the excretory-secretory material of *Trichinella* stichocytes. Studies of the surface antigen, conducted with the use of various techniques, demonstrated heterogeneity of the antigen, depending upon developmental stage of the parasite (8–10). Using autoradiography and polyacrylamide gel electrophoresis, glycoprotein antigens demonstrate variable molecular weights at various stages of *Trichinella* development.

The excretory-secretory antigen originates mainly from alpha granules of stichocytes of the invasive larvae. Studies of several authors (10,11) using modern molecular techniques have demonstrated that the strongly immunogenic and specific antigen isolated from excretory-secretory material of *Trichinella* larvae is a glycoprotein of 49 kDa molecular weight. This antigen has proven valuable in the immunodiagnosis of trichinellosis.

## **III. DISEASES**

### **A. Disease Incubation Period**

Ingestion of *Trichinella* larvae by humans initiates the incubation period, which ends when the syndrome of clinical symptoms appear. The incubation period exhibits a variable length that reflects several factors: size of the invasive dose (number of larvae), frequency of consumption of infected meat, type of meat consumed (raw, semiraw), as well as the involved species and strain of *Trichinella*. The length of the incubation period ranges from 2 to 45 days. It is generally thought that the shorter incubation period is correlated with a more severe disease course. According to some authors who studied large numbers of such patients, severe trichinellosis exhibits a short incubation period ( $7 \pm 3.2$  days), while the moderate, benign, and abortive forms of the disease are associated with mean latent periods of  $16 \pm 5.9$ ,  $21 \pm 9.1$ , and  $30 \pm 30$  days, respectively. In recent years, clinicians have not always detected such a relation, and in some epidemics, prolonged incubation periods (18–30 days) are followed by severe illness.

The incubation period is not always asymptomatic. In approximately 10% of patients, development of typical symptoms is preceded by loose stools containing no mucus or blood. They reflect the self-cure phenomenon, typical for the acute stage of the invasion and parasite development in the small intestine. The intestinal symptoms usually last for 4–6 days, but they occasionally persist and associate with the syndrome of typical clinical signs/symptoms of trichinellosis. Intestinal symptoms may be accompanied by flatulence, moderately intense abdominal pain, loss of appetite, and, sometimes, vomiting.

### **B. Clinical Picture of Acute Trichinellosis**

Most patients manifest a sudden and dynamic beginning of the disease. General weakness, chills, and headache are accompanied by fever (occasionally up to 40°C) and tachycardia (corresponding to elevated body temperature). Excessive sweating is typical for trichinellosis. Symmetrical eyelid

and periocular edema develops (Fig. 1); frequently the edema includes the entire face. Conjunctivae demonstrate inflammation of blood vessels, and approximately 25% of patients exhibit petechiae and intraconjunctival hemorrhages (12–15) or hemorrhages to nail beds. Pains in various muscle groups, which may restrict the patient's motility, follow. Early symptoms develop at varying intervals and intensity. They also do not disappear in a defined standard sequence. The pattern can be fully appraised and the severity of disease estimated only after further observation.

Fever, eyelid and periocular edema, and muscle pains form the principal set of clinical signs/symptoms. They are always accompanied by hypereosinophilia and usually by high white blood cell counts. Fever represents one of the most frequent and earliest signs of trichinellosis. It increases rapidly, sometimes reaching 39–40°C. Fever may be continuous and persist up to 3 weeks in severe clinical forms. In mild cases subfebrile body temperatures are frequently noted, which disappear after a few days of effective treatment. The frequency of fever varies from 41 to 100% of cases in various foci of the disease.

Periocular and facial edema represent a typical sign, but their intensity varies depending upon the allergic reactivity of the host (Fig. 2). The edema is symmetrical and usually vanishes in 5 – 6 days, particularly with glucocorticoids treatment. Eyelid edema is usually accompanied by conjunctival hyperemia, itching, lacrimation, and, occasionally, light intolerance. Analysis of numerous epidemic foci of trichinellosis showed that periocular swelling develops in 17–100% of patients. A particularly low incidence of ocular signs has been documented in epidemic foci of trichinellosis, resulting from consumption of horse meat infected with *T. spiralis*, *T. britovi*, or *T. murrelli*. Such signs were observed in only 26–35% of patients (16–18). Eye pains upon ocular movements have been observed in around 77% of patients (13). Also, disturbed sight acuity and sometimes bilateral exophthalmia deserve attention. Nystagmus, reflecting involvement of ciliary muscles of patients (13), may occur. This pain may be accompanied by disturbances in visual acuity and bilateral exophthalmia.

Muscle pain involves various groups of muscles, and the intensity reflects the severity of the disease course. Pain develops in nuchal and trunk muscles, in the muscles of the upper and lower extremities, and less frequently in masseter muscles. It affects patients during execution of movements (particularly in lower extremities), while spontaneous pain is less frequently observed. According to some authors (19,20), spontaneous muscle pain troubles patients with a severe course



**FIGURE 1** Periorbital edema and conjunctivitis in patient with acute stage of trichinellosis. (From Ref. 99.)



**FIGURE 2** Periorbital edema and mouth swollen on 18 day of experimental Trichinellosis in *Macaca mulatta* monkey. (From Ref. 38.)

of the disease or due to complications in the form of thrombophlebitis. In some patients with exceptionally grave course, adynamia dominates, which may persist for a long time, reflecting pronounced intensity of angiomyositis type pathology or neuromuscular disturbances. Itching and numbness or tingling sensation in various muscle groups frequently manifest together with muscle pain. Restricted motility due to muscle pain associated with movements may lead to contractions, particularly in knee and elbow joints, nuchal pseudorigidity, and difficulties in opening the mouth. The signs gradually disappear during convalescence, and their complete regression may be obtained with physiotherapy.

Clinical analysis of some trichinellosis foci in Europe (16–19,21–26) has shown that frequency of muscle pain manifestation depends upon the source of infection and species or isolate of *Trichinella*, and it ranges between 59 and 97% of patients. According to Viallet et al. (27), who studied trichinellosis foci in Canada resulting from consumption of walrus meat, muscle pains have been observed in a low proportion of patients (25–60%). No periocular edema was observed in the patients, while the clinical pattern was dominated by diarrhea (seen in 100%) of patients.

Apart from those mentioned above, other signs and symptoms may accompany trichinellosis originating in the alimentary tract, cardiovascular system, or nervous system. Signs from the alimentary tract most frequently include loose stools (sometimes up to 10–15 times a day). The stools are greenish-brown in color, frequently with an admixture of mucus but containing no blood. Persistent diarrhea usually causes deterioration in the patient's general condition, leading to dyselectrolytemia and marked hypoproteinemia. These prolong the convalescence period.

Nausea and vomiting usually appear during the first days of the disease and subside after a few days. Frequency of intestinal disturbance is estimated at 6–60% of cases in various foci of

trichinellosis. In some epidemics, however, these may dominate the clinical pattern and be present in all affected patients (27,28). Biopsies of intestinal mucosa performed in patients with persistent diarrhea demonstrate pathomorphology. The lesions involve deformation of intestinal villi, enterocyte proliferation that reaches apices of the villi, deepening of Lieberkühn crypts, and intense cell infiltrates in lamina propria. Mononuclear cells, plasma cells, and eosinophils dominate (14) the cell infiltrates.

Disturbances of the cardiovascular system are particularly evident in the severe and moderate courses of the disease. These develop usually between the third and the fourth weeks of the disease. Intensity of the signs/symptoms depends upon the time at which therapy was started and the health condition before the *Trichinella* invasion began. Pains in the heart region (6.2%) and tachycardia (22.1%) occur. Auscultation may detect dull heart tones (36.6%), heart murmurs (23.9%), and, occasionally, arrhythmia (21,22).

Alterations in ECG records in patients at the acute stage of trichinellosis include flattened T, lowered S-T, and lowered QRS and disturbed atrioventricular or interventricular conductance. The ECG records persist despite subsidence of other clinical signs/symptoms of trichinellosis. This reflects persisting hypoproteinemia and hypopotassemia. Potassium ion deficit in heart muscle cells leads to polarization disturbances that are expressed by pathological ECG record (29). Compensation of potassium deficit in such cases promotes rapid normalization of ECG. Biochemical exponents of heart muscle injury also include released muscle enzymes (CPK, LDH), the augmented activity of which is important for clinical evaluation of the heart muscle, particularly when skeletal muscle pain has already subsided.

Disturbances of the nervous system are of a heterogeneous type. The most frequent include headaches, which are vascular in origin and accompany almost every case of trichinellosis, including mild ones. In patients with the severe course of trichinellosis, blurring of consciousness or excessive excitation, frequently somnolence, or apatia is observed. In some cases, signs of cerebrospinal meningitis or encephalopathy are seen (30–32). The alterations in the cerebrospinal fluid are of little known significance. Slight increases in protein content and moderate cell levels are found, the latter not exceeding 250 cells/mm<sup>3</sup> and comprising lymphocytes and eosinophils (possibly important in differential diagnosis). Dizziness, nausea, and tinnitus are transient and subside in parallel with improvement in general condition. Neuromuscular disturbances, i.e., decreased muscle strength, lowered tendon reflexes, disturbed swallowing, lockjaw, and muscle contractions, are usually only encountered between the second and fourth week of the disease, but in patients affected by the severe form of the disease they may persist up to 2 months.

In patients with a severe course of trichinellosis, Fourestie et al. (31) described a neurocardiological syndrome consisting of Central Nervous System (CNS) pathology and cardiac disturbances. Significantly, neurological signs and symptoms in the form of a diffuse encephalopathy appeared a few days after signs of trichinellosis became evident. Computed tomography (CT) of the head demonstrated hypodense foci in the cortex and white matter of both cerebral hemispheres. The neurological symptoms were accompanied by heart muscle pathology, manifested by a pathological ECG record and by high muscle enzyme activity with peak levels of eosinophils in peripheral blood. In such cases, the cytotoxic action of eosinophils on the muscle is pronounced, mediated by eosinoperoxidase (EP) and by eosinophil cationic peroxidase (ECP). When manifested with the neurocardiological syndrome, trichinellosis is frequently lethal. The frequency of neurological signs and symptoms in the course of trichinellosis has decreased since glucocorticoids and anthelmintics were introduced as part of treatment. Within the past 25 years, neurological signs and symptoms in Polish epidemic foci have been noted in 3.1–46.3% of cases. In France (23), neurological signs were noted in approximately 60% of trichinellosis foci. Prior to the introduction of corticosteroids, neurotrichinellosis was lethal in 46% of cases; after their introduction, the rate dropped to 17%

### C. Morphological Pattern of Pheripheral Blood

High eosinophil and leukocyte levels in peripheral blood characterize the clinical pattern of trichinellosis. Pronounced eosinophilia represents an inherent trait of the disease. Eosinophilia develops

in each case of *Trichinella* invasion. It appears early, sometimes before symptoms characteristic for trichinellosis disease; therefore, it represents a valuable diagnostic criterion. Eosinophil level varies; tested by Carpentier's technique, it amounts to 1000–19,000 per mm<sup>3</sup>. This corresponds to 30–60% and, in some cases, up to 86% leukocytes.

Eosinopenia observed at the beginning of the acute stage of the disease or abruptly decreasing levels of acidophilic granulocytes in the course of the disease correspond to immunosuppression and are associated with severe forms of the disease. Eosinopenia forecasts an unfavorable course of the disease (32) and may be lethal. Some patients with a severe course of trichinellosis may also demonstrate lymphopenia (33). Increase in blood eosinophil level has been found to correlate with an invasive dose of *Trichinella* larvae. However, it is not correlated with the intensity of muscle invasion, severity of the clinical course, or increase in eosinophil level (28).

Leukocytosis is characteristic of the acute stage of trichinellosis and reflects developing inflammation in the course of the disease. Leukocyte levels may increase to 15,000–30,000 per mm<sup>3</sup>. Leukocytosis appears early and develops in parallel with increasing eosinophilia, although it regresses more rapidly with vanishing acute signs of the disease while augmented eosinophil levels remain high and may persist for 6–8 weeks. Reappearance of leukocytosis points to development of complications (e.g., thrombophlebitis, pneumonia), which are typical for severe forms of trichinellosis (19).

#### **D. Metabolic Disturbances**

Metabolic disturbances in trichinellosis appear as enzymatic disturbances, disturbances in water and electrolyte turnover, and disturbed albumin-to-globulin blood ratios. The disturbances develop in parallel at the acute stage of the disease and increase in severity with the progressing pathological process, particularly in patients untreated in the early stages of the disease.

Enzymatic disturbances pertain mainly to the muscle enzymes: creatinophosphokinase (CPK), lactate dehydrogenase (LDH) and its isoenzymes (LDH<sub>4</sub>, LDH<sub>5</sub>). They were demonstrated in both clinical and experimental studies (22,24,34–36). Increase in CPK activity was observed most frequently between the second and fifth weeks of the disease in 75–90% of examined patients. The increase was multifold and, in some cases, even greater. However, no relationship could be discovered between the severity of clinical course and increase in CPK or LDH activities. Increase in LDH activity and in LDH<sub>4</sub>, LDH<sub>5</sub> activities were observed between the first and sixth weeks of invasion, with maximum values reached earlier than peak levels of anti-*Trichinella* antibodies (36).

Proteinase activity was examined in muscles at various stages of experimental trichinellosis (37), and it increased between the first and fifth weeks of invasion. Proteinase may provide a useful laboratory parameter for the evaluation of trichinellosis course in humans.

Water and electrolyte disturbances involve mainly potassium (K) ion deficit in blood. Disturbed electrolyte levels are particularly evident in patients with persistent diarrhea, in vomiting patients, and in patients with profuse sweating. The hypopotassemia participates in decreasing muscle strength and in development of disturbances in heart function.

Disturbances in plasma proteins develop later and are particularly typical for severe or moderate course of disease. The disturbances involve a pronounced deficit in serum proteins (hypoproteinemia) and hypoalbuminemia. They develop due to progressive development of *Trichinella* larvae and their increasing mass, consumption of proteins and of albumin needed for reconstruction of the host tissues, as well as a translocation of albumin and liquids from the intravascular to the intercellular compartment. The extent of hypoalbuminemia correlates with the clinical severity of trichinellosis. It is manifested by orthostatic edema on shanks and, occasionally, on the trunk and upper extremities as well as by transudate to body cavities.

The above-mentioned metabolic disturbances in trichinellosis motivated recommendations for implementation of rational therapy, aimed at counterbalancing the water, electrolyte, and protein deficits by appropriate supplementation.

Bioelectric disturbances in muscles develop because of failing function of invaded muscle cells with the subsequent pathomorphological and ultrastructural alterations. The disturbances are ex-

pressed in an altered electromyographic (EMG) record, which exhibits lowered amplitude and shortened duration of motor unit potentials. The alterations are typical for the primary type of muscle injury noted in the course of trichinellosis in experimental animals and in humans (38–40). The pathology in EMG records disappears within a few months in parallel to clinical improvement and histological healing of muscle tissue.

## E. Complications

Complications may develop at both the early and late stages of the disease and may involve multiple systems. They often affect patients with a severe course of trichinellosis and are frequently linked to lowered immunity. They also tend to affect patients improperly treated at the early stage of the disease. The complications pertain to the respiratory system, organ of sight, cardiovascular system, and nervous system.

Respiratory disturbances at the early stage of the disease exhibit immune and vascular bases. Most frequently they manifest the form of antigenic pneumonitis and disappear rapidly under corticoid treatment (21). At a later stage of the disease—between the third and seventh week—pneumonia and plueritis of bacterial aetiology may develop in patients with a severe clinical form. In some patients lung infarct may manifest itself due to circulatory disturbances. Hypoproteinemia may result in an accumulation of liquid in pleural cavities.

Sight disturbances appear as a sequel of intense invasion of eye muscles, followed by increased intracranial pressure and involvement of cranial nerves. This results in eyeball pain of long duration (evident upon eye movements), fatigability during reading, strabismus and exophthalmus, which may persist for a few weeks after other morbid signs have disappeared (12,13,19).

Cardiovascular disturbances are most frequent and develop in 20% of hospitalized patients between the third and fifth weeks of the disease, mostly in those with a severe course of trichinellosis or in patients in whom treatment was delayed. Patients may manifest myocarditis-type symptoms; these represent the most frequent cause of death (21,22,31). Pulmonary artery emboli or paroxysmal tachycardia may cause sudden death even after all other morbid signs have disappeared. Deep vein thrombophlebitis is a frequent complication of trichinellosis that significantly prolongs the convalescence.

Neurological complications affect mainly the central nervous system of patients with a severe course of the disease. Immunopathological abnormalities are encountered that produce focal or diffuse injuries in various parts of the brain or spinal cord and less frequently in peripheral nerves. Diverse types of paresis and paralysis follow this, sometimes by hemianopia and seldom anisocoria, transient aphasia, and signs of involvement of cranial nerves (19,21,30). While the neurological disturbances, which appear at early stages of the disease, represent no contraindication to application of corticosteroids and most frequently vanish with treatment, complications appearing at late stages (after the third or fourth week) may lead to permanent consequences and require consultation with a neurologist in designing treatment.

## F. Differential Diagnosis

Differential diagnosis is particularly important at the acute stage of trichinellosis when the decision has to be made to implement appropriate treatment. This is particularly difficult with isolated cases and in untypical course cases. Due to the presence of periocular and facial edema and fever, it is advisable to differentiate trichinellosis from diseases such as acute glomerulonephritis, serum sickness, toxic-allergic reactions to drugs or allergens, and dermatomyositis.

In cases of intense headaches and nuchal rigidity with blurred consciousness and overexcitability, trichinellosis should be differentiated from various forms of cerebrospinal meningitis, encephalopathy, and neuroinfections with elevated body temperature. In cases of trichinellosis associated with intraconjunctival hemorrhages and intradermal petechiae, the differentiation should consider leptospirosis (*L. icterohaemorrhagiae*) and exanthematic fever.

In patients in whom diarrhea dominates the clinical pattern, the disease should be differentiated

from shigellosis or salmonellosis and other infections of the gastrointestinal tract (viral, bacterial, or parasitic, e.g., induced by *Giardia intestinalis* infection). In patients with a particularly grave course of the disease but free of periocular edema, the clinical pattern may include neurological disturbances (confusions or overexcitability), lowered white blood cell (WBC) count, lowered eosinophil levels in blood, and decreased antibody production. The syndrome should be differentiated from typhoid fever. Muscle biopsy is the decisive test in such differentiation. Detection of *Trichinella* larvae and typical muscle cell pathology with signs of basophilic transformation unequivocally confirms the invasion.

#### IV. EPIDEMIOLOGY

Although trichinellosis has been reported to have both a sylvatic and a domestic cycle (41), it is principally a zoonosis of sylvatic animals with cannibalistic and scavenger behavior. All warm-blooded animals are receptive to this infection, yet its spread in nature depends on those animals in which muscle larvae are transmitted from individual [i.e., from carnivore to carnivore, from carnivore to omnivore, or in unusual cases from omnivore to herbivore (horse, sheep, deer, etc.)] (42,43). Furthermore, non-encapsulated *Trichinella* larvae have been detected in naturally infected crocodiles (44) and in experimentally infected snakes, varans, and tortoises.

In the genus *Trichinella*, all currently recognized species (*Trichinella spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. nelsoni*, *T. murrelli*, *T. papuae*, and *T. zimbabwensis*) (44,45) are transmitted by a sylvatic agent that is widespread on all continents (except Antarctica). Cannibalistic and scavenger behavior are the key factors in transmission, yet this feeding behavior occurs only in carnivore populations living in remote wild regions, including mountain areas and natural parks (43). In fact, animal carcasses of cogener animals are not an attractive food source for carnivores living in or near human habitats, where there are abundant food sources, such as rubbish dumps and domestic animals (46), and which can be considered free from sylvatic trichinellosis. Nonetheless, when humans fail in the management of wild and domestic animals (43), sylvatic species of *Trichinella* may invade the domestic habitat. However, the domestic habitat does not represent a good environment (except in the case of *T. spiralis*) because the reproductive capacity of these parasites in swine and rodents is low. Thus a domestic cycle (pig-pig, pig-rat-pig, etc.) cannot be maintained and domestic animals infected with sylvatic species represents a “dead end” for the sylvatic cycle.

The only exception to the above is *T. spiralis*, for which there exists a domestic cycle, mainly in pig and rat populations. This parasite is maintained in the domestic habitat primarily when humans fail to properly dispose of carcasses of domestic and game animals and allow uncontrolled garbage dumps to exist. *T. spiralis* is the only species that shows a high infectivity for swine and rodents (42), and the main features of domestic trichinellosis are as follows: poor sanitary conditions, presence of sylvatic and/or synanthropic animals infected with *T. spiralis*, low level of industrialization in the pig farms, and lack of veterinary controls. The worldwide distribution of sylvatic species of *Trichinella* is related to isotherms, except in the cases of *T. spiralis* and *T. pseudospiralis*, which are cosmopolitan in distribution. This observation suggests that there is a “free-life” stage in the life cycle that depends on environmental temperature (42,43,47,49). This stage corresponds to the time of survival of muscle larvae in tissues of host carcasses, when larvae are no longer protected by the homeothermy of the host. This enhances the role of animals with cannibalistic and scavenger behavior as the main hosts and the importance of the nurse cell in protecting larvae in decaying muscle tissues, and it explains why the metabolism of the nurse cell–larva complex is anaerobic.

*T. spiralis* shows a cosmopolitan distribution due to the passive introduction of domestic pigs and synanthropic rats. This parasite is currently prevalent in all continents except Antarctica: North and South America (Argentina, Canada, Chile, Mexico, and the United States), Europe (Bulgaria, Byelorussia, Finland, former Yugoslavia, France, Germany, Hungary, Latvia, Lithuania, Moldavia, Poland, Romania, Russia, Spain, Sweden, The Netherlands, and Ukraine), Africa (Egypt), Asia (China, Indonesia, Russia, Thailand, and Vietnam), and Australia (New Zealand). In the domestic habitat the main reservoir is the pig, which can acquire the infection through cannibalism or by eating infected pork scraps and rats. However, infected rats are present only if the infection is



widespread among pigs or synanthropic and sylvatic animals. The parasite *T. spiralis* cannot be maintained in nature in a rat population for a long period of time (i.e., infected rats are present because there are infected pigs and not vice versa). Most human *T. spiralis* infections come from consumption of domestic pig, wild boar, and horse meat (42,43).

*T. nativa* is the etiological agent of sylvatic trichinellosis in arctic and subarctic regions of North America, Europe, and Asia. The isotherm-5°C in January seems to be southern limit of distribution of this parasite (47). The main reservoirs are carnivores living in frigid zones (polar bear, grizzly bear, arctic fox, wolf, wolverine, and walrus). There have been very few reports of this parasite in domestic and sylvatic swine (48) because these hosts, like rats, are resistant to this infection. Humans acquire *T. nativa* by consuming raw or undercooked meat from bears, walruses, or other infected carnivores. Muscle larvae can survive in frozen muscle tissues of carnivores for years (49).

*T. britovi* is the etiological agent of sylvatic trichinellosis in temperate areas of the Palearctic region. The isotherm-6°C in January seems to be the northern limit of distribution (42,43). The main reservoirs are carnivores living in the temperate zone (fox, wolf, jackel, raccoon dog, mustelids, etc.). This parasite can infect domestic and sylvatic swine and sometimes rodents, but it cannot survive in the domestic habitat because of its low reproductive capacity in these animals. Humans acquire this infection by consuming wild boar meat or, on rare occasions, dog and horse meat (26,42,43). Muscle larvae of *T. britovi* can survive in frozen muscle tissues of carnivores for several months (50), whereas they survive in frozen meat from wild boars only 2–3 weeks (50).

*Trichinella pseudospiralis* is the most peculiar species in the genus *Trichinella* since muscle larvae are two-thirds the size of those of the other species, they do not induce muscle cells to change into nurse cells, and because muscle larvae can infect both mammals (marsupials, rodents, wild boars, domestic pigs) and omnivorous and carnivorous birds. This parasite shows a cosmopolitan distribution, though it has rarely been detected in animals [e.g., single cases in Kazakhstan, India, Russia, Kamchatka, Thailand, Italy, France, Tasmania, and the United States] (51) or in humans (e.g., a single case in Tasmania and outbreaks in Thailand, Kamchatka, and France) (51–55).

*Trichinella nelsoni* is the etiological agent of sylvatic trichinellosis in sub-Saharan Africa. The main reservoirs are large carnivores living in natural parks and protected areas (spotted hyena, striped hyena, lion, leopard, etc.), although omnivores (warthog and bush pig) can sometimes acquire this infection and transmit it to humans (56,57). The low level of *T. nelsoni* infection in sylvatic swine, the practice of eating only well-cooked meat, and religious laws that forbid the consumption of pork probably account for the rarity of human trichinellosis in sub-Saharan Africa. In fact, fewer than 100 cases have been documented, and those only in Kenya, Tanzania, Senegal, and Ethiopia (57,58).

*Trichinella murrelli* is widespread among carnivores from the eastern to the western coast of the United States (black bears, red fox, bobcat, coyote, raccoon, etc.) (59). This parasite was detected in a horse imported from Connecticut to France, where it was the source of human infections. In the United States, humans acquire this parasite eating raw or undercooked bear meat. This species is unable to infect domestic pigs and rats (59,60).

*Trichinella papuae*, the second non-encapsulated species, was discovered in domestic and sylvatic swine of Papua New Guinea (61). Experimental infections have shown that this species can also infect reptiles, but not birds. Human infections have been documented with a prevalence of 28% in the endemic areas, where young hunters frequently acquire this parasite eating raw or undercooked wild pig (62).

*Trichinella zimbabwensis*, the third non-encapsulated species, was discovered in 40% of farm crocodiles (*Crocodylus niloticus*) of Zimbabwe (44). Experimental infections have shown that this species can infect mammals including several species of monkeys, domestic pigs, rats, and mice, but not birds (44). Infection in humans has not been documented.

## V. ISOLATION AND IDENTIFICATION

Artificial digestion can be used to isolate larvae in the infecting stage from muscles of infected hosts for the purpose of diagnosis and identification of the species or genotype.

## A. Isolation of Larvae from Fresh Muscle Tissues

Apparatus needed includes blender, magnetic stirrer, incubator at 38–39°C, 1–2 L beaker, suction pump, and scale; chemicals needed include hydrochloric acid (HCl), pepsin 1:10.0000, tap water at 38–40°C, and distilled water.

- Step 1. *Preparation of tissues for digestion*: Remove as much as possible the fat, perymysium, and parts of the tendons that are present in the sample, being careful not to remove muscle tissues, especially those near the tendon insertion. Cut the muscle tissues into very small pieces (as small as possible).
- Step 2. *Digestive fluid*: Add HCl to tap water (38–40°C) until obtaining a 0.5% concentration (vol/vol). Add pepsin until obtaining an 0.5% concentration (wt/vol), and stir gently until dissolved.
- Step 3. *Digestion*: Place the small pieces of the muscle sample in the digestive fluid until reaching a 1/1 concentration (wt/vol). Blend for 30–60 seconds. The objective is to obtain the smallest possible pieces of muscle without cutting and killing muscle larvae. Pour the blended muscle tissue mixture into 1–2 L beaker. Rinse the blender with additional digestive fluid and add this to the beaker. Add enough additional fluid to obtain approximately 30–40 mL digestive fluid per gram of muscle tissue. Place the beaker on a magnetic stirrer in an incubator at 38–39°C.
- Step 4. *Collection of larvae*: Stop the magnetic stirrer after 30–40 minutes (if the muscle samples are already partially decayed, the time of digestion must be shortened). Wait for 15 minutes to allow larvae to sediment. Remove the supernatant with a pump, being careful not to suck out the sediment. Add tap water (30–40°C). Wait for 15 minutes to allow larvae to sediment. Remove the supernatant with a pump. Wash larvae 2–3 times. Place the sediment in a Petri dish and examine under a dissection microscope at a 20–50 magnification. Transfer larvae to another Petri dish containing distilled water at 38°C with a pipette.
- Step 5. *Preservation of larvae*: Place motile larvae, either singly or in groups, in 15 mL conical tubes containing 5 mL distilled water. Immediately store the conical tubes at –30°C. Alternatively, place clean motile larvae in absolute ethyl alcohol and store them in a 0.5 mL conical tube at +4°C.

## B. Isolation of Larvae from Frozen Muscle Tissues

Cut the frozen muscle sample into small pieces to facilitate fast thawing. Immediately after thawing, digest the sample as above but for no longer than 15–20 minutes. All procedures (i.e., sedimentation, washing, and collection of larvae) must be performed on ice. Immediately store the conical tubes at –30°C.

## C. Identification of Larvae at Species or Genotype Level

The lack of morphological species-specific characters and the fact that the biological and biochemical features do not allow the species to be easily or rapidly identified on the basis of the availability of one or few dead larvae, stress the importance of methods based on polymerase chain reaction (PCR). A plethora of PCR-derived analyses have been developed to identify the species or genotype of these parasites (RAPD, PCR-RFLP, PCR-SSCP, multiplex-PCR, CFLP) (63–66) but at the present time, only two assays (multiplex-PCR and PCR-RFLP) allow single larvae to be identified.

1. Multiplex PCR—This analysis can unequivocally distinguish all currently recognized species of *Trichinella* (the five encapsulated and the three non-encapsulated species) as well as the three genotypes of *T. pseudospiralis* from the Palaearctic, Nearctic, and Australian regions, and *Trichinella* T6 a genotype of *T. nativa* present in the Rocky mountains from

Idaho and Montana up to Alaska (66). This analysis utilizes five primer sets generated from the expansion segment five (ESV) region of the large subunit ribosomal DNA, and from internal transcribed spacer (ITS) regions 1 and 2. One or two PCR fragments with a definite bp size characterize each species and genotype. The ability to perform this test on individual larvae makes it ideal for epidemiological samples where often only a single larva or few larvae are available. Single fresh, dead, frozen, or in absolute ethyl alcohol larvae can be used (66).

2. PCR-restriction fragment length polymorphism (RFLP)—PCR-RFLP has an excellent discriminating power in genetic analysis, generating a unique set of DNA fragments that allow for the identification, using a single larva, of six species (all the encapsulated species and *T. pseudospiralis*) and the genotypes *Trichinella* T8 and *Trichinella* T9 of *T. britovi*, which cannot be differentiated by the multiplex-PCR analysis described above (66). The PCR-RFLP also allows intraspecies polymorphism to be identified (64,66).

## VI. PATHOLOGY

The complex picture of pathological changes in the host organism during trichinellosis depends on the changes caused by the two generations of *Trichinella*: adults in the host intestine and encapsulating larvae in the host muscles.

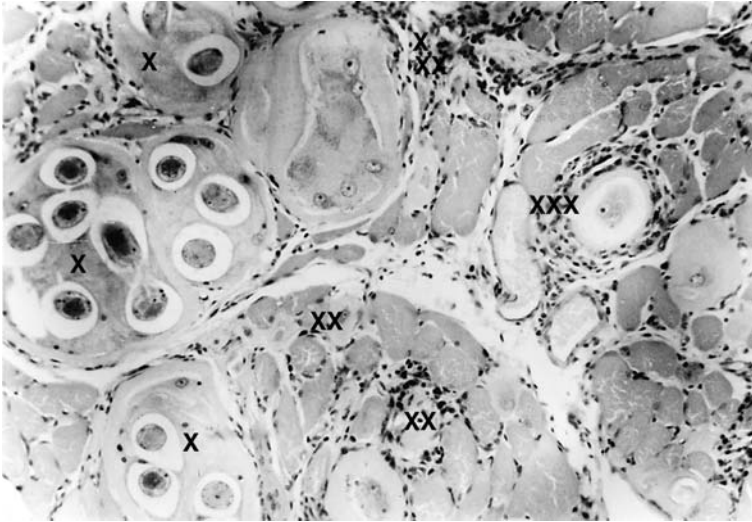
The immunological response (proportional to the original number of ingested cysts) is strongly dependent on individual host hypersensitivity of type I, which plays a major role in an early intestinal stage of trichinellosis. The adult forms of *Trichinella* induce severe intestinal pathology, including villus atrophy and crypt and goblet cell hyperplasia. This pathology is directly correlated with the activity of T-cell cytotoxic subsets. The production of cytokines and antibodies in lymphocyte Th subpopulations is very important for the mechanism of an early immunological response (67). This response varies with the host species.

The immune response includes the activation of T lymphocytes; these are involved in inflammatory changes in the intestine responsible for the spontaneous expulsion of adult forms from this location (self-cure). Following their activation, the Th2 cells produce a factor that induces stem cells to differentiate preferentially along the goblet cell lineage (68). Treatment with anti-IL-4 antibody is responsible for the delayed expulsion of the *Trichinella* larvae whereas treatment with anti-IL-5 for the accelerated expulsion (69). The stem cell factor (SCF) is the most important factor in the induction of mast cells production that occurs in an immediate hypersensitivity reaction of type I.

The immunopathology of the enteric phase of trichinellosis and specific mucosal adaptations in this phase of trichinellosis as important factors in “host-parasite interplay” have been investigated for many years at the Texas Health Science Center (70,71). Although transient, mucosal inflammation in *T. spiralis*-infected host is the causative agent of long-lasting (up to 42 days postinfection) alterations in enteric neuromuscular function (72).

The specific mechanism involving the modifications of the cuticle of newborn larvae (NBL) is now considered a factor decreasing to a certain degree the number of invasive larvae entering the muscle tissue (73). Most of the NBL pass very rapidly through the lung barrier, and their cuticle is modified during this passage (74). The cells adhering to 2-hour-old NBL are mostly eosinophils and those adhering to 20-hour-old NBL are macrophages. From this observation it is evident that eosinophils play a determinant role in the cytotoxic reaction at this stage of trichinellosis and during tissue eosinophilia. Eosinophils with lower density have higher cytotoxic activity (75). The idea of a specific, protective role of nitrogen free radicals in lungs in *Trichinella spiralis*-infected guinea pigs has not been confirmed and is still being investigated.

In the acute, muscular phase of trichinellosis, which may start about 10 days postinfection (pi) in severe cases and may last up to some weeks in cases with low intensity of infection, the encysting larvae invade the following groups of host active muscles: tongue, diaphragm, neck, ribs, gastrocnemius. Thus, in trichinellosis muscle fibers with predominantly oxidative metabolism are objects of alteration (76). The encystment of larvae in red fibers of these muscles might easily be the result



**FIGURE 3** *T. spiralis*-infected human muscle tissue. (x) Inside capsulae; basophilic transformed muscle cell without *T. spiralis* larvae; (xx) basophilic transformed muscle cell with *T. spiralis* larvae; (xxx) cellular infiltrate with cell-like lymphocytes. (Rutin stain H+E. Magnification 120 $\times$ . From L. Gustowska, unpublished results.)

of the following: (a) easily induced disorders in the oxidative process, and in particular the uncoupling of the oxidative phosphorylation (77,78); (b) damage to the contractile apparatus as a result of the lack of energy caused by the disorders mentioned in (a); (c) the originally greater number of mitochondria, which ensure the energy for cyst formation and larval growth.

The participation of reactive oxygen species (ROS) in the biochemical protective mechanism during the muscular phase of infection with *T. spiralis*, which attracted a lot of attention, has already been described (79). It has recently been proven, using both histochemical and biochemical methods, that nitrogen free radicals (RNI) may play a role in the protective mechanism of the host muscles against *Trichinella spiralis* larvae (81). During infection a bimodal stimulation of the activity of inducible nitrogen oxide synthase (iNOS) by day 21 pi and 70 pi has been observed (82,83).

Unlike other muscle parasites, *Trichinella* transforms the muscle cell, adjusting cell metabolism of the host to its own requirements. The transformation of muscle cell into the nurse cell—first stage larva complex is indispensable for larvae survival, development, and maturation (7,84). Studies of many authors show that the transformed muscle cell represents a viable unit. The transformations are the result of signals emitted by the parasite and the host. Four features are typical of basophilic transformation first defined by Gabryel and Gustowska (85,86): (a) decrease of the number of myofilaments starting on day 2 pi; (b) development of sarcoplasmic reticulum in various spatial relations to mitochondria; (c) proliferation of the rough endoplasmic reticulum; and (d) transformation and translocation of the cell nuclei (87) (Fig. 3).

Comparison of the elaborate network of blood vessels surrounding the nurse cell with the vessels in the surrounding uninfected muscle suggests that this network is the result of de novo angiogenesis induced by *T. spiralis* larvae. The parasite may elicit angiogenesis directly or indirectly by inducing change in nurse cells, which, in turn, stimulates the enlargement of blood vessels network (88).

Pathogenesis of the changes in other tissues (heart muscles, CNS, lungs, liver, or kidney) in trichinellosis has not yet been precisely described, because the parasites—although they cause transient inflammations—die in those tissues in a short period of time.

While in the heart muscle *T. spiralis* induces transient myocarditis, the CNS damage may be far more severe. Pathogenesis of CNS is due to infiltration of the pia-arachnoid with lymphocytes,

macrophages, fibroblasts, and gitter cells. Nodules of glial cells and small hemorrhages may be present in the periventricular and other areas of the white matter (89).

## VII. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

Two genetic factors related to parasite genotype and one related to host species have been found to contribute to the virulence of infection with *Trichinella*. The first factor is the number of newborn larvae produced by each female. *Trichinella spiralis* is the most fecund species—1500 larvae during its life span—whereas females of a sylvatic species or genotype can produce no more than 200 larvae. The second factor has been identified only on the basis of clinical sequelae observed in human infections, but the genetic and biological mechanisms are unknown. Fifteen *T. spiralis* muscle larvae/per gram in an individual were lethal, whereas 1468 *T. britovi* larvae/per gram induced no symptomatology in an individual under corticosteroid therapy. Furthermore, a boy with 3000 *T. nelsoni* muscle larvae/per gram recovered from the infection, whereas a man with 4000 *T. nelsoni* muscle larvae/per gram died (42). Severe diarrhea and abdominal pain have been reported in about 98% of individuals infected with *T. nativa* (90,91), whereas this percentage drops to 40% in *T. spiralis* infections and to 20% in *T. britovi* and *T. nelsoni* infections (92,93). The host species also influences the virulence of the parasite. For example, there is a natural resistance of swine and rats to all species and genotypes of *Trichinella*, with the exception of *T. spiralis* and *T. pseudospiralis*; indeed, very few larvae of these parasites are able to develop to adulthood in the gut, and very few newborn larvae are able to develop in muscles, and the few larvae that settle in muscles are rapidly calcified. By contrast, larvae of sylvatic species can survive in muscles of carnivores for a long period of time (94).

## VIII. CONTROL METHODS

Laboratory methods to demonstrate *Trichinella* infection in humans or animals can be divided in two categories: direct methods, which demonstrate the parasite in tissues, and indirect methods, which suggest the presence of the parasite in a particular host because of a demonstrable immunological response. Example of direct methods are trichinostomy and a variety of artificial digestion methods in muscle samples of slaughtered animals. Often so-called predilection sides are sampled, such as diaphragm, tongue, or musculi extensor of the front legs. The choice of predilection muscle is depending on the host species under examination and should be based on solid experience by experimental infection and search for the most infected muscles.

In humans muscle biopsy is usually followed by sectioning of formalin-fixed and paraffin-embedded material. Such method are not very sensitive and often fail to detect light infections. Indirect methods such as serological techniques (humoral immune response) and skin testing (cellular immune response) have the advantage of testing animals that are still alive. Furthermore, the sensitivity of such methods as compared to direct methods is much greater, identifying infections with just a few larvae in more than 100 g of predilection side musculature. Of course, the quality of the reagents used and the standardization of the tests largely influence the reliability of these methods. Serological tests can be used either as diagnostic tools to confirm a supposed infection in individuals or as screening (monitoring) tools in a population (95). At the individual level sensitivity and specificity play a major role in the diagnostics and accuracy of the methods used, but at the population level predictive values (positive or negative) must be assessed before methods can be used reliably. The latter criteria are directly related to the prevalence of trichinellosis in the population under examination.

The actual methods and their principles have been exhaustively described in the literature and handbooks. International organizations have described such methods in detail (EU directives, OIE handbook on *Trichinella* control) and have greatly contributed to transparent meat inspection regulations. Recent developments and debate on control measures are updated every 4 years in the proceedings of the scientific meetings of the International Commission on Trichinellosis (ICT

proceedings). It is not the intention of this chapter to repeat and review existing laboratory methods. Here a variety of public health objectives will be discussed in relation to possible control measures.

## **A. Veterinary Public Health Objectives**

### *1. Prevention of Disease in Humans*

After the recognition of *T. spiralis* as a human pathogen that could be recognized in the musculature of infected pigs by relatively easy microscopic examination, regular inspection methods were introduced at the abattoir level in some places. With the introduction of trichinoscopy, human disease could largely be prevented. Private slaughtering without veterinary control, illegal slaughtering, or disbelief in parts of the world that trichinellosis is a preventable infection are some of the reasons why trichinellosis has remained a public health problem in many countries. In some areas with no tradition of consuming improperly cooked pork (products), the recognition of a public health problem only started after introduction of other consumption habits or after immigration of people from other cultures.

The goals of the European directives on fresh meat were prevention of disease in humans. In livestock animals only relatively heavily infected carcasses can be identified using routine direct methods. These identified carcasses are removed from the meat-production process. In endemic areas, humans and animals with light and subclinical infections have been identified by the use of serological methods. Trichinellosis as an infection in livestock and wildlife has been maintained since *Trichinella* evolved. This indicates that in endemic areas humans and animals may harbor *T. spiralis* larvae without knowing it.

### *2. Prevention of Infection*

Only after the introduction of sensitive serological methods and extensive attempts to compare the efficacy of direct and indirect systems to prevent human disease discussions were started as to whether meat should be free from parasites or if prevention of disease in humans (<3 larvae/g) would be sufficient. These recent considerations paralleled the discussion on alternative routes of prevention of trichinellosis: i.e., freezing or irradiation of carcasses as methods of rendering the parasite noninfectious.

In countries with modern or industrialized animal production facilities, similar objectives were aimed at with a much wider perspective: the raising and fattening of pigs on a large scale under high containment conditions to keep out animal infectious diseases and to produce standard quality, safe, and wholesome food. Under such conditions trichinellosis simply disappeared as a source of infection via pork. This international debate continues (3).

### *3. Epidemiology Versus Individual Carcass Control*

With the availability of reliable serological tools, reassessment of control procedures became realistic. Particularly in geographical areas where human trichinellosis is not a regular reported disease, it is questionable whether routine carcass control at the abattoir is still necessary. Seropidemiological monitoring systems have been established for a number of animal (pig) diseases in many countries. Monitoring the actual presence or absence of *Trichinella* infection in a given pig population is therefore feasible.

Because the generation time of pigs is very short and the infection pressure from *Trichinella* in wildlife may exist permanently, a continuing monitoring program is to be preferred over regular-interval surveillance. It goes without saying that a proper identification and administration system should exist for all livestock in such areas. Such an epidemiological control system in a given area should be based on certification of herds/farms. This implies that the negative predictive value of the testing system should be high (>99%), and therefore the most sensitive serological methods should be employed.

## B. Control Systems

The International Commission on Trichinellosis has recognized since 1980 that none of the existing control methods is infallible. This is still true today. Failure of humans, equipment, administration, etc. will always result in a certain level of infection. Training, permanent education of personnel, and implementation of quality control program are a prerequisite for minimizing the risks in any country.

### 1. Control in Endemic Areas

Since endemic areas are defined as areas where irregular outbreaks of trichinellosis are reported, either as small family outbreaks or large-scale urban-type outbreaks, it is obvious that meat inspection at the abattoir level is necessary. The prevention of human disease is the primary goal. Individual carcasses should be examined with methods equally sensitive as the classical trichinology. Generally direct methods to demonstrate the absence of *Trichinella* larvae in predetermined amounts of muscle are to be preferred as preventive measures. Serology is not acceptable for preventive control of carcasses at the abattoir level, since individual immunological responses may be different and early infections become detectable only after several weeks (4–6), depending on the initial infectious doses of the animals.

### 2. Control in Nonendemic Areas

Nonendemic areas are defined as geographically determined areas where human trichinellosis has not been reported for more than 10 years because of the consumption of meat (products) produced in that area. Nevertheless, existing legislation often requires individual control at the abattoir level using relatively insensitive methods (see above). It may be questioned whether public health authorities in such areas should request information on the real status of *Trichinella* infection in humans and animals. Epidemiological surveys should be carried out with sufficiently sensitive methods before concluding that an area is really free from *Trichinella* infection. Such surveys should include humans and animals, including livestock and wildlife species such as wild boars, foxes, or other relevant (semi) wildlife species such as cats, martens, lynx, badgers, bears, etc.

In particular regions indicator wildlife animal species may be identified (e.g., fox), which represent an infection pressure in that area. If such indicator animals show no or limited *T. spiralis* infection levels (<0.1% of the population), individual control of slaughter pigs originating from modern farms does not make sense (44). The individual control may be replaced by monitoring of farms/herds followed by certification of absence of trichinellosis (see 96).

## C. *Trichinella*-Free Farming

Under strict conditions it is possible to guarantee *T. spiralis*-free farming. It has been demonstrated that the domestic life cycle of *T. spiralis* will not occur in modern farming. Modern farming and industrialized farming with hygienic measures and strict rules of good manufacturing practice (GMP) combined with good veterinary practice (GVP) will exclude the risk of transmitting trichinellosis. The hygiene measures include:

- Keeping animals indoors throughout life

- The use of hygiene barriers before the actual stables can be entered, by authorized people only (e.g., change of clothes and boots, washing facilities in preentrance area)

- Blocking of free entrance of farming areas (e.g., fences, canals) and keeping the direct environment free from rough vegetation

- Rodent and bird control programs

- The use of qualified (sterile) feed

- Admission of new animals at the farm only after proper certification of *Trichinella* freedom

- No garbage dumps near farm area

Regular control by public or animal health authorities is required to maintain a *Trichinella*-free status as mentioned above, preferably together with the results of a monitoring system (97). Animals originating on farms with a *Trichinella*-free status do not have to be examined at the abattoir level.

#### D. Other Sources of *Trichinella* Infection in Humans

In recent decades large outbreaks in Europe were reported after the consumption of infected horse meat (98). Wild boars and other wildlife species are also sources of human trichinellosis. All animals that have access to the environment and/or potentially infected feed (swill, carcasses, etc.) will always constitute a potential public health threat with regard to trichinellosis. Consequently, all those animals (swine, horses, wildlife) must undergo proper inspection at the individual level before they are declared suitable for consumption.

### IX. FINAL REMARKS

Human trichinellosis continues to be a public health problem that cannot be ignored in any part of the world. Even in areas where the problem has hardly existed for many years, it may reoccur for a variety of reasons mentioned in the introduction of this chapter. Therefore adequate control should always be maintained in any area. In many parts of the world *none* of the existing methods of control are applied because of lack of equipment, reagents, or trained personnel. Education of the public as to the potential risks of consuming improperly controlled meat of any kind that is not fully cooked before consumption should be part of the effective control of human trichinellosis.

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# Variant Creutzfeldt-Jakob Disease and Bovine Spongiform Encephalopathy

**Andrea S. Vicari**

*Louisiana Office of Public Health, Infectious Diseases, New Orleans, Louisiana, U.S.A.*

**William D. Hueston**

*Center for Animal Health and Food Safety, University of Minnesota, St. Paul, Minnesota, U.S.A.*

**Dominic A. Travis**

*Lincoln Park Zoo, Chicago, Illinois, U.S.A.*

## I. BACKGROUND

Bovine spongiform encephalopathy (BSE) is a neurological disease of adult cattle with an insidious onset and progressive, invariably fatal course. It was first recognized in Great Britain in November 1986 (1), where it surged to epizootic levels over the following decade (2). On March 20, 1996, a new form of human spongiform encephalopathy, variant Creutzfeldt-Jakob disease (vCJD), was announced in the British Parliament. Although there was no direct reference to an alimentary pathway of transmission, exposure of humans to BSE-infected tissue prior to 1989 was considered the likeliest explanation (3). Soon thereafter, a scientific paper describing the disease was published by Will et al. (4). Evidence is mounting that this human disease is linked to the occurrence of BSE in cattle (5–7).

Both BSE and vCJD are members of a group of animal and human diseases called transmissible spongiform encephalopathies (TSEs). These diseases share several similarities. They are caused by an unconventional, infectious agent and are characterized by progressive microscopic degeneration of the brain. Clinically, neurological signs predominate, and the course is always fatal with no known effective treatment options. Finally, an immunological response is missing. Other animal TSEs include scrapie in sheep and goats, transmissible mink encephalopathy, and chronic wasting disease of mule deer and elk. Notably, scrapie was first reported over 200 years ago (8) and is commonly considered the prototype of all the TSEs. The causal agent of BSE has been shown to infect a wide spectrum of animal species. In particular, cases following natural transmission have been reported in domestic cats and zoo animals. Other than vCJD, four forms of human TSE are known: classical Creutzfeldt-Jakob disease (sporadic, familial, and iatrogenic), kuru, Gerstmann-Straussler-Scheinker syndrome, and fatal familial insomnia (9–11). A sporadic form of fatal familial insomnia has been recently reported (12).

BSE is principally of concern as an animal disease. Unlike other animal TSEs, it assumed endemic dimensions because of feedborne spread. Epidemiological analysis in Great Britain found that the earliest suspected cases occurred in April 1985. The only exposure common to all of the farms with BSE cases has been the incorporation of meat-and-bone meal, a rendered animal protein, into the cattle feed. Exposure appears to have begun in 1981/82 (13).

Current theories connecting BSE and vCJD are based on three pieces of evidence taken together:

1. vCJD emerged in the same country (United Kingdom) as the BSE epidemic in cattle, and the vast majority of cases have occurred there (4).
2. The strain characteristics of the agent encountered in vCJD patients were found to have physicochemical properties resembling those of BSE transmitted to mice, the domestic cat, and the macaque, while different from those of other human TSEs (5).
3. The incubation period and the pattern of neurological lesions observed in mice experimentally infected with the agent of vCJD are remarkably similar to those caused by the BSE agent, while very different from those seen in other animal and human TSEs (6).

This body of evidence supports a link between BSE and vCJD but cannot be considered as definitive proof of the zoonotic character of the BSE agent. Nevertheless, given the ethical constraints regarding conducting human challenge studies, the findings to date provide compelling evidence that BSE must be treated as a zoonotic disease. There is no evidence that other animal TSEs represent a threat to public health.

## II. AGENT CHARACTERISTICS

The nature of TSE agents remains incompletely understood, but three major hypotheses have been advanced. Two of these concern a virus and virino, respectively (14,15). The third and most cited hypothesis associates TSEs with a proteinaceous infectious particle called a prion (16,17). This particle is believed to cause a structural change in a normal cellular protein (PrP<sup>c</sup>), which consequently tends to aggregate and is proteinase resistant (PrP<sup>RES</sup>).

TSE agents, and by inference the BSE agent, are exceptionally resistant to heat (18), ultraviolet and ionizing radiation, and chemical disinfection (18–20). A measure of heat resistance is given by the recommendation that inactivation of CJD agent in hospitals and laboratories should involve porous load autoclaving at 134–138°C for 18 minutes at 30 psi (=207 hPa)(21). An experimental study on commercial rendering processes used in Europe for the production of meat-and-bone meal and tallow was conducted (22,23), and it led the European Union to enact a regulation providing that all ruminant protein waste be treated at 133°C, 3 bar for 20 minutes (24). Sodium hypochlorite providing 2% (20,000 ppm) available chlorine acting for one hour at 20°C has been shown to be completely effective in inactivation studies (19,20,25). A useful alternative disinfectant is 1 N (4%) or 2 N (8%) sodium hydroxide acting for one hour at 20°C (18–20).

The origin of the BSE agent is still unexplained, and two major hypotheses have been proposed (13). The first theory suggests adaptation of the scrapie agent to a strain transmissible from sheep or goats to cattle. An alternative hypothesis states that BSE has existed in cattle populations in an unrecognized or clinically silent form for a long time. The emergence of the epidemic under either hypothesis is linked to enhanced survival of the agent in ruminant-derived protein subsequent to changes in the rendering process. The enhanced survival of the agent allowed sufficient exposure so that cattle not only became infected, but also developed clinical signs of the disease within their lifespan. The epidemic was amplified by the recycling of infected cattle via ruminant-derived protein in feed prior to the recognition of the disease's epidemiology and the implementation of effective control measures.

## III. vCJD DISEASE CHARACTERISTICS

Clinical features and diagnostic test results for the first 14 cases of vCJD in the United Kingdom have been reported (26,27). Eight cases were women. Mean age at onset of clinical signs was 29 years (range 16–49 years), and the median duration of illness was 14 months (range 9–35 months). The first symptom was psychiatric in 9 patients, including anxiety, depression, withdrawal, and behavioral changes. The remaining 5 cases also had early psychiatric features. Eight patients developed early sensory symptoms (persistent painful sensation in the limbs and/or face). As illness evolved, all cases developed neurological signs, including ataxia and involuntary movements. Towards the end of the illness, most patients had severe cognitive impairment and a state of akinetic

mutism. Electroencephalogram findings were abnormal in most patients, but cerebral imaging was usually normal or showed nonspecific abnormalities.

A definitive diagnosis of vCJD remains dependent on neuropathological confirmation. Spongiform changes, astrocytic gliosis, and neuronal loss are neuropathological features common to all animal and human TSEs and are most evident in the basal ganglia and thalamus. Multiple “florid” plaques extensively distributed throughout the cerebral and cerebellar cortex distinguish vCJD from several other human TSEs (4).

CJD is an incurable disease. At present patients can only be treated supportively.

#### IV. EPIDEMIOLOGY OF vCJD

As of March 1999, 42 definite and probable cases of vCJD have been reported worldwide. Forty-one cases have been reported in the United Kingdom (28); one case was diagnosed in France (29). Monthly updates on the number of CJD and vCJD cases in the United Kingdom are available online (30).

A case-control study of CJD has been carried out in the United Kingdom since 1990 to investigate potential risk factors for CJD (31). In particular, medical history, consumption of different meats after 1985, and occupation are under investigation. With the occurrence of vCJD, this disease was also considered in the study. As of 1998, analyses concerning vCJD included data from 35 cases and 25 age- and sex-matched hospital controls. While no evidence of either iatrogenic or occupational risk factors was found, cases did appear to eat beef more frequently than controls. However, this finding has to be interpreted with caution due to the limitations of case-control studies.

Prior to the prohibition of human consumption of specified bovine offal in November 1989, BSE-contaminated tissues entered the human food chain in the United Kingdom (32). Because BSE infectivity is limited to certain tissues and the origin of a cattle may reflect BSE risk, the potential threat of a given food may be assessed by considering which bovine tissues it contains and its origin. Bioassay is the only available method to demonstrate BSE infectivity, and it has been applied to study the pathogenesis of the disease. Experimental challenges of cattle have been carried out in the United Kingdom, and preliminary findings have been published (33–35). Calves were orally challenged with brain homogenate from BSE-affected cows at 4 months of age, and groups were then sacrificed sequentially from 2 to 40 months after inoculation. The earliest onset of clinical signs occurred after 35 months, and diagnostic histopathological changes in the brain after 36 months. This indicates that the onset of clinical signs and pathological changes in the central nervous system occur at approximately the same time. Through mouse bioassay, infectivity was first detected in distal ileum 6 months after inoculation. Beginning 3 months before clinical onset (from 32 months after inoculation), infectivity was demonstrated in the brain, spinal cord, retina, dorsal root ganglia, and the trigeminal ganglia. Furthermore, limited infectivity has also been detected in sternal bone marrow during the clinical phase. The results of this experimental challenge are consistent with what is known about scrapie pathogenesis. The scrapie agent shows a predilection for tissues of the lymphoreticular system, where it replicates before invading the central nervous system. In naturally infected sheep, replication begins in the tonsil, retropharyngeal lymph node, and Peyer’s patches, which probably reflects an oral route of infection (36,37). In tissues from naturally occurring cases, BSE has only been detected in brain and spinal cord. The discrepancy between findings of naturally occurring BSE and experimental challenges should be interpreted with caution because the experimental dose of 100 g of brain homogenate from BSE-affected cows appears to be 10–100 times greater than the levels most cattle will have been exposed to via feed. Also, because mice appear to be around 1000-fold less sensitive to BSE than are cattle, mouse bioassay has a limited sensitivity.

BSE cases in native cattle have been reported in nine European countries (Table 1); five other countries have detected cases in imported animals (2). However, BSE has assumed an epidemic dimension only in the cattle population of the United Kingdom, where 177,690 cases of BSE—the vast majority of all cases reported worldwide—had been diagnosed as of April 1999. Figure 1 shows the yearly distribution of BSE cases in the United Kingdom. After its emergence mid-1980s, the number of cases reached a peak in 1992, when up to 1000 new cases were being reported each

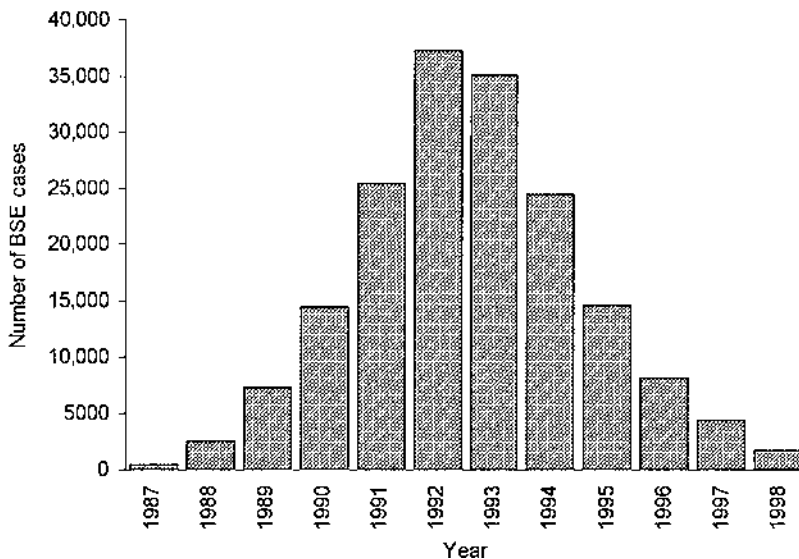
**TABLE 1** Number of Bovine Spongiform Encephalopathy Cases in Native Cattle, Worldwide as of Spring 1999

Country	No. of Cases
United Kingdom	177,690
Republic of Ireland	371
Switzerland	308
Portugal	297
France	65
Belgium	9
Netherlands	6
Liechtenstein	2
Luxembourg	1

Source: Ref. 2.

week. The number of cases has gradually decreased since then, and the British BSE epidemic is expected to die out over the next decade.

Although at dramatically lower rates than the United Kingdom, cases in native cattle have been reported in eight other European countries: Republic of Ireland, Switzerland, Portugal, France, Belgium, the Netherlands, Liechtenstein, and Luxembourg (Table 1). Epidemiological studies in some of these countries have shown that the likely cause of infection was exposure to feed containing BSE-contaminated protein products of animal origin, much of it originating from the United Kingdom and not necessarily imported through the most direct route (38). In addition, some cattle exported from the United Kingdom have succumbed to BSE in Canada, Denmark, the Falkland Islands, Germany, the Sultanate of Oman, and Italy (2).



**FIGURE 1** Number of bovine spongiform encephalopathy cases reported in the United Kingdom, 1987–1998. (From Ref. 2.)



Whereas it can be assumed that products of British origin might have represented the highest hazard (especially prior to the implementation of measures of animal and public health), products from a country that has reported fewer or no BSE cases cannot be assumed to be free of infectivity. BSE diagnosis in cattle relies on reporting of suspect cases, and it is known that the level of disease awareness and surveillance varies from country to country (39). Epidemiological investigations suggest that large numbers of potentially exposed cattle and large amounts of presumably contaminated ruminant-derived protein were exported from the United Kingdom, principally to European countries. Consequently, more countries may be experiencing BSE than is currently recognized (40).

## V. IDENTIFICATION

Bioassay in laboratory animals is the only reliable method that allows replication of the TSE agents. Similarly, few methods exist to identify their presence in organs and tissues. Scrapie-associated fibrils can be purified from brain of BSE-affected cattle and detected by electron microscopy (41). Methods for the immunodetection of PrP<sup>RES</sup> have been popular tools for diagnosis and research studies. Immunohistochemistry methods to identify PrP<sup>RES</sup> in tissues from the central nervous system are commonly applied for the diagnosis of several human and animal TSE, including BSE in cattle (42). Immunoblot analysis and ELISA have been used for research purposes. Applications of those methodologies on brain tissue for detecting or confirming clinical cases and for screening dead or slaughtered animals for BSE have recently been developed and are under validation (43). The results indicate that some tests show both excellent sensitivity and specificity and would thus allow for detection or confirmation of clinically BSE-affected cattle. However, since knowledge of the relationship between infectivity titer and PrP<sup>RES</sup> concentration throughout the incubation period is limited and PrP<sup>RES</sup> concentration in tissues other than brain is likely to be smaller by a factor of several logs, detection of BSE-infected cattle prior to clinical onset or of infectivity in their tissues remains problematic. This reasserts the primacy of prevention and control measures.

## VI. GENETIC FACTORS CONTRIBUTING TO VIRULENCE

Whereas more than 20 strains of scrapie agents have been identified by the distinctive neuropathological patterns and incubation periods in mouse bioassay, BSE and vCJD appear to be caused by a single strain.

The host genotype is recognized to play an important role in several human or animal TSEs. In particular, the inherited human TSEs (familial CJD, Gerstmann-Straussler-Scheinker syndrome, and fatal familial insomnia) are caused by mutations in the PrP gene located on the short arm of chromosome 20 (44). Methionine homozygosity at codon 129 of the PrP gene is a recognized risk factor for the development of sporadic CJD (45). A consistent body of literature shows that the length of the incubation period of scrapie in sheep is determined by the type of alleles combination of the *Sip* (scrapie incubation period) gene (46). However, a predisposing genetic phenotype has never been identified for BSE in cattle.

All cases of vCJD that have actually been tested resulted in methionine homozygotes at codon 129 of the PrP gene (47), although the prevalence of methionine homozygosity in normal population is reported to be around 40% (48). The statistical improbability of this circumstance may be explained by one of three alternative mechanisms: methionine/valine heterozygosity and valine/valine homozygosity are protective, the incubation period for these genotypes are longer, or clinical features of CJD in these patients are significantly different.

## VII. CONTROL MEASURES

Because of the tenacity of the BSE agent, the likely small infectious dose, and the inevitably fatal course of the disease, the primary food safety concern is to prevent TSE agents from entering the

human food chain. While no single measure alone can achieve this goal, a concerted series of animal and public health prevention and control measures is necessary. Since scrapie in sheep has been suggested potentially to mask the occurrence of BSE in that species (49), similar measures should also be extended to sheep and goats.

In the United Kingdom, the most critical control measures leading to the control of BSE have been disease awareness and surveillance, mandatory notification of suspect cases in cattle and subsequent compensation of farmers, reliable animal health diagnostic capabilities, control of live cattle movements, prohibitions on feeding ruminant-derived rendered protein to all ruminants, stringent rules regarding disposal of those tissues and organs likely to contain the highest infectivity levels, and rendering conditions (50,51).

Surveillance of the cattle population for clinical signs compatible with BSE is the best approach for increasing the ability to detect the disease. Since BSE causes clinical signs that are not pathognomonic, individual animals with symptoms compatible to BSE should be observed in all countries with cattle populations. Surveillance must focus on cattle over 24 months of age displaying neurological signs or cattle moribund without signs of infectious or traumatic illness. The brains of those animals should be submitted to a qualified laboratory for neuropathological examination. This work-up may lead to alternative diagnoses such as cerebral listeriosis, rabies, or brain tumor (52). An adequate level of compensation to farmers for the disposal of suspect or confirmed cases and complimentary laboratory examination are considered important elements in favoring reliable reporting. Carcasses and products of suspect and confirmed cattle should be destroyed in order to avoid infectivity entering the human food chain or being recycled in animals through contaminated feedstuffs.

The BSE epidemic in the United Kingdom was amplified by the recycling of infected cattle via ruminant-derived protein in feed (13). Therefore, prohibition on feeding ruminant-derived protein to ruminants, adequate disposal of specified bovine offal, and regulation of rendering conditions are essential measures to avoid the spread of the diseases. Because of possible cross-contamination, an effective ban on feeding ruminant-derived proteins can only be achieved through careful separation and processing of ruminant offal. This separation starts at the slaughter plant and continues through rendering, feed preparation and transportation, and on the farm. Due to the practical challenges of effectively excluding ruminant proteins from ruminant feed, several countries have opted for more stringent rules. For instance, prohibition of all mammalian-derived protein in ruminant feed and incorporation of meat-and-bone meal in any farmed livestock feed was enacted in the United Kingdom (51). In the United Kingdom, bovine tissues and organs likely to contain the highest infectivity levels were originally assessed based on scrapie infectivity distribution in sheep tissues. They were defined as "specified bovine offal" and included the brain, spinal cord, spleen, thymus, tonsils, and intestines of bovines aged 6 months or over (50). More recently, the designation of "specified risk material" has been introduced, referring to those tissues of cattle, sheep, and goats that are known to, or might potentially, harbor detectable BSE infectivity. This material should be excluded from the animal feed chain (53). Rendering conditions should be such as to guarantee adequate inactivation of TSE agent. In Europe, all ruminant protein waste must undergo a heat treatment at 133°C, 3 bar for 20 minutes if the rendering products are intended for use in feed (24). Studies to assess the inactivation potential of different rendering systems have been conducted for both BSE and scrapie (22,23).

Besides animal health measures, measures specifically aimed at safeguarding public health are also applicable. Careful ante mortem inspection of adult cattle, in particular of animals with ambulatory problems, should be performed at the slaughter plant, since it can lead to the detection of BSE-affected animals. Care should be taken during the slaughter process to avoid cross-contamination with central nervous system tissues. Critical procedures are stunning, pitting, and longitudinal splitting of the carcasses. As the finding of brain emboli in the lungs of cattle after stunning indicates (54), those procedures may lead to contamination with highly infective tissues. Finally, the exclusion of specified risk material from the human food chain represents an important measure for minimizing the exposure of consumers to TSE agents (51).

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## Incidence of Foodborne Infections in Northern Europe

**Maija Hatakka and Pekka Pakkala**

*National Food Agency, Helsinki, Finland*

This chapter deals with the incidence of foodborne illnesses in the five Nordic countries—Denmark, Finland, Iceland, Norway, and Sweden—which have a total population of around 24 million (Fig. 1). In 1998, the population was 5.3 million in Denmark, 5.2 million in Finland, 275,000 in Iceland, 4.4 million in Norway, and 8.9 million in Sweden. Denmark, Sweden, and Finland are member countries of the European Union (EU). All the Nordic countries joined the WHO Surveillance Program for the Control of Foodborne Infections and Intoxications in Europe at an early stage, at the beginning of the 1980s.

### I. ESTIMATED RATES OF ILLNESS

The most frequently reported causes of intestinal infections in humans in the Nordic countries are *Salmonella* spp. and *Campylobacter* spp. Figure 2 (1–9) shows the incidence rates of registered cases of salmonellosis and campylobacteriosis in different Nordic countries between 1985 and 1999. In the Nordic region, *Salmonella* infections were the most frequent zoonoses in the 1980s, but *Campylobacter* infections rose during the 1990s and exceeded *Salmonella* infections. In Sweden campylobacteriosis became more frequent than salmonellosis beginning in the early 1990s. The same occurred somewhat later in Norway, Iceland, and Finland (between 1995 and 1998) and in Denmark in 1999.

#### A. *Salmonella enterica*

The number of registered cases of salmonellosis increased in the Nordic countries during the 1980s. A sharp rise in the numbers of travel-related infections was observed in Norway, Sweden, and Finland. In Finland, for example, the annual number of salmonellosis cases was as high as 7500 in 1998, when 1.5 million out of 5.1 million Finns spent their holidays outside Finland. The rate of infections then plummeted owing to the recession in the early 1990s. Since 1995, the annual number of cases of salmonellosis has been around 3000 (5). The number of reported cases of salmonellosis corresponded well with growing charter tourism in the mid-1980s (7).

Between 1993 and 1999, the incidence rate of salmonellosis varied between 95 and 24 cases/1000,000 inhabitants/year in the Nordic countries, with Denmark showing the highest incidence and Norway the lowest (Fig. 2). *Salmonella* Enteritidis was the dominating serotype in all countries (42–67%), followed by *S. Typhimurium* (7–17%)(1,3,5,7,9).

Over the past few years, human infections caused by multiresistant *Salmonella* spp. have occurred in the Nordic region, *S. Typhimurium* DT104 being the most frequent (1,3,4,10,11).



**FIGURE 1** The Nordic countries.

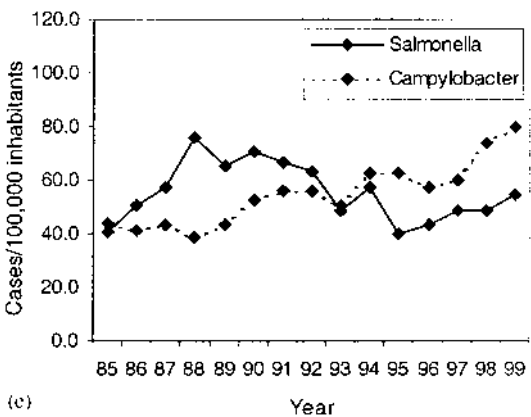
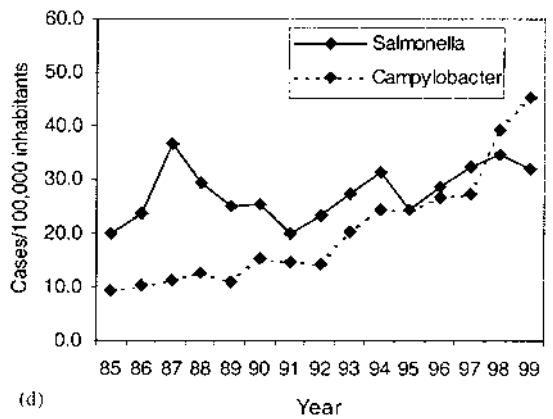
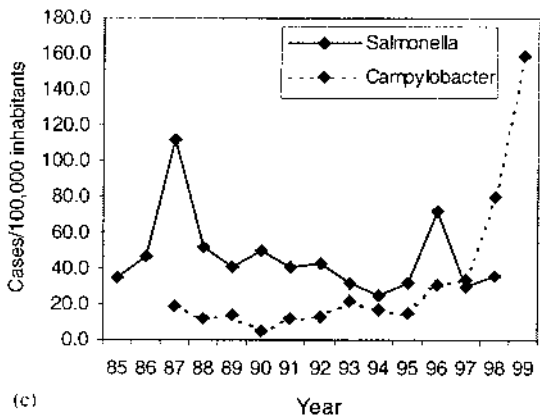
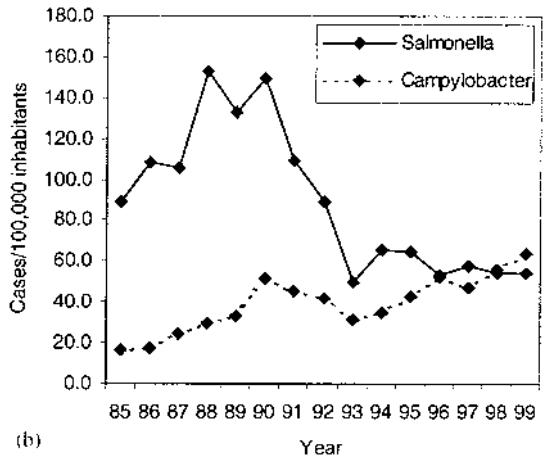
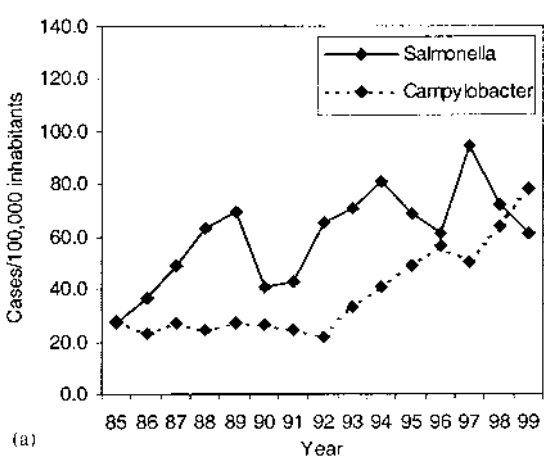
### **B. *Campylobacter* spp.**

The incidence of registered human *Campylobacter* infections has increased significantly over the past 10 years and now exceeds the incidence of human salmonellosis in the Nordic region (Fig. 2). *C. jejuni* was the most common species isolated from humans, followed by *C. coli*. Incidence rates continued rising in each of the Nordic countries during 1999, when the highest incidence, 159 cases/100,000 inhabitants, was reported in Iceland (1,12).

In Denmark around 20% of the cases were associated with recent travel abroad, whereas 30–50% of the Swedish cases, 50% of the Norwegian cases, and 80% of the Finnish cases were considered travel-related (13). In Iceland around 50% of cases were associated with travel until 1997, but from 1997 to 1999 the number and proportion of domestic cases increased constantly and was 75% in 1999 (M. Geirsdottir, personal communication). *Campylobacteriosis* shows a marked seasonal incidence in all Nordic countries. The incidence of both domestic and travel-related cases peaks in July–September.

### **C. Other Causative Agents of Foodborne Infections**

The third most common enteropathogenic bacterium reported in human cases in the Nordic countries was *Yersinia enterocolitica* (3,4,6,8). The dominating serotype was O:3. The incidence was highest in children under the age of 5. In Finland, the incidence (12–13 cases/100,000 inhabitants) was higher than in other Nordic countries, where 3–9 cases/100,000 inhabitants were observed at the



**FIGURE 2** (a) Incidence of salmonellosis and campylobacteriosis in Denmark, 1985–1999. (b) Incidence of salmonellosis and campylobacteriosis in Finland, 1985–1999. (c) Incidence of salmonellosis and campylobacteriosis in Iceland, 1985–1999. (d) Incidence of salmonellosis and campylobacteriosis in Norway, 1985–1999. (e) Incidence of salmonellosis and campylobacteriosis in Sweden, 1985–1999.



end of the 1990s. In Finland, 30–42 human infections caused by *Y. pseudotuberculosis* per year have been reported, and in recent years some foodborne outbreaks have been identified (4,14,15).

The incidence of *Listeria monocytogenes* varied between 3 and 9 cases per 1 million inhabitants in the Nordic countries. This level was the same as in many other industrialized nations (1,3,4,6,8).

There has been a low number of enterohemorrhagic *Escherichia coli* (EHEC) infections in the Nordic region. The incidence rate observed varied from 0 to 1 cases per 100,000 inhabitants per year in 1998 and 1999, with Norway showing the lowest and Denmark the highest incidence (1,3,4,6,8). The methods for detecting EHEC were improved in the mid-1990s. A few outbreaks have been identified since 1995. In recent years cases have been mainly sporadic.

## II. REPORTED OUTBREAKS

Although collecting data on human infectious diseases seems to be similar in the Nordic countries, there are differences between countries in collecting data on foodborne outbreaks. For instance, all foodborne outbreaks are reported in Finland, whereas obligatory reporting in Sweden involves definite communicable diseases, and reporting of traditional foodborne agents such as those causing intoxications occurs voluntarily. This means that not all the information is comparable (1,16).

Based on the data presented by the World Health Organization (WHO) (1), foodborne outbreaks in the Nordic countries between 1993 and 1998 are shown by causative agents in Table 1. The total

**TABLE 1** Foodborne Outbreaks by Causative Agents in Nordic Countries, 1993–1998

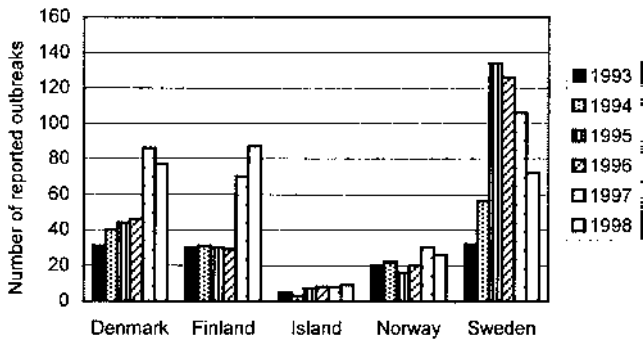
Causative agent	Denmark	Finland	Iceland	Norway	Sweden	Total	
						N	%
<i>Bacillus cereus</i>	13	22	5	30	25	95	12.8
<i>Campylobacter</i> spp.	7	3	2	8	34	54	7.3
<i>Clostridium botulinum</i>	0	0	0	5	3	8	1.1
<i>Clostridium perfringens</i>	22	30	6	19	16	93	12.5
EHEC	0	1	0	1	1	3	0.4
<i>Listeria monocytogenes</i>	0	1	0	1	0	2	0.3
<i>Salmonella enterica</i>	104	29	8	15	32	188	25.3
<i>Shigella</i> spp.	6	0	0	0	22	28	3.8
<i>Staphylococcus aureus</i>	12	13	4	40	19	88	11.8
<i>Yersinia</i> spp.	0	5	0	0	1	6	0.8
Pathogenic vibrio	0	1	0	0	1	2	0.3
Hepatitis A	1	2	0	0	6	9	1.2
Norwalk-like viruses <sup>a</sup>	4	14	NR	NR	46	64	8.6
Viruses (not identified)	2	9	0	0	0	11	1.5
Bean lectine	0	13	0	0	0	13	1.7
Biogenic amines	13	9	0	1	4	27	3.6
Shellfish poisoning	3	5	0	0	0	8	1.1
Other known <sup>b</sup>	<u>22</u>	<u>2</u>	<u>1</u>	<u>14</u>	<u>5</u>	<u>44</u>	<u>5.9</u>
Total	209	159	26	134	215	743	100.0

<sup>a</sup> Sweden: 11 outbreaks of Norwalk-like virus and 35 outbreaks of other calicivirus were included in this group.

<sup>b</sup> Sweden: three outbreaks due to both *B. cereus* and *C. perfringens* were included in this group.

NR, Not reported.

Source: WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe 7th Report, 2001 (Denmark: the register of Veterinary and Food Administration, Norway: laboratory confirmed outbreaks).



	Denmark	Finland	Iceland	Norway	Sweden
1993	31	30	5	20	32
1994	40	31	3	22	56
1995	44	30	7	16	134
1996	46	29	8	20	126
1997	86	70	8	20	106
1998	77	87	9	26	72

**FIGURE 3** Reported foodborne outbreaks in the Nordic countries, 1993–1998. (From WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe 7th Report, 2001 (Denmark: the register of Veterinary and Food Administration, Norway: laboratory confirmed outbreaks)).

number of registered outbreaks in the Nordic region was 1301, of which the causative agent was identified in 743 cases. Compared with 1993, the total number of outbreaks more than doubled in 1997 and 1998 (Fig. 3). The reason for this may be more active surveillance and reporting, but a real increase in foodborne illness may also be a fact. In Finland, the surveillance and reporting of food- and waterborne outbreaks was reorganized and improved in 1997 (17). This resulted in a marked increase in numbers of outbreaks in Finland in 1997 and 1998 (Fig. 3) (14–16). In Sweden, the peak in 1995 reflects the increased reporting activity of the capital area, whereas the decreasing trend in following years may show a lack of interest in notification (Fig. 3) (P. Norberg, personal communication). Around 3500–6500 cases of foodborne illness connected with outbreaks were reported annually in the Nordic region, and this figure peaked in 1997 (1).

It is well known that annual reports of foodborne outbreaks provide a poor reflection of the reality, showing only the tip of the iceberg (18,19). Although reporting activity is rather high in the Nordic countries, outbreaks are still underreported. Swedish studies have clearly shown that only a small proportion of foodborne outbreaks as well as sporadic cases are reported to the official registries, and an estimate of half a million human cases of foodborne illness per year has been presented in Sweden (20,21).

### III. FREQUENCY AND RELATED FOODS

According to many national epidemiological registries, the number of outbreaks of foodborne salmonellosis tends to dwarf that reported for other bacterial pathogens (22). Likewise, *Salmonella* spp. were the most frequent cause of reported foodborne outbreaks in the Nordic region in 1993–1998 (Table 1). *Salmonella* spp. caused 188 of the 743 (25%) outbreaks in which the causative agent was identified. *Bacillus cereus* came second (95/743; 13%), followed by *Clostridium perfringens* (93/743; 13%), *Staphylococcus aureus* (88/743; 12%), viruses (80/743; 11%), and *Campylobacter* spp. (54/743; 7%) (1).

**TABLE 2** Ranking of Foodborne Microbial Pathogens in Nordic Countries, 1993–1998

Country	Number of foodborne outbreaks					Norwalk-like virus <sup>a</sup>
	<i>Salmonella enterica</i>	<i>Bacillus cereus</i>	<i>Clostridium perfringens</i>	<i>Staph. aureus</i>	<i>Campylobacter</i> spp.	
Denmark	104	13	22	12	7	4
Finland	29	22	30	13	3	14
Iceland	8	5	6	4	2	NR
Norway	15	30	19	40	8	NR
Sweden	32	25	16	19	34	46

<sup>a</sup> Sweden: 11 outbreaks of Norwalk-like virus and 35 outbreaks of other caliciviruses were included in this group.

NR, not reported.

Source: WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe 7th Report, 2001 (Denmark: the register of Veterinary and Food Administration; Norway: laboratory confirmed outbreaks).

Some differences occurred between countries with respect to the order of common causative agents (Table 2) (1), although different reporting of foodborne outbreaks among the Nordic countries may also contribute to the order. *Salmonella* spp. were the most frequently reported agents in Denmark and Iceland, followed by *C. perfringens*, *B. cereus*, and *S. aureus*. In Sweden *Campylobacter* spp. were the leading bacterial agents, preceding *Salmonella* spp. In Norway, the leading causative agents were traditional food poisoning bacteria such as *S. aureus* and *B. cereus*, followed by *C. perfringens* and *Salmonella* spp. In Finland, *C. perfringens* was the most frequent agent, the second was *Salmonella* spp., followed by *B. cereus*. Outbreaks caused by Norwalk-like viruses were reported in Denmark, Sweden, and Finland. In Sweden, Norwalk-like virus and calicivirus accounted for the most cases of food poisoning, causing 46 (21%) out of all outbreaks in which the causative agent was known.

### A. *Salmonella* spp.

Denmark has experienced three waves of human salmonellosis, in which the majority of cases was caused by three distinct sources: chicken in the late 1980s, pork in the mid-1990s, and eggs in the mid-late 1990s. In Denmark in 1998, the estimated incidence of human cases per 100,000 inhabitants that could be attributed to various sources was as follows: eggs 35, travel 10, pork 10, Danish-produced broilers 1, other poultry 5, beef 0.2 (23). The falling incidence of *Salmonella* infections during 1998 and 1999 was attributed to the *Salmonella* action plans implemented (3,10).

Between 1993 and 1998, most of the Danish outbreaks were caused by *S. Enteritidis* (82/104; 79%), and the figure peaked in 1997 (1). These outbreaks were closely connected to the consumption of raw table eggs used for desserts and other non-heat-treated foods containing raw eggs. The source of infection in Denmark in this period was contamination of layer-breeder flocks by *S. Enteritidis* (3,10). *S. Typhimurium* was another serotype frequently associated with outbreaks in Denmark, where it was mostly traced to meat products, especially pork.

Unlike the situation in Denmark, most salmonellosis in Sweden, Norway, Iceland, and Finland is acquired abroad. Correspondingly, the number of domestic outbreaks was lower in the other Nordic countries than in Denmark (1). The number of *Salmonella* outbreaks was especially low in the other Nordic countries in 1998, when five rather small outbreaks were registered—four in Sweden and one in Finland—involving some tens of persons altogether. However, the figures for Sweden and Finland showed a rising trend in terms of both outbreaks and ill people in 1999, when Sweden

and Finland reported 10 and 8 outbreaks, respectively, with hundreds of affected people in both countries (8,15). The sources of the largest outbreaks were traced to filled sandwiches, Béarnaise sauce, cheese made from unpasteurized milk, and alfalfa sprouts. Eggs and egg products were very seldom found to be the vehicle for foodborne illness in Sweden, Norway, and Finland (0.1–1%), whereas eggs were the major source (45–50%) of salmonellosis in Denmark (1). Poultry and pork products were also mentioned as common vehicles.

Several *Salmonella* outbreaks connected to the consumption of bean sprouts were reported in the Nordic countries in the 1990s (1,15,24,26–28). Alfalfa sprouts were the most common vehicle in these outbreaks. Bean sprouts contaminated by *S. enterica* serovars Newport, Stanley, and Bovismorbificans were reported to be associated with international outbreaks (Table 3).

Traveling by ship, especially between Finland and Sweden, is very popular. There are many restaurants on the ships, in which a wide range of different dishes are served. Several *Salmonella* outbreaks traced to food served on these ships were recorded during the 1980s. In 1993, *S. Infantis* caused such an outbreak involving approximately 250 passengers (Table 3) (29). The investigations showed that many different dishes, especially cold served dishes, were found to be or suspected of being contaminated.

Multiresistant *S. Typhimurium* DT104 was found to be the reason for one outbreak in Finland in 1995, where the vehicle was found to be imported salami (4). A similar outbreak was recorded in Sweden during the same year (29). In 1997, an outbreak caused by multiresistant *S. Typhimurium* DT112 occurred in Finland involving 100 people. The source of this infection was not found (4). The first outbreak of *S. Typhimurium* DT104 was reported in Denmark in 1998. It included 25 culture-confirmed cases and was traced back to pork of Danish origin (23). In 1999, Sweden reported one outbreak of *S. Typhimurium* DT104 associated with imported smoked turkey, with 33 clinical cases including two deaths (8). In Iceland, the first outbreak caused by multiresistant *S. Typhimurium* DT204b was reported in 2000. During this large outbreak 180 laboratory-confirmed cases were reported. On the basis of the epidemiological study, the vehicle suspected was imported iceberg lettuce (Table 3) (M. Geirsdottir, personal communication).

## **B. *Campylobacter* spp.**

Although *Campylobacter* spp. emerged as the leading bacterial cause of acute gastroenteritis in humans in the Nordic region in the 1990s, identified foodborne outbreaks of *Campylobacter* spp. (54/743; 7%) were rather uncommon (Tables 1, 2) (1). The most common sources for major outbreaks in Denmark and Sweden were identified or suspected as being contaminated poultry, but cases in Norway and Finland involved contaminated drinking water. Raw milk and the consumption of pork and beef were also found to be or suspected of being sources (30). In Iceland, the number of human *Campylobacter* spp. infections has risen dramatically since 1995. This could be associated with the consumption of poultry. Before 1996 only frozen poultry products were allowed to be sold in Iceland. The significant, continuing increase in domestic cases was mainly linked to the high increase in marketing and consumption of fresh poultry products with a much higher contamination level of *Campylobacter* spp. than frozen products (1,30).

## **C. *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus***

The most prominent contributory factor leading to *B. cereus* and *C. perfringens* outbreaks was slow refrigeration. Meat and meat products were the major vehicles for *C. perfringens* outbreaks. A major outbreak caused by *C. perfringens*, in which over 400 people fell ill after eating a lamb stew, was reported in Finland in 1998 (14). *S. aureus* was the leading causative agent in Norway only. The more common occurrence of *S. aureus* in Norwegian tank milk than in other Western countries may explain the high frequency of *S. aureus* outbreaks in Norway. The risk is linked to the production of unpasteurized milk products (31). Until the mid 1980s, *S. aureus* was the most common reported

**TABLE 3** Some Major *Salmonella* Outbreaks in Nordic Countries, 1990s

Year	<i>Salmonella enterica</i>	No. of cases	Location of outbreak	Source	Ref.
1993	<i>S. Infantis</i>	253	Sweden	Dishes served on ship	29
1993	<i>S. Typhimurium</i>	70	Norway	NK	7
1993	<i>S. Infantis</i>	550	Denmark	Pork	1
1993	<i>S. Saintpaul</i>	130	Denmark	Turkey	1
1994	<i>S. Santiago</i>	230	Denmark	NK	1
1994	<i>S. Bovismorbificans</i>	492	Sweden, Finland	Alfalfa sprouts	26, 27
1995	<i>S. Stanley</i>	242	Finland, US	Alfalfa sprouts	25
1995	<i>S. Newport</i>	~300	Denmark, Canada	Alfalfa sprouts	1, 28
1995	<i>S. Enteritidis</i>	190	Finland	Eggs	4
1995	<i>S. Typhimurium</i> DT104 <sup>a</sup>	40	Finland	Italian salami	4
1995	<i>S. Typhimurium</i> DT104	58	Sweden	Salami	29
1996	<i>S. Typhimurium</i> DT12	170	Denmark	Pork	1
1996	<i>S. Enteritidis</i>	124	Iceland	Bakery product	*
1997	<i>S. Typhimurium</i> DT112 <sup>a</sup>	100	Finland	NK	4
1997	<i>S. Typhimurium</i> DT124	160	Finland	Pork	4
1999	<i>S. Typhimurium</i> FT193	71	Finland	Alfalfa sprouts	15
1999	<i>S. Typhimurium</i> FT1	170	Finland	Unpasteurized milk	4, 15
1999	<i>S. Typhimurium</i> FT104	33	Sverige	Imported turkey	8
1999	<i>S. Blockley</i>	20	Sverige	Alfalfa Sprouts	9
1999	<i>S. Enteritidis</i>	87	Sverige	Béarnaise sauce	8, 9
1999	<i>S. Enteritidis</i> 11 out- breaks <sup>b</sup>	312	Denmark	Several foods (also raw eggs)	3
1999	<i>S. Enteritidis</i> 69 out- breaks <sup>c</sup>	NK	Denmark	Several foods (also raw eggs)	3
2000	<i>S. Typhimurium</i> DT204b <sup>a</sup>	180	Iceland	Iceberg lettuce	G. Geirsdottir; personal com- munication, 2001

<sup>a</sup> Multiresistant.

<sup>b</sup> From: Danish Veterinary and Food Administration.

<sup>c</sup> From: Statens Serum Institut, Dept. of Epidemiology.

NK, Not known.

agent in Finland, too, and the outbreaks were mainly associated with smoked herring (32). In the 1990s, there was a marked decrease in the role of *S. aureus* as a causative agent in Finland.

#### D. Other Causative Agents

A large outbreak of *Shigella sonnei* infecting hundreds of people was reported in Sweden and Norway in 1994 (1,33). The outbreak was caused by contaminated iceberg lettuce imported from Spain. Baby maize was shown to be the source of a *Shigella sonnei* outbreak involving 80 people in Denmark in 1998 (1).

Between 1993 and 1998, foodborne outbreaks caused by *Yersinia* spp. were reported in Finland and Sweden. Between 1997 and 1999 *Y. pseudotuberculosis* serotype O:3 caused three outbreaks in Finland. Based on epidemiological study, the probable cause was iceberg lettuce (15,34).

Most *Clostridium botulinum* outbreaks were reported in Norway, where small outbreaks of botulism are usually associated with the consumption of home-made “rakefisk,” a traditional half-fermented fish dish, or home-made smoked cured ham (7). Five cases of infant botulism were reported in the Nordic countries between 1997 and 1999. Honey acted as a vehicle in all of these cases (7,35,36).

Processed ready-to-eat products have been identified as a source of listeriosis in the Nordic countries, where four foodborne outbreaks of listeriosis have been recorded (Table 4). In 1992, an outbreak involving eight cases was traced to contaminated, vacuum-packed cold cuts from a Norwegian meat producer (6). Vacuum-packed fish products such as cold-salted and/or cold-smoked rainbow trout were found to be the vehicles for listeriosis outbreaks in Sweden in 1994–1995 and in Finland in 1997 (37,38). Butter was found to be the vehicle in an outbreak of listeriosis in Finland in 1999. A total of 25 patients, most of them immunocompromised, were identified. The same pulsed-field gel electrophoresis type of *L. monocytogenes* was detected in samples taken from ill people, the butter, and dairy equipment (39,40).

Foodborne outbreaks caused by enterohemorrhagic *E. coli* have been rare in the Nordic region, and only a few outbreaks have been reported. An *E. coli* O157 outbreak occurred in Sweden with about 120 confirmed cases in 1995. Human infections were for the first time traced to the presence of *E. coli* O157 in a cattle herd (8). Finland reported one cluster of *E. coli* O157 with five cases in 1998, but the source of infections remained unclear (14). Sweden reported in 1999 that 11 persons were infected with *E. coli* O157 after a common meal, but the source of infection remained unknown (8). In Norway, an outbreak of *E. coli* O157 involving 4 patients was reported in 1999. Epidemiological investigations identified domestically produced lettuce as the most likely source of infection (6). No domestically acquired food-associated outbreaks have been identified in Denmark (3).

Pathogenic *Vibrio* have been very uncommon causative agents of foodborne illness in the Nordic region. However, a rapidly growing international trade, including import of exotic seafood, has brought this new agent to this latitude, too. Sweden reported one large outbreak of *Vibrio parahaemolyticus* involving 350 persons in 1995 (1). The source of infection was traced to Chinese crayfish. In Finland, one case of *Vibrio cholerae* was found in 1998. The source of contamination was traced to mussels smuggled from Thailand (14).

**TABLE 4** *Listeria monocytogenes* Outbreaks in Nordic Countries, 1990s

Year	Country	Vehicle	Number of cases	Ref.
1992	Norway	Vacuum-packed cold cuts	8	6
1994–95	Sweden	Vacuum-packed fish product	6	37
1997	Finland	Vacuum-packed cold-smoked rainbow trout	5	38
1998–99	Finland	Butter	25	39,40

Norwalk-like viruses were not commonly implicated as foodborne disease agents in reports before 1994 (24). However, improved analytical capacity to detect viruses together with better epidemiological knowledge has resulted in Norwalk-like viruses being among the most commonly reported agents in foodborne outbreaks (Table 1) (1). Finland, Sweden, and Denmark reported large foodborne outbreaks involving hundreds of people in the late 1990s (1,9,14). A Finnish investigation proved a strong correlation between a large outbreak of gastroenteritis and eating imported frozen raspberries (41). Other vehicles reported in connection with foodborne outbreaks caused by Norwalk-like viruses were oysters and mussels (1). Outbreaks caused by hepatitis A virus were reported in Sweden, Finland, and Denmark between 1993 and 1998. In some of these outbreaks the suspected source was traced to contaminated vegetables.

Biogenic amines were the most common chemical substances causing foodborne illness, although the chemical was produced by bacteria. Biogenic amines were the reason for 27 (4%) rather than small outbreaks. Canned tuna fish, salami, and cheese were mentioned as incriminated foods (1).

A causative agent was shown in 41–65% out of the reported outbreaks in different Nordic countries between 1993 and 1998 (1).

#### **IV. RISK ASSESSMENT**

Although work on scientific quantitative risk assessment is in the early stages in various countries, several estimates, evaluations, and qualitative assessments have already been done using the data available. Efforts have especially focused on *Salmonella* and *Campylobacter*.

##### **A. *Salmonella***

Although *Salmonella* spp. have been the leading causative agent in outbreaks, there is great variation between countries. *Salmonella* is more common in Denmark and in Iceland than in Finland, Norway, and Sweden. Registers in Finland, Iceland, Norway, and Sweden show that a large majority, 80–90%, of human *Salmonella* infections are still acquired abroad, whereas most of the infections in Denmark were domestically acquired (1,3,4,6,8,10).

In the 1980s the risk of contracting salmonellosis abroad was 30 times greater than that of acquiring it in Finland. Tourists traveling to Asia or North Africa were most at risk. Although traveling has increased over the past few years, salmonellosis rates have remained stable in Finland, Sweden, and Norway. Either tourists have learned to avoid risky food items, or the rate of *Salmonella* spp. has fallen in those countries.

Food of animal origin, especially poultry meat, other meat, and meat products and eggs and egg products, is the most common source of salmonellosis. In Denmark, eggs are the major source of human salmonellosis, whereas in Finland, Norway, and Sweden, eggs are very seldom (less than 1%) the vehicle. Over the past 10 years fruits and vegetables have played an increasingly greater role. Bean sprouts in particular have been the source of many large outbreaks. The reason for this is contaminated seed, and similar outbreaks have occurred in Finland and Sweden caused by alfalfa seed from the same exporter (26).

##### **B. *Campylobacter* spp.**

A quantitative risk assessment of *Campylobacter jejuni* in chicken products has been carried out in Denmark (42). Three distinct ways of reducing the probability of exposure and illness were analyzed: by reducing the flock prevalence, by reducing the concentration of *Campylobacter* on contaminated chickens, or by improving the level of food hygiene in private kitchens. The Finnish National Salmonella Control Program and the risks caused by the foodstuffs included in the program are currently being assessed at the National Veterinary and Food Research Institute. The first models have been made, and after additional data collection with collaborators, the full risk assessment is expected to be completed in 2001.

The Nordic countries have also joined forces to assess the risk of *Campylobacter* spp. Workshops were held in 1997 and 2000 in order to establish contacts between experts and to exchange and discuss information regarding the risk assessment of *Campylobacter*. Conclusion from the meeting in June 2000 are as follows (30):

The number of human *Campylobacter* infections in most of the Nordic countries is still growing.

The reason for this remains uncertain and might vary from one country to another.

*Campylobacter* in poultry in the Nordic countries would seem to differ not just in the prevalence between countries, but also in the verified serotypes. Whereas O2 and O4 are the most common serotypes found in poultry and humans in Denmark and Sweden, O144 and O4 are the dominating serotypes in Finland.

Drinking water remains a problem in some countries and may pose a potential outbreak risk.

Although available methods to isolate and identify *Campylobacter* are sufficient to identify the different species involved in human campylobacteriosis and to match victims and sources, they are insufficient to ascertain the quantitative number of *Campylobacter*. Neither can we use existing methods to verify source specific strains. In Europe, pulsed-field gel electrophoresis is the preferred method, while sequencing is used in the United States.

### C. Other Microbes

Processed ready-to-eat products, especially those with prolonged shelf life, create a potential risk of *Listeria monocytogenes*. Vacuum-packed, cold-salted, and cold-smoked fish products have caused listeriosis epidemics in Sweden (1994–1995) and Finland (1997) (Table 4). In Norway, vacuum-packed cold meat cuts caused an outbreak in 1992. Butter was found to be the vehicle in Finnish outbreak caused by *Listeria monocytogenes* serotype 3a. A risk-assessment model for *Listeria monocytogenes* involving vacuum-packed ready-to-eat fish products has been carried out in Sweden and in Finland.

In the future, the risk of listeriosis will probably increase due to the aging of the population and the ensuing greater numbers of susceptible people. Another reason will be the increase in refrigerated food products with a longer shelf life.

Improved analytical capacity, together with improved epidemiological knowledge, has resulted in a huge increase in reported Norwalk-like virus outbreaks. Imported frozen raspberries in particular caused several large outbreaks in Finland and Sweden in 1997–1999. Epidemiological investigations have shown Norwalk-like viruses to be the causative agent.

### D. Contributing Factors

Microbes usually require favorable conditions to grow and to produce toxin in a food before the food can cause an infection or poisoning. Microbes need a conducive temperature to be able to grow and produce toxin. It is therefore not surprising that poor temperature control is the most common contributory factor in outbreaks every year. The most common factors falling within this category are improper cooling or refrigeration and inadequate reheating (1,2). Other contributory factors include contaminated raw material and equipment and contamination by staff. Although a food handler is often found to be infected in association with outbreaks, it is difficult to show if he or she is the source.

## V. ONGOING AND PROPOSED MEASURES TO REDUCE FOODBORNE ILLNESS

In combating *Salmonella*, it is essential that control covers the entire production chain, including primary production on the farm. Animal feedstuffs have already been checked for *Salmonella* for



many decades in Finland, Sweden, and Norway. Additional guarantees concerning *Salmonella* were given to Finland and Sweden when they joined the European Union (EU) in 1995. A precondition for these guarantees was that Finland and Sweden have a national *Salmonella* control program relating to *Salmonella* in meat in order to provide for equivalent guarantees concerning meat delivered from other member states and meat produced in Finland and Sweden. A similar program has been approved in the agreement between the EU Commission and Norway. The control program aims at keeping the incidence of *Salmonella* in production animals and products obtained from these at a level below 1% and below 5% in individual abattoirs and cutting plants. In Denmark, an intensive plan to control *Salmonella* in whole table-egg production commenced in 1996 and was fully implemented in 1998 (3,10).

The reason for the continuing increase of *Campylobacter* infections remains uncertain and is a concern for public health in the Nordic countries. Considerable research and method development are in progress in the Nordic countries in a bid to trace the source of infection (30). Sweden has been carrying out a surveillance system for *Campylobacter* in broilers for many years (8). In Iceland, a *Campylobacter* control program was established following a significantly increased number of campylobacteriosis in 1998. This increase occurred at the same time that the selling of fresh broilers began. Measurements resulted in a marked fall in human cases in 2000.

According to European Council Directive 93/43/EEC (43), food business operators shall ensure that food handlers are supervised and instructed and/or trained in food hygiene matters commensurate with their work activity. In Denmark, for example, 8–24 hours of hygiene training for employees has been compulsory since 1997. In Sweden, such training is organized by the National Food Administration and companies together. In Sweden this course is voluntary.

Since autumn 2001 in Finland, employees have been able to take an exam to show they have sufficient knowledge of food handling. The exam is held by the National Food Agency. Information received about foodborne outbreaks, especially contributory factors, is analyzed and actively used in food handler education and training.

Swedish and Finnish authorities have recommended that retail sale temperatures should be lowered and sell-by dates shortened in a bid to reduce the risk of *C. botulinum* and *L. monocytogenes* associated with vacuum-packed ready-to-eat fish products. Hygiene measures in fishery establishments in Finland in 2000 were followed by a research project of ready-to-eat fish products at the retail level.

The slaughtering practice of pigs has been changed in Nordic countries in order to avoid infections by *Yersinia* spp. With respect to beef production, recommendations on slaughtering hygiene and meat hygiene have been given in order to eliminate EHEC contamination.

On the basis of several identified Norwalk-like virus infections via frozen raspberries, it has been recommended to the institutional kitchens in Finland to heat frozen raspberries that have been imported from East European countries to at least 90°C for 2 minutes.

The Nordic countries are actively publishing food safety leaflets and other types of information to distribute to consumers.

## VI. ESTIMATED EFFECTIVENESS OF THE MEASURES

The national *Salmonella* control programs of Sweden, Finland, and Norway were mentioned earlier, as were their objectives, which were successfully achieved in 1995–1999 (23). As a consequence of the control program in Denmark, reported outbreaks caused by *S. Enteritidis* fell sharply during 1998 and 1999. A fairly comprehensive cost-benefit analysis of the *Salmonella* control program has been made in Finland. This analysis clearly demonstrates how it pays to keep the incidence rate at such a low level. This type of analysis involves several uncertainties, and the benefit-cost ratio can be estimated in many different ways. However, in each case, the ratio was >1 and at best 268 (44).

Sweden and Iceland both have a program to control *Campylobacter*. However, the incidence rate of campylobacteriosis is still high in Sweden (80.2 cases/100,000 inhabitants), whereas in Iceland the figures have dropped.

The requirements concerning the education of food handlers are so new that it is impossible to say how effective they are. However, all the parties involved—the authorities, trade and industry, and teachers—have high expectations.

The strong recommendations relating to controlling the risk of *C. botulinum* and *L. monocytogenes* seem to have had a positive effect. In Finland, the occurrence of *L. monocytogenes* in vacuum-packed fish products was much lower in the studies performed in 2000 (5%) than in the 2 previous years (45).

Changing porcine slaughtering practice has been reflected in the decreasing incidence of yersiniosis, especially *Y. enterocolitica*, in the Nordic region. The sources and contamination routes concerning *Y. pseudotuberculosis*, however, are not clear.

Outbreaks of Norwalk-like viruses via frozen raspberries have significantly fallen in Finland after the recommendation to institutional kitchens on heat treatment of raspberries imported from Eastern European Countries (15).

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## Incidence of Foodborne Illness in Central Europe

**Maria Barna and György Biró**

*Semmelweis University, Budapest, Hungary*

For this chapter, the following countries ranging from north to south are included as representative of Central Europe: Poland, The Czech Republic, The Slovak Republic, Austria, Hungary, Croatia, and Slovenia. In spite of their geographical closeness, these countries are rather heterogeneous with regard to their nutritional habits, choice of consumed foodstuffs, and cooking methods used in general. The incidence of foodborne illness is therefore described separately for each country. The territory and the population of each country is described for general information and for consideration of the magnitude of outbreaks and cases. The life expectancy at birth, which seems to be one of the important features concerning the health status of the population and health care, is also noted.

It is important to note that the incidence of foodborne illnesses differs among the different Central European countries, although the morbidity rate appears to be fairly similar through the years in the same country. This is due to factors such as (a) differences in the principles and practices of reporting and registration; (b) differences in the epidemiological investigations and evaluations (e.g., whether a gastrointestinal illness is regarded as foodborne); (c) the efficacy of the reporting system (it is the opinion of several experts that no more than 4–10% of actual foodborne illnesses are generally reported) (1). However, in a country with a well-functioning public health system, the proportion of registered foodborne illnesses might be much higher.

### I. ESTIMATED RATE OF ILLNESS

#### A. Poland

Poland is approximately 312,700 km<sup>2</sup> with about 38.7 million inhabitants. Life expectancy at birth is 68.9 years in men and 77.3 years in women.

The “collective” outbreaks of foodborne illnesses are reported and registered. The term “collective outbreak” means four or more cases (2). [Table 1](#) gives an overview of the frequency of illnesses that are or may be transmitted by foods.

#### B. Czech Republic

The Czech Republic is approximately 78,900 km<sup>2</sup> in size with about 10.3 million inhabitants. Life expectancy at birth is 71.1 years in men and 78.1 years in women.

Between 1993 and 1997 the number of cases caused by bacterial foodborne intoxication fluctuated between 332 and 924 ([Table 2](#)). In 1997, the distribution of causative agents was as follows: *Bacillus* spp. and *Clostridium perfringens* type A, 10–10%; *Staphylococcus aureus*, 9%; nothing found, 26%; not examined, 45%.

**TABLE 1** Overview of Possible Foodborne Intestinal Diseases (Cases) Caused by Bacteria or Bacterial Toxins, Poland, 1985–1998

Year	Salmonellosis (altogether)	Staphylococcal enterotoxin	Botulism	Other bacteria
1985	39,072	700	551	3506
1986	47,743	1000	417	4926
1987	49,242	428	284	4370
1988	61,522	517	357	4232
1989	53,534	489	314	3425
1990	49,507	538	328	3128
1991	52,127	235	173	2103
1992	42,061	609	165	2565
1993	31,154	374	143	2483
1994	36,344	268	116	2166
1995	30,093	760	118	2990
1996	26,106	213	107	2497
1997	23,206	450	81	4234
1998	26,739	375	93	3372

### C. Slovak Republic (Slovakia)

The size of Slovakia is about 49,000 km<sup>2</sup>, with approximately 5.4 million inhabitants. The life expectancy at birth in men is 69.6 years and in women, 76.7 years.

In Slovakia the incidence of salmonellosis is increasing: in 1975 there was a morbidity rate somewhat below 100 per 100,000 inhabitants, in 1985 about 120, and in 1997 almost 350 per 100,000 (3). The highest age-specific morbidity has been shown to be in infants and children 1–4 and 5–9 years of age. There is no information on the role of food and other factors in the transmission of infection by *Salmonella* spp., but the incidence of other gastrointestinal bacterial infections also appear to have a slightly increasing tendency (morbidity rate: 15 per 100,000 inhabitants in 1975, 40 per 100,000 in 1997). However, in 1991 the incidence rate of shigellosis, which can be foodborne, decreased from about 250 per 100,000 inhabitants in 1975 to approximately 30 in 1997.

### D. Austria

The size of Austria is approximately 83,900 km<sup>2</sup>, with a population of roughly 8.1 million. The life expectancy in men is 73.9 years and in women, 80.2 years.

In Austria, data on bacterial foodborne infections and intoxications are available (4). These are summarized in Table 3. There is a peak in 1992 and 1993 followed by a decrease in the following 2 years, but since then there appears to have been a slight, steady increase.

**TABLE 2** Foodborne Intoxications of Bacterial Origin, Czech Republic, 1993–1997

	1993	1994	1995	1996	1997
Number of cases	924	406	916	625	332
Morbidity rate per 100,000 inhabitants	8.9	3.9	8.9	6.1	3.2

**TABLE 3** Foodborne Diseases of Bacterial Origin, Austria, 1986–1998

	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998
Number of cases	1845	2239	2710	4973	8671	9362	11,125	10,014	8706	8705	9306	9451	9995

## E. Hungary

The size of Hungary is approximately 93,000 km<sup>2</sup> with a population of 10.1 million. The life expectancy at birth in men is 66.1 years and in women 75.2 years (the lowest levels in Central Europe).

The number of foodborne outbreaks and cases fluctuated in the last 15 years from year to year without a definite tendency. However, between 1993 and 1996 (and even considering the number of outbreaks until 1998) there was an increasing trend as shown in Table 4 (5,6). It is noteworthy to mention that in 1996 a large outbreak of salmonellosis in a school catering establishment caused 5243 cases (Table 4).

## F. Croatia

Croatia is about 56,500 km<sup>2</sup> in size, with approximately 4.5 million inhabitants. Data on life expectancy are not available.

The total number of foodborne illnesses in Croatia decreased during the mid-1990s (1993–1996), but since 1997 there has been almost no change in the number of illnesses (7).

## G. Slovenia

Slovenia is about 20,300 km<sup>2</sup> in size and has a population of approximately 1.98 million people. Life expectancy in men is 71.1 years and in women, 78.7 years. The data on foodborne illnesses reported between 1990 and 1999 are summarized in Table 5. The frequency of outbreaks fluctuated between 22 and 33, without a definite trend. However, the number of cases appeared to be decreasing, with minimal mortality (8).

## II. REPORTED ILLNESSES

### A. Poland

The causative agents for foodborne and waterborne illnesses are registered in one group. However, in 1998, drinking water was the vehicle in two outbreaks involving 54 cases. The overwhelming

**TABLE 4** Foodborne Outbreaks and Cases, Hungary, 1985–1998

	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998
Outbreaks	358	295	466	561	316	313	321	242	351	504	659	716	544	772
Cases	4964	3970	7856	4719	5872	3679	5268	2928	5779	4377	4983	10364	4490	4362
Cases/outbreak	13.9	13.4	16.8	8.4	18.6	11.7	16.4	12.1	16.5	8.4	7.6	14.5	8.2	5.6

**TABLE 5** Overview of Foodborne Outbreaks and Cases, Slovenia, 1990–1999

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999
Outbreaks	33	26	33	39	26	23	27	22	26	24
Cases	2823	2387	2805	1955	1582	1300	2087	1275	1789	876
Deaths	5		1		1				1	

**TABLE 6** Causative Agents of Foodborne and Waterborne Diseases, Poland, 1997–1998

Causative agent	1997				1998			
	Outbreak		Case		Outbreak		Case	
	No.	%	No.	%	No.	%	No.	%
<i>Salmonella</i> spp.	274	85.5	4817	80.0	325	78.9	6662	78.2
<i>C. botulinum</i>	2	0.6	8	0.1	1	0.2	4	0.04
<i>E. coli</i> (unspecified)	8	2.4	271	4.5	14	3.4	348	4.1
<i>Shigella</i> spp.	9	2.7	164	2.7	10	2.4	275	3.2
<i>S. aureus</i>	12	3.7	395	6.6	13	3.2	371	4.4
Other					1	0.2	5	0.06
All bacteria	305	93.0	5655	93.9	364	88.3	7665	89.9
<i>Trichinella</i>	1	0.3	8	0.1	3	0.7	24	0.3
Rotavirus					1	0.2	26	0.3
Mushrooms	2	0.6	9	0.1				
Mycotoxin					1	0.2	13	0.2
Chemicals					1	0.2	9	0.1
Unknown	20	6.1	348	5.8	42	10.2	787	9.2
Total	328	100.0	6020	100.0	412	100.0	8524	100.0

majority of the outbreaks have been caused by bacteria, including salmonellae. The role of viruses, mycotoxins, and chemicals is negligible. Cases of taeniasis fluctuated during the late 1980s and early 1990s between 1400 and 1850 per year; in 1997 the numbers decreased to 763 cases, and in 1998, to 634 cases. There was a total number of 20 cases of trichinosis in 1997, and 33 in 1998, but those implicated in foodborne illnesses were lower. Toxic mushrooms have seldom been implicated in causing illness. The number of unknown causative agents seems rather low. The distribution of the causative agents is shown in Table 6.

Among the salmonellae, *S. enteritidis* is the predominant serotype. In 1997 this serotype caused 96.7% of the outbreaks and 95.5% of cases and, in 1998, 94.5% and 92.5% of outbreaks and cases, respectively. The second most prevalent agent was *S. indiana* in 1997 (0.4% of outbreaks, 2.4% of cases) and *S. virchow* in 1998 (1.5% of outbreaks and 2.7% of cases).

## B. The Czech Republic

As in Poland, salmonellosis appears to be the most important foodborne illness in the Czech Republic (9). The total number of cases, the number of outbreaks, the number of cases per outbreak, and the morbidity rate for 1993–1997 are shown in Table 7. In 1997 *S. enteritidis* was isolated in 95.3% of the stools of infected persons, *S. typhimurium* from 2.9%, and other *Salmonella* spp. from 1.8%. Over 90% of the salmonelloses were transmitted by foods. In 1997, 93.5% of all the illnesses were

**TABLE 7** Salmonellosis Cases, Outbreaks and Morbidity Rate per 100,000 Inhabitants, Czech Republic, 1993–1997

	1993	1994	1995	1996	1997
Number of cases	43147	50734	54554	48143	39862
Number of outbreaks	31	32	40	23	34
Cases per outbreak	42.1	50.3	36.8	27.4	31.8
Morbidity rate	417.7	490.8	518.0	466.4	385.9



**TABLE 8** Proportion of Vehicle Foods in 12,669 Salmonellosis Cases, Czech Republic, 1997

Foodstuff	Percent
Confectionery	15.8
Poultry meat	14.9
Desserts	11.7
Meat products	7.8
Dairy products	4.6
Sausages, salami	11.6
Eggs	27.7
Fish	2.6
Vegetables, fruits	0.2
Ice cream	2.1
Others	1.0

of foodborne origin, 3.7% were transmitted by contact, and 0.1% by water. Specific foods have been incriminated in roughly one third of foodborne salmonellosis (Table 8).

The number of reported campylobacteriosis cases is much lower than salmonellosis (in 1996 2545 cases; in 1997 3623 cases), but it is the second most frequent zoonosis in the Czech Republic. The morbidity rate was the lowest in 1993 (21/100,000 inhabitants) and the highest in 1997 (37/100,000 inhabitants). Campylobacteriosis was the most frequently isolated organism in the 1- to 4-year age group (Table 9). In 1997 the food vehicle was identified in 47% of cases, with the mode of transmission remaining unclear for the same proportion of cases.

In 1997, 325 cases caused by *Citrobacter* spp. were reported. In one outbreak 122 people were exposed and 31 became ill. The incriminated food was a salami spread left several hours at room temperature. The highest age-specific morbidity rate was found in the 0- to 4-year age group (42.5/

**TABLE 9** Age-Specific Morbidity Rate per 100,000 Inhabitants of 3623 Campylobacteriosis Cases, Czech Republic, 1997

Age (years)	Morbidity rate
0	2.0
1-4	9.6
5-9	5.3
10-14	3.2
15-19	3.0
20-24	3.3
25-34	3.0
35-44	1.8
45-54	1.6
55-64	0.7
65-74	0.9
> 74	0.6

**TABLE 10** Foodborne Diseases (Cases) by Causative Bacteria, Austria, 1996–1998

Year	Causative bacterium							
	<i>C. botulinum</i>	<i>Campylobacter</i> spp.	EHEC	<i>Salmonella</i> spp.	<i>Shigell</i> spp.	<i>Staphylococcus aureus</i>	<i>Yersinia enterocolyticá</i>	Not identified
1996	4	1131	1	7209	112		52	797
1997		1667	13	7488	204	4	70	5
1998		2454	17	7236	167	16	94	11

100,000 inhabitants in 1996; 34.4/100,000 inhabitants in 1997); in the other age groups the morbidity rate was 1.7–4.1/100,000 inhabitants). There are no data on the mode of transmission.

With regard to yersiniosis, 206 cases were reported in 1996 and 155 in 1997. The highest incidence was reported in children below 9 years of age. Here again, there is no information on the mode of transmission.

### C. Slovakia

In the course of bacterial investigations of food samples (roughly 20,000 samples/year), the following strains were isolated in 1998: 138 *Salmonella* strains, 57 *Aeromonas* strains, 89 *Pseudomonas aeruginosa* strains, 12 *C. perfringens* strains, 764 *S. aureus* strains, and 1 *Listeria monocytogenes* strain. Among the salmonellae, *S. enteritidis* and *S. typhimurium* have been the most frequently isolated serotypes. The role of *S. enteritidis* appears to be increasing, and that of *S. typhimurium* appears to be decreasing. In 1999 the contribution of pathogens in the gastrointestinal infections other than salmonellosis was as follows: *Campylobacter* spp., 57.96%; *E. coli*, 23.1%; *B. proteus*, 3.56%; *Citrobacter*, 3.19%; *Klebsiella*, 2.99%; *Pseudomonas aeruginosa*, 1.85%; *Yersinia enterocolitica*, 1.52%; *Enterobacter*, 1.25%; *B. cereus*, 0.28%; *S. aureus*, 0.28%. In 1991 the incidence rate of shigellosis — the disease is partly of foodborne origin — decreased from about 250/100,000 inhabitants in 1975 to approximately 30 in 1997 and about 5 in 1999. The age-specific morbidity is similar to that of salmonellosis.

### D. Austria

Among the causative agents, the salmonellae are the most predominant and campylobacters the second most predominant, but the latter appear to have a significant increasing tendency. The details are shown in Table 10.

### E. Hungary

Between 1985 and 1998, a total of 122 persons died due to foodborne illness, mostly as a consequence of intoxication caused by toxic mushrooms. In Hungary, people not very familiar with toxic mushrooms often gather these in the woods and fields, prepare meals at home containing both edible and toxic mushrooms, and become ill. The mortality of foodborne illness here is very low (0.16 %) except for the intoxications caused by toxic mushrooms (2.1%) (Table 11).

**TABLE 11** Mortality Caused by Foodborne Diseases, Hungary, 1985–1998

<i>Salmonella</i> spp.	Causative agent							Total
	<i>C. botulinum</i>	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	Toxic mushrooms	Toxic plants	Chemicals		
15	4	2	1	91	1	8	122	

**TABLE 12** Foodborne Diseases by Causative Agents, Hungary, 1997–1998

Causative agent	1997				1998			
	Outbreak		Case		Outbreak		Case	
	Number	%	Number	%	Number	%	Number	%
<i>Aeromonas</i> spp.	1	0.2	9	0.2				
<i>Campylobacter</i> spp.	23	4.2	75	1.7	47	6.1	207	4.7
<i>Enterococcus faecalis</i>	3	0.5	163	3.6	1	0.1	22	0.5
<i>E. coli</i> (unspecified)					1	0.1	13	0.3
<i>Klebsiella</i> spp.					1	0.1	126	2.9
<i>Salmonella</i> spp.	389	71.5	3412	76.0	485	62.8	2535	58.1
<i>B. cereus</i>	4	0.7	218	4.8	5	0.6	177	4.1
<i>C. perfringens</i>	1	0.2	97	2.2	1	0.1	83	1.9
<i>C. botulinum</i>	10	1.8	18	0.4	10	1.3	19	0.4
<i>S. aureus</i>	4	0.7	20	0.4	7	0.9	64	1.5
All bacteria	435	80.0	4012	89.3	558	72.3	3246	74.4
Toxic mushrooms	76	14.0	175	3.9	156	20.2	382	8.8
Toxic plants	1	0.2	1	0.0				
Toxic animal					1	0.1	3	0.1
Chemicals	2	0.4	4	0.1	1	0.1	3	0.1
Nothing found	24	4.4	279	6.2	34	4.4	661	15.1
Not investigated	6	1.1	19	0.4	22	2.8	67	1.5
Total	544	100.0	4490	100.0	772	100.0	4362	100.0

*Salmonella* spp. appear to be the most important causative agents, although their contribution to foodborne illness slightly diminished in 1998. However, the causative role of *Campylobacter* spp. is on the increase (Table 12).

## F. Croatia

The percentage of cases caused by *Salmonella* spp. fluctuated between 44.7% and 64.5% (Table 13). Among the causative agents in outbreaks, salmonellae are as predominant as in other Central European countries. In 1999, 50.2% of cases in outbreaks were caused by *Salmonella* spp. There is a high incidence of trichinellosis (23.8% of cases in outbreaks in 1999) with an apparent increasing trend. The number of cases with unknown causative agents seems relatively low (Table 14).

**TABLE 13** Foodborne Outbreaks and Cases, Croatia, 1993–1999

	1993	1994	1995	1996	1997	1998	1999
Number of outbreaks	56	67	47	47	56	65	66
Cases/outbreak	2028	1811	1433	928	581	1492	1223
Number of sporadic cases	8954	7237	6294	5553	7660	6828	7021
Total number of cases	10982	9048	7727	6481	8241	8320	8244
Salmonellosis	7087	4931	3642	2899	4204	4288	4120
Salmonellosis, percent of cases	64.5	54.5	47.1	44.2	51.0	51.5	50.0

**TABLE 14** Cases in Foodborne Outbreaks by Causative Agents, Croatia, 1993–1999

Causative agent	1993	1994	1995	1996	1997	1998	1999
<i>Salmonella</i> spp.	1374	1240	900	403	378	723	614
<i>Shigella</i> spp.	192	7				4	4
<i>C. jejuni</i>			14				
<i>E. coli</i> (unspecified)				19	15		
<i>S. aureus</i>	73	14	7		84		30
<i>C. perfringens</i>			50	65		16	57
<i>C. botulinum</i>		3		5	3	9	12
<i>B. cereus</i>		10					
HAV							7
Trichinella	25	63	115	156	49	298	291
Mycotoxin	35				6		
Histamine		17					
Unknown	329	457	347	280	46	54	208

## G. Slovenia

In 1997 the main causative microorganisms were *B. cereus* and coronavirus; in 1998 *S. pyogenes*, *S. aureus*, and *Salmonella* spp.; in 1999 *Salmonella* spp. and *B. cereus*. The pattern of causative organisms in this country is different from that seen in other Central European countries (Table 15).

## III. FREQUENCY AND RELATED FOODS

### A. Poland

We have an interesting picture when we look at the role of different foods in the transmission of causative agents. The most important vehicles include cakes, cream, and ice cream, followed by mayonnaise and different meals with eggs. The details are shown in Table 16. The category “ready-to-serve meals” includes mayonnaise, croquettes, pancakes, dumplings, and vegetable salads. “Raw

**TABLE 15** Foodborne Diseases by Causative Agents, Slovenia, 1997–1999

Causative agent	1997		1998		1999	
	Outbreak	No. cases	Outbreak	No. cases	Outbreak	No. cases
<i>S. aureus</i>	5	105	4	146	1	17
<i>C. botulinum</i>		2		1	2	6
<i>B. cereus</i>	1	195		1	1	68
<i>E. coli</i> (unspecified)	1	22				
<i>Salmonella</i> spp.	3	58	6	145	10	170
<i>S. pyogenes</i>			1	350		
<i>C. perfringens</i>	1	49	1	14		
Shellfish poisoning			1	21		
<i>Campylobacter</i> spp.			1	81		
Coronavirus	1	194				
Enterovirus	1	n.d.			1	n.d.
Nonidentified	9	650	12	1030	9	609

n.d. = No data.

**TABLE 16** Foodborne Disease Incidents by Incriminated Foods, Poland, 1998

	Milk, dairy products	Meals from milk and eggs	Eggs and egg products	Cakes, creams, ice cream	Ready- to-serve meals	Meals from eggs	Raw minced meat, eggs	Meals from meat and eggs	Meat, meat products	Meals from meat and vegetables	Poultry meat	Meals from poultry and eggs	Venison, venison products	Fish and fish products	Soups and sauces	Mushrooms and edible plants	Others and meals from ≥2 raw materials	Unknown
Number of outbreaks	5	6	4	155	48	16	9	27	18	1	7	13	1	2	2	4	41	51
Percent of outbreaks	1.2	1.5	1.0	37.6	11.6	3.9	2.2	6.5	4.4	0.2	1.7	3.2	0.2	0.5	0.5	1.0	9.9	12.4
Number of cases	98	163	44	2719	728	250	296	656	261	5	216	216	8	42	23	96	1403	1245
Percent of cases	1.1	1.9	0.5	31.9	8.5	2.9	3.5	7.7	3.1	0.1	2.5	2.5	0.1	0.5	0.3	1.1	16.4	14.6

minced meat” indicates a dish made of ground or minced raw meat mixed with raw eggs, spices, and onion.

## B. Slovakia

In the early 1990s the foods serving as vehicles for illness were ranked as follows, in order of highest to lowest: cold plates (salads), meat and meat products, ice cream, eggs and eggs products, confectionery, poultry, meat (10). The most common place where food was prepared and/or consumed implicated in foodborne illness was the catering trade (school catering, canteens, hospitality industry), with households coming in second.

## C. Austria

The majority of outbreaks caused by salmonellae (98–99%) comes from household (family) outbreaks. Poultry, hens’ eggs, and food containing eggs are the most important sources of human infections. In the early 1990s the serotypes *S. enteritidis* and *S. typhimurium* were the most frequently isolated salmonellae.

## D. Hungary

Regarding the incriminated foods, eggs and egg-containing meals, different kinds of meat, cold plates, confectionery products, and meals containing mushrooms play a major role (Table 17). Among the places where foods were contaminated, acquired, or eaten, households where there was mass catering ranked the highest (Table 18).

## E. Croatia

The incriminated food was not found in 5.3–19.1% of outbreaks between 1993 and 1999. Egg cream cakes accounted for the major portion of incriminated foods, with 23.8% of outbreaks. Eggs and eggs products were involved in 9.4% of the outbreaks, indicating that eggs played a role in a total of 33.2% of outbreaks. Meat courses came second (17.5%) and meat products third (17.5%) (Table 19).

**TABLE 17** Foodborne Incidents by Incriminated Foods, Hungary, 1997–1998

Food	1997		1998	
	Outbreak	Case	Outbreak	Case
Milk	1	1	2	68
Dairy products			2	21
Eggs	160	483	242	697
Meat	57	418	108	377
Meat products	5	64	3	5
Chitterlings	6	32	6	24
Pork cheese	8	64	8	25
Offals	3	30	6	6
Ice cream	3	42	1	4
Pastry filled with custard	21	297	22	127
Floating islands	39	469	27	114
Noodles	18	386	11	135
Cold plates	35	515	33	256
Confectionery	22	252	22	209
Meals from mushrooms	87	193	183	434
Others	50	996	74	1568
Unknown	29	248	22	292

**TABLE 18** Foodborne Incidents by Place Where Food was Contaminated, Acquired, or Eaten, Hungary, 1997–1998

Place	1997		1998	
	Outbreak	Case	Outbreak	Case
Catering trade	22	560	39	671
Workplace canteen	8	498	5	144
Kindergarten, school catering	20	903	32	1414
Medical care facilities	5	85	6	156
Household	459	1631	665	1706
Meat-processing plant	4	49		
Confectioner's shop	13	448	9	103
Other food-processing plant	1	3		
Retail store, market	3	6	5	9
Holiday resort, camp	4	95	2	25
Social welfare institutions	2	57	3	70
Others	2	154	3	60
Unknown	1	1	3	4

With respect to places where the incriminated foods were acquired, eaten, or contaminated, the major risks were represented either by households (41.3% of outbreaks 1993–1999) or by mass catering establishments (13.4% in the hospitality industry, 11.9% social gatherings, 11.1% in canteens) followed by medical care facilities (7.9%). (Table 20).

## F. Slovenia

The most frequent places where the foods were contaminated or eaten include the food and hospital-ity industries and households (Table 21).

**TABLE 19** Foodborne Disease Outbreaks by Incriminated Foods, Croatia, 1993–1999

Food	1993	1994	1995	1996	1997	1998	1999
Beef	2	1	2				
Pork	3	5	4	3	5	5	3
Chicken meat	6	4	1		6	4	
Unspecified meat	5	1	3	2		1	2
Minced meat	4	2				3	1
Sausages		2	2	7	5	8	16
Other meat product	1	5	1	2	4	4	4
Fish, shellfish	1	4	1	2		1	1
Eggs, egg products	1	6	6	5	10	6	4
Bean salad	2	1		1			1
Potato salad, coleslaw	7	4	1	2	2		1
Confectionery, ice cream	7	7	3		2	1	2
Egg cream cakes	14	14	14	8	12	18	16
Milk, dairy products	2	1	3	2	3	3	4
Ready-to-eat meals	1	2	3	4	1	4	6
Mushrooms	2				1		
Unknown	3	8	9	9	5	7	5

**TABLE 20** Foodborne Outbreaks by Place Where Food Was Contaminated, Acquired, or Eaten, Croatia, 1993–1999

Place	1993	1994	1995	1996	1997	1998	1999
Canteen	12	8	8	5	5	2	5
Catering trade	10	11	6	8	2	7	10
Confectioner's shop	7	6	4	2	2	3	4
Private household	14	24	13	15	36	37	28
Holiday resort		1		1		1	
Medical care facilities	3	4	5	10	3	4	3
Refugee camp	1	2	2	2		2	
Kindergarten	5	4	1	1	1	1	2
Social gathering	4	6	8	3	7	7	13
Unknown		1				1	1

## IV. RISK FACTORS

### A. Poland

For the purposes of assessing risk, we can analyze the distribution of places where food was prepared or takeout food was acquired, where food was eaten, and where food was contaminated (Tables 22–24). In 1998 2438 persons were hospitalized, i.e., 28.6% of all the cases (8524), because of foodborne (or waterborne) illnesses. There were no deaths. The number of subjects at risk was estimated to be 28,656, i.e., 336.2% of cases.

### B. Hungary

As contributing factors to foodborne illness incidents, inadequate heating, infected or toxic raw materials (toxic mushrooms), and inadequate cooling are to be emphasized (Table 25).

### C. Croatia

Contributing factors in most cases included inadequate heating, reheating, use of contaminated raw materials, and contamination by infected persons. Improper warm holding and inadequate thawing of deep-frozen foods plays a minor role in causing foodborne illness (Table 26).

**TABLE 21** Number of Outbreaks by Place Where Food Was Contaminated or Eaten, Slovenia, 1997–1999

Place	1997	1998	1999
Industry	9	7	7
Restaurant	5	7	3
Private household	1	3	7
Hospital	1	1	
Nursing home	2	3	2
School	1	3	2
Kindergarten	2		
Holiday resort		1	
College		1	1
Other			2



**TABLE 22** Foodborne Diseases by Places Where Incriminated Food was Prepared or Acquired, Poland, 1998

	Private household	Holiday resort	Canteen	Kindergarten, school catering	Sanatorium	Hospital	Welfare home	Orphanage	Restaurant	Café, confectionery	Excursion	Other	≥2 places	Unknown
Number of outbreaks	234	9	10	37	5	11	4	3	10	38	4	20	23	4
Percent of outbreaks	56.8	2.2	2.4	9.0	1.2	2.7	1.0	0.7	2.4	9.2	1.0	4.8	5.6	1.0
Number of cases	3178	420	195	1120	162	554	89	71	181	949	55	891	789	70
Percent of cases	37.3	4.9	2.3	13.1	1.9	6.5	1.0	0.8	2.1	11.1	0.6	8.1	9.3	0.8

**TABLE 23** Foodborne Diseases by Places Where Food was Eaten, Poland, 1998

	Private household	Holiday resort	Canteen	Kindergarten, school catering	Sanatorium	Hospital	Welfare home	Orphanage	Restaurant	Café, confectioner's shop	Excursion	Other	≥2 places	Unknown
Number of outbreaks	210	8	11	41	5	12	5	4	15	6	6	33	56	
Percent of outbreaks	51.0	1.9	2.7	9.9	1.2	2.9	1.2	1.0	3.6	1.5	1.5	8.0	13.6	0.0
Number of cases	2315	312	203	1194	162	560	139	82	342	117	66	993	2039	
Percent of cases	27.2	3.7	2.4	14.0	1.9	6.6	1.6	1.0	4.0	1.4	0.8	11.6	23.9	0.0

**TABLE 24** Foodborne Diseases by Place Where Food was Contaminated, Poland, 1998

	Private farm	Collective farm	Private household	Holiday resort	Kindergarten, school catering	Hospital	Welfare home	Orphanage	Restaurant	Excursion	Others	Uncertain	≥2 places	Unkonwn
Number of out- breaks	126	4	4	1	4	1	1	1	1	1	32	61	49	125
Percent of outbreaks	30.6	1.0	1.0	0.2	1.0	0.2	0.2	0.2	0.2	0.2	7.8	14.8	11.9	30.3
Number of cases	2185	76	37	45	121	83	26	25	13	7	695	699	1514	2960
Percent of cases	25.6	0.9	0.4	0.5	1.4	1.0	0.3	0.3	0.1	0.1	8.1	8.2	17.8	34.7

**TABLE 25** Foodborne Incidents by Contributing Factors, Hungary, 1997–1998

Contributing factor	1997		1998	
	Outbreak	Case	Outbreak	Case
Inadequate heating	171	1411	269	1076
Inadequate cooling	37	226	49	492
Improper warm holding	6	207	3	288
Improper storage	1	48		
Improper preparing	5	205		
Infected raw material	106	630	120	619
Poisonous raw material (mushrooms, weed seeds, etc.)	80	178	178	430
Contamination by infected person	5	116	1	19
Contamination by infected animal			1	26
Contaminated equipment	7	134	3	196
Use of illegal ingredients	2	4	1	3
Unknown	124	1331	147	1213

## D. Slovenia

The most recurrent foods identified as transmitting factors in outbreaks ranged from poultry through ground meat to confectionery products (Table 27).

## V. ONGOING OR PROPOSED MEASURES TO REDUCE FOODBORNE ILLNESS

### A. Poland

In Poland outbreaks of foodborne infections and intoxication are required to be reported by the practitioner to the Regional Institute of Hygiene and Epidemiology, from there to the District Institute of Hygiene and Epidemiology, and finally to the National Institute of Hygiene, which makes

**TABLE 26** Foodborne Outbreaks by Contributing Factors Croatia, 1993–1999

Contributing factor	1993	1994	1995	1996	1997	1998	1999
Too long between cooking and consuming	7	2	3	1	2	5	5
Improper warm holding	1		1	1	1	2	1
Improper storage	8	6	2	2	5	3	5
Inadequate thawing	1			3		2	1
Inadequate cooling	4	6	2	3	7	9	3
Inadequate heating, reheating	6	6	9	5	14	10	8
Infected raw material	1	9	3	8	2	10	9
Contamination by infected person	8	10	10	6	6	4	5
Contaminated equipment	5	4	3	8	3	1	5
More factors	5	5	2	1	5	6	6
Other factors	2	5	3	5	6	9	10
Unknown	8	14	9	4	5	4	8

**TABLE 27** Number of Outbreaks by Incriminated Food, Slovenia, 1997–1999

Food	1997	1998	1999
Minced meat	2	1	2
Poultry	2	1	4
Smoked meat			2
Meat products		1	
Confectionery	1	1	3
Eggs, mayonnaise	1		1
Ice cream	1		
Dairy products	1		
Vegetables	1		
Shellfish	1	1	
Other	3	2	1
Unknown	9	19	11

the central registration and directs the epidemiological activity (10). This reporting is obligatory if the consumption of food is suspected or known to be the cause of acute intestinal illness or other specific symptoms of a foodborne illness. All outbreaks are epidemiologically investigated when at least four people are involved, but in the case of botulism, even a single case must be examined. The laboratory methods used in the course of investigations are prescribed and controlled by the National Institute of Hygiene.

## B. The Czech Republic

In the Czech Republic the National Institute of Public Health is the central organization responsible for the control and the registration of foodborne illnesses (10). The eight regional Institutes of Hygiene and Epidemiology govern the 85 District Institutes of Hygiene and Epidemiology. There is obligatory notification of communicable illnesses, including foodborne illnesses. Two cases connected with the same source of infection are considered an outbreak. Cases not connected are regarded as sporadic. The primary care physician who tends to the ill person(s) is obliged to report the incident to the local District Institute.

## C. Slovakia

In Slovakia there is a statutory notification of foodborne and other infectious illnesses (10). The first step is reporting by the practitioner to the District Institute of Hygiene and Epidemiology. The central National Institute of Hygiene and Epidemiology acts as a guiding center of the countrywide network: it prepares proposals and provides expertise for the Ministry of Health. The District Institutes carry out epidemiological investigations for each case.

## D. Austria

In Austria bacterial foodborne illnesses (diagnosed or suspected) are notifiable (10). Practitioners must report the case(s) immediately to the local health authority, which then forwards the report through the state health authorities to the Ministry of Labour, Health and Social Affairs. The National *Salmonella* Center of Austria in the Federal Institute for Bacteriology and Serology identifies all the *Salmonella* strains isolated in the country. The local and state authorities, after obtaining information from practitioners, conduct epidemiological investigations and take the necessary steps together with the food-controlling authorities and the trade board to control the outbreak.

## E. Hungary

Data on foodborne infections and intoxications are collected in Hungary according to two reporting systems (10). The first is that of foodborne illnesses: each outbreak in which food or beverages are suspected or have been confirmed has to be reported, no matter the number of ill persons or the causative agent. The second reporting system takes into account all cases of notifiable communicable illnesses, including foodborne infections. The first notification is that of the practitioner who detects the illness, but managers of food production establishments, trade, and catering are also obliged to report if they have information about persons becoming ill. Laboratory investigations have to be made of each outbreak.

## F. Croatia

Before 1991 Croatia was a federal unit of the former Yugoslavia and the reporting system for foodborne illnesses was part of that of Yugoslavia, but since then the data for Croatia have been registered and published separately. As an independent state, Croatia has developed its own reporting system according to the international requirements whereby foodborne diseases are notifiable.

## G. Slovenia

Until 1991 Slovenia was a member state and a federal unit of the former Yugoslavia. Like Croatia, the Yugoslavian public health system, including foodborne disease control, was applied in Slovenia. The independent Slovenia has developed its own organization complying with the requirements of WHO. Foodborne illnesses are notifiable and registered.

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# Incidence of Foodborne Illness in Southern Africa

**John Frean, Karen Keddy, and Hendrik Koornhof**

*National Institute for Communicable Diseases and the University of the Witwatersrand,  
Johannesburg, South Africa*

## I. ESTIMATED RATE OF ILLNESS

The southern African region comprises Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Zambia, and Zimbabwe. With a few exceptions there is a paucity of information on foodborne diseases from this region for a number of reasons. First, there is a shortage of resources for adequate investigation of disease outbreaks; second, enteric infections are predominantly waterborne due to inadequate water supply or treatment in many areas, and as such take precedence in reporting; and third, countries that are dependent on foreign currency from agricultural exports may be reluctant to convey negative perceptions relating to their products. Disease statistics of most countries do not differentiate between foodborne and waterborne diseases or other routes of transmission. For instance, reports of anthrax in ministerial bulletins may not clarify whether the disease was cutaneous, inhalational, or gastrointestinal.

Food poisoning is notifiable in South Africa but, as is the case in most countries worldwide, is markedly underreported, and accurate incidence rates are not available. [Table 1](#) summarizes notification data on food poisoning and food-related diseases in South Africa from 1993 to 1997 (1).

Only incomplete reports are available from health ministries in South Africa, Zimbabwe and Zambia (Y. Motarjemi, personal communication). Neither the Zambian nor the Zimbabwean data are available beyond 1988. Large ranges in the data that are reported suggest that reporting is largely a function of who is working in the field at the time. Given that the rate of reporting in a developed country such as the United States is estimated to be between 1/10 to 1/100 of the actual incidence (2), it is reasonable to assume that the South African figures are gross underestimations.

## II. REPORTED ILLNESSES

The following foodborne pathogens are known to occur in the region from published or unpublished studies or cases, surveillance activities, or laboratory reports.

**Viruses:** hepatitis A, hepatitis E; all cosmopolitan enteric viruses

**Bacteria:** *Aeromonas hydrophila*, *Bacillus* spp. including *B. anthracis*, *Brucella abortus*, *Campylobacter* spp., *Clostridium perfringens*, *Escherichia coli* (including enterotoxigenic and verocytotoxigenic strains), *Helicobacter pylori*, *Listeria monocytogenes*, *Plesiomonas shigelloides*, *Staphylococcus aureus*, *Salmonella* Enterica serovars including Typhimurium and Typhi, *Shigella* spp., *Vibrio cholerae*, other *Vibrio* spp., *Yersinia enterocolitica*

**Protozoa:** *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium parvum*, *Toxoplasma gondii*, microsporidia, *Balantidium coli*, *Sarcocystis* spp., *Isospora belli*

**Helminths:** *Ascaris lumbricoides*, *Trichuris trichiura*, *Fasciola hepatica*, *Echinococcus granu-*

**TABLE 1** Notified Cases of Food-Related Disease in South Africa, 1993–1997

Year	Food poisoning		Hepatitis A		Typhoid fever		Cholera		Brucellosis	
	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
1993	334	0	1048	1	1414	12	89	2	28	0
1994	27	0	115	0	864	11	4	0	11	0
1995	6	0	22	0	590	14	0	0	0	0
1996	188	0	188	0	1202	2	631	11	0	0
1997	39	1	648	3	425	17	3	0	0	0

Source: Ref. 1.

*lopus*, *Taenia solium*, *Taenia saginata*, *Dipylidium caninum*, *Hymenolepis* spp., *Spirometra* spp., *Toxocara canis*, *Inermicapsifer madagascariensis*, *Gnathostoma* spp., *Bertiella studeri*

**Toxins:** aflatoxins, dinoflagellate-associated toxins, scombroid

### III. FREQUENCY OF INFECTION AND RELATED FOODS

A wide spectrum of food-associated infections has been recognized in the region, and some large outbreaks have occurred. The pathogens associated with these outbreaks are well recognized as causes of foodborne diseases. Much of the work has never been published, and the associations are therefore anecdotal or based on unpublished laboratory reports. One disease that appears to be virtually absent is human botulism, of which only ten cases have been diagnosed on the basis of toxin detection (3), although animal botulism is well known in the region.

Over the past few years, several new foodborne pathogens have emerged in the southern African region. The most notable was a large epidemic of *Escherichia coli* O157:H – infection in eastern Swaziland in 1992 (4), which affected thousands of patients and spilled over into the eastern areas of South Africa. Although the primary transmission of this outbreak was waterborne, the organism was also isolated from fly-infested cooked maize. There has been a subsequent anecdotal report of a small family outbreak associated with hamburgers from a fast-food outlet.

*Salmonella* Enteritidis has been associated with a number of outbreaks in poultry and has also been isolated from eggs. A large outbreak in poultry was recognized by the Onderstepoort Veterinary Institute in South Africa, but no human cases were described. More recently, an outbreak in poultry due to *S. Enteritidis* phage type 4 occurred in Mpumalanga, South Africa. Although it was not adequately documented, this may have caused human cases and resulted in the death of a 2-year-old child (data not published).

A further interesting outbreak of salmonellosis due to *S. Typhimurium* associated with the consumption of biltong (a dried meat product similar to jerky) has been described in the Free State, South Africa. Extensive molecular typing confirmed that at least three different strains were concurrently causing foodborne disease (5). This case is not unique, and a previous report has appeared in South African literature of an outbreak of salmonellosis at a school, following consumption of biltong contaminated with *Salmonella* Newport (6). There was one fatality among the 21 hospitalized cases. It was interesting to note that 2 years later, the organism could still be isolated from the dried meat, which had been stored at room temperature (6). Currently, there are no legislated microbiological standards related to the preparation of dried meats in South Africa. Statistics pooled from 11 major abattoirs in South Africa over 3 years (1993–1995) indicated an estimated 0.75%–3% of cattle had cysticercosis; the prevalence in pigs was 0.25%. The overall prevalence rate of human cysticercosis in South Africa is estimated at 3.3%–7.4%, although prevalences of up to 20% occur in some areas (for example, the former Transkei) (7). Hydatids were detected in sheep and goats



at an infection rate of 0.001% in the same survey. The incidence of hydatid disease in humans is unknown but cases are regularly diagnosed in some areas of South Africa.

An outbreak of salmonellosis associated with an exported herbal tea resulted in the agricultural authority specifying international standards for this product for the export market. Over 100 different serotypes were identified, most of which were type II which had previously been associated with animals only. The source of the contamination was never found, but was thought to be workers' hands, contaminated water, rodents, reptiles or insects (8). Since the introduction of decontamination, the problem appears not to have recurred.

The full extent of milkborne disease in southern Africa is probably also underestimated, as many cases are sporadic. Cases of brucellosis, usually due to *Brucella abortus*, are identified occasionally. Bovine tuberculosis has largely disappeared with extensive use of pasteurization procedures and the system of accreditation of tuberculosis-free herds. Previously, occasional milkborne typhoid fever outbreaks occurred. The predominance of animal pathogens seen in developed countries has not been described, probably due more to lack of investigation than to a real absence. Salmonellae and campylobacter are the most important pathogens isolated in these outbreaks, often in association with raw milk consumption, but postpasteurization contamination has also been recognized. A survey of fresh milk quality at point of sale, organized by the Department of Health in South Africa in 1995, showed that only 36% of pasteurized milk samples and 4% of raw milk samples complied with all the legislated microbiological specifications (9). Bovine tuberculosis and brucellosis remain problems in other southern African countries as well, and human cases have been described in association with drinking unpasteurized milk from infected herds.

Outbreaks of anthrax continue to occur in cattle herds in southern Africa. The last major documented epidemic was in the 1970s in Zimbabwe, associated with the breakdown of vaccination of cattle herds due to the war. This epidemic was associated with human disease (10). The annual incidence rate of anthrax (all forms) in South Africa between 1938 and 1987 varied between 0 and 16 per 1,000,000 population (11). More recently, there have been reports of anthrax outbreaks in humans in the Northern Cape Province (unconfirmed) and in cattle in the North-West Province, South Africa, in 1998. Although farm workers in this last outbreak ate infected meat, no human cases of enteric disease resulted.

A major epidemic of cholera occurred in southern Africa in the early 1980s, and there are periodic resurgences in the region, most recently in Mozambique. Although the disease is primarily waterborne (12,13), occasional foodborne cases have been identified (D. Dürreim, personal communication, 1998).

Foodborne shigellosis has also been described in southern Africa. Most cases are poorly documented, but an outbreak of *Shigella flexneri* associated with consumption of orange juice on a South African private game park suggests that food at all classes of establishments may be at risk (14). A foodborne outbreak of *S. boydii* involving 437 cases occurred in a military camp in 1984 (J. Frean, unpublished data). *S. dysenteriae* type 1 has affected the southern African population since 1990 as a Central African epidemic extended southward (15). Although the outbreaks have been primarily waterborne, cases of foodborne transmission have also been recognized (K. Keddy, unpublished observations, 1999). Anecdotal reports of shigellosis have been obtained from Zimbabwe, Swaziland, Lesotho, and most provinces in South Africa. A nosocomial outbreak of dysentery in a psychiatric ward due to *S. dysenteriae* type 1 may have been due to contamination of foodstuffs because of poor personal hygiene (16), and another psychiatric hospital-associated outbreak of *S. flexneri* in the Western Cape Province resulted in several deaths (G. Coetzee, personal communication, 1999). An outbreak of diarrhoeal disease that affected more than 100 inmates in an old age home in Northern Province resulted in more than 30 deaths. It was postulated that a shigella was responsible, because of the recent outbreaks in the country and its association with dysentery in this type of patient, but this was never proven.

Information on foodborne outbreaks, both laboratory-based and from local authorities, was most readily available in the Western Cape Province. A general problem is that numbers of cases are often unknown because of poor follow-up and disease notification. Noteworthy laboratory-based

reports of outbreaks of foodborne disease include an outbreak of *Clostridium perfringens* food poisoning affecting over 1000 people at a factory canteen. It was not possible to implicate a particular food item. This outbreak occurred in 1995 and was not reflected in the Department of Health notifications (1), supporting the premise that an estimation of the full extent of foodborne disease in southern Africa is crippled by underreporting. A visiting international cricket team and the accompanying officials the following year also succumbed to *C. perfringens* type A food poisoning identified in a lasagne from a specific supplier. The team lost the cricket match.

#### **IV. RISK ASSESSMENT**

For meaningful and ongoing assessment of the risk of food spoilage or food poisoning and other diseases following ingestion of contaminated food, the following should be in place:

- Appropriate legislation
- A suitable infrastructure at administrative, inspection, and laboratory levels
- Implementation of hazard analysis critical control point (HACCP) systems
- Construction of risk assessment models or projects to evaluate HACCP systems

Basic critical control point (CCP) principles are being utilized to varying degrees in southern African countries through inspection of food processing by environmental health officers (formerly called "health inspectors") with or without suitable qualifications and training and generally without adequate laboratory backup.

Although the expertise to perform highly sophisticated techniques in food microbiology such as DNA fingerprinting exists in the major teaching and research institutions in South Africa and Zimbabwe, the sophistication and standards applied in most southern African countries do not at present compare well with those of the developed world. In a recent publication, the International Commission for Microbiological Specifications for Foods (ICMSF) set out principles on which national objectives involving microbiological levels of food safety and related control measures should be based (17). The following steps in the management of hazards in foods in international trade were proposed:

- Provision of an estimate of the public health impact associated with hazards in food, based on principles and guidelines for the conduct of microbiological risk assessment as proposed by the Codex Alimentarius Commission (CAC) in 1997 (18)
- Application of general principles of food safety management (19)
- Establishment of a food safety objective (FSO)
- Confirmation of achievability of FSOs through the application of HACCP and good health practices (GHP)
- Development of control measures by risk managers in industry and/or government

In South Africa attempts at following the abovementioned approach, including the application of HACCP principles, have been client driven, and investigations to meet safety requirements requested by firms importing or exporting foods are being performed by laboratories in the major centers of the country. Enabling legislation for the introduction of HACCP on a regular basis in the public health sector is in preparation, with a firm intention of early promulgation. Legislation will be followed by incremental implementation steps based on needs and availability of trained personnel.

HACCP-based risk assessment models or projects are not yet operational in the southern African region, but with the implementation of new legislation in South Africa and neighboring countries, the construction of risk assessment models will hopefully follow in due course. As the development of models is still at an early stage, even in developed countries, it is to be expected that models designed for the developing world, including southern African countries, will take some time before

implementation can be achieved. Ideally, conditions prevailing in third world countries should be considered in such models, taking into account scarce resources coupled with high risks in these countries.

Expansion of the informal food vendor trade in South Africa is receiving the attention of health authorities in South Africa. A potential risk in the rural setting was demonstrated in Mpumalanga Province, where a statistically significant association was found between the acquisition of typhoid fever and the eating of food sold by vendors at schools (20). The incidence of typhoid fever is declining in South Africa but this disease is still a major problem in rural areas. The application of *Salmonella typhi* Vi antigen vaccine in an endemic region was shown in a local study to be approximately 60% effective during the first 2 years after immunization (21). This vaccine is a candidate for use in travellers to countries where typhoid fever is endemic.

The risk of travellers' diarrhoea and other food-related illness, although dependent on many factors, of which safety of products of food processing is but one, has been used in several studies as an indicator of food safety (22–23). Attack rates of diarrhoea during the first 14 days of stay in a country varied from approximately 30% to more than 50% in east, west, and north African countries. Statistics for southern African countries are not available.

Enterotoxigenic *E. coli* (ETEC) was by far the commonest cause of diarrhoea in these studies, while salmonellae, shigellae, and campylobacter were less frequently encountered. All these pathogens have been isolated from patients with diarrhoea in southern African countries, but the extent of their relative roles in travellers' diarrhoea in this region has not been established.

Statistics based on compulsory notification of foodborne diseases may give an indication of the risks involved in food consumption (Table 1).

## V. ONGOING OR PROPOSED MEASURES TO REDUCE FOODBORNE ILLNESS

Little information regarding food safety control in countries bordering on South Africa was forthcoming. However, four southern African countries (Botswana, Mozambique, South Africa, Zimbabwe) were among 15 African Codex member countries that participated in a survey of food regulations and controls (25). While it is not possible to isolate the responses of the southern African group from those of the others, the results are likely to be broadly representative of the region and are summarized here. Codex standards form the most popular basis for regulations/standards on traded food. Only 22% of respondents currently have HACCP-related requirements in their regulations/standards, while 90% of the remainder indicated an intention to include HACCP requirements by 2003.

At present there is no integrated national food control system in South Africa, and its components are currently divided between several authorities. However, there is recognition of the importance of food safety and the need for effective quality assurance and management of food production, harvesting, processing and handling (by applying HACCP principles) and of promoting awareness of food safety in the community (26,27). The national health plan adopted in principle by the South African government includes measures for ensuring the safety of foods at three levels of public health service delivery (27). At national level, the Food Control Directorate is directly responsible for food safety via:

- Compilation of legislation and regulations in line with international standards
- Monitoring and auditing functions
- Informing and educating consumers, producers and law enforcers

At the provincial level, health departments are responsible for inspecting imported foods through Port Health Services, as well as general support, monitoring, coordination, and planning of matters related to food safety. At the district level, local authorities are responsible for rendering environmental health services to communities by means of inspection, investigation of complaints or foodborne

disease outbreaks, law enforcement, and control of health hazards. Advising and educating food handlers, processors, and consumers, especially in the informal sector, are other important functions.

Current or recent activities of the Food Control Directorate include (28):

Initiatives to improve the safety of street-vended foods

Updating food standards legislation to comply with Codex Alimentarius requirements

Coordinating activities of role players in aviation food safety

Developing a National Food Safety Program Auditing System for imported as well as domestic food

Arranging surveys of specific foodstuffs as part of a routine monitoring program, eg. microbiological safety of fresh milk (1995), microbiological quality of street-vended foods (1995), and aflatoxins in groundnuts (1997)

## VI. ESTIMATED EFFECTIVENESS OF THESE MEASURES

In the previously-mentioned survey of African countries (25), all respondents indicated that they test food (mainly by government organizations) before import and export. The following constraints in relation to food trade were identified by respondents, in order of severity:

Insufficient financial resources for food control activities

Inadequate testing and inspection facilities

Inadequate trained manpower in the food industry

Inadequate standards and/or regulations

Inefficient food processing technologies

The need for training was identified as a major priority and delegates from South Africa and other southern African countries have attended several FAO- and WHO-sponsored meetings in recent years in order to keep abreast of international developments in this field.

The control of food safety in South Africa is presently somewhat fragmented and divided between a number of entities (29). At least 13 acts have provisions related to food. A single food product is therefore often controlled by several different authorities in terms of a number of sets of legislation, which leads to inefficiency, duplication, and, sometimes, a lack of control. For example, meat is controlled by the Department of Health, the South African Bureau of Standards, local authorities, and three components of the Department of Agriculture. It is recognized at the central government level that there is a need to comply with international standards related to South Africa's membership of the World Trade Organisation and FAO/WHO Codex Alimentarius Commission. A new national food control program, and an accompanying auditing system, are currently being drafted to address current deficiencies.

## NOTES ADDED IN PROOF

1. Two fatal cases of foodborne botulism occurred in 2002, after the victims consumed a damaged tin of fish (a). This brings the number of laboratory proven cases in southern Africa to 10.

2. The South Africa/Swaziland *Escherichia coli* O157:H- outbreak of 1992 has now been fully reported (b).

3. A large scale outbreak of food poisoning at an international sporting event occurred in 2000. Although no pathogen could be directly linked to the outbreak, over 500 children were affected (c).

4. Cholera re-emerged in epidemic form in KwaZulu-Natal, South Africa in 2000 (d). To date, over 200,000 cases have been reported.

5. Three children acquired *Salmonella* Newport enteritis from eating meat from a dead horse

donated by a local farmer to inhabitants of an informal settlement near Johannesburg, South Africa, in October 2002 (KHK, personal communication).

6. Outbreaks of anthrax are intermittently reported in South Africa and other southern African countries, almost invariably after local communities eat dead cattle (e, f).

These cases serve to emphasize that poverty frequently plays a critical role in outbreaks of foodborne disease in southern Africa.

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# Incidence of Foodborne Illness in Southeast Asia

**Punnee Pitisuttithum**

*Faculty of Tropical Medicine, Bangkok, Thailand*

## I. EPIDEMIOLOGICAL BACKGROUND, PREVALENCE, AND INCIDENCE

Foodborne illness is one of the most widespread health problems in the world, especially in Southeast Asia (1). Because of the absence of good foodborne disease surveillance systems in the region, the information on these issues is incomplete and limited, especially from Cambodia, Laos, Burma, and Vietnam.

The mortality rate due to cholera reported for the Southeast Asian countries from 1994 to 1999 is shown in [Table 1](#). The number of cases reported from this region seemed to be low because of underreporting, but there is a tendency of actual decline in the number of cholera cases in this region (2–5). However, some cholera outbreaks have been occurring in this region up until the present.

### A. Thailand

The number of cases of foodborne or food-related illness per 100,000 population reported from Thailand between 1990 and 1995 is shown in [Table 2](#) (6), and the reported cases and deaths per year from 1992 to 1998 are shown in [Table 3](#) (6). It appears that cases of acute diarrhea and food poisoning are increasing in spite of improvement in food hygiene. This may be due to a better surveillance system by the Ministry of Public Health, Thailand, rather than an actual increase in cases.

The majority of cases of acute diarrhea that is mainly food related per 100,000 population occurred in the southern part of Thailand. The maximum number of cases was reported to occur in June; this peak coincided with the maximum number of cases of food poisoning reported, which occurred in the summer, as shown in [Figure 1](#) (6). The majority of cases of food poisoning was reported to occur in the northeast of Thailand. An etiological organism was reported for only 1.31, 5.62, and 6.50% in the years 1993, 1994, and 1995, respectively. Among these, 45.55% were caused by *Vibrio parahaemolyticus*, 29–39% by *Salmonella* spp., and 11–15% by *Staphylococcus aureus* (toxin) (6).

#### 1. Severe Diarrhea

It has been reported that *Vibrio cholerae* is the main cause of severe diarrhea in Thailand. Cases of severe diarrhea per 100,000 population reported from the capital, Bangkok, are shown in [Figure 2](#) (7). The maximum number of cases was reported in 1993; thereafter the numbers declined gradually. The number of cases per 100,000 population in 1996 was only 0.14 (323 cases reported), with the maximum number of cases reported for March and April ([Fig. 3](#)). A study was conducted between November 15, 1993, and June 3, 1994, at Samutsakorn, in the eastern part of Thailand, 30 km southwest of Bangkok to identify the source of *Vibrio cholerae* O1 and O139 infection in Thailand (8). A total of 366 patients were confirmed to have cholera by culturing the organism: 165 (45%) with O139, 191 (52%) with O1 Ogawa, and 10 (3%) with both groups.

**TABLE 1** Cases of Deaths from Cholera Reported from Southeast Asian Countries, 1994–1999

Country	1994	1995	1996	1997	1999
Cambodia	3085 (211)	4190 (123)	740 (20)	155 (0)	193 (18)
Indonesia	47 (0)	—	—	66 (0)	—
Lao PDR	9640 (606)	1365 (174)	720 (33)	—	—
Malaysia	534 (0)	116 (0)	1486 (2)	389 (4)	87 (0)
Myanmar	421 (4)	1296 (6)	—	—	—
Philippines	3340 (27)	874 (4)	1402 (14)	605 (0)	265 (17)
Singapore	41 (1)	14 (0)	19 (0)	19 (0)	24 (2)
Thailand	3487 (5)	—	—	—	—
Vietnam	5776 (68)	5953 (59)	566 (2)	4 (0)	—

Risk factors for contracting cholera identified by case-control comparisons were similar for the two sero-groups, as shown in Tables 4 and 5, and included consumption of untreated water, uncooked seafood, and food served at group gatherings (8).

## 2. Dysentery

The number of reported cases of dysentery per 100,000 population per year in Thailand from 1986 to 1995 is shown in Figure 4 (6). The maximum number of cases occurred in 1987 during an outbreak of multidrug-resistant *Shigella dysenteriae* type 1. Thereafter there was a marked decline from 1989 to 1995. The etiological agents identified included amoebae, 2.0%; bacillary dysentery, 4.1%; and unspecified, 93.9% (6). The maximum number of cases per 100,000 population in 1995 was reported from Rayong in the east, as well as from the northern and northeastern parts of Thailand. The high-risk area was along the Thai-Myanmar border, specially Mae-houson province (9).

A study conducted during 1985–1993 in a rural hospital in the central part of Thailand showed that *Shigella* spp. was found throughout the year, with a peak incidence in June and July. The most common type isolated was *S. flexneri* (74.43%). These *Shigella* isolates had a high rate of resistance

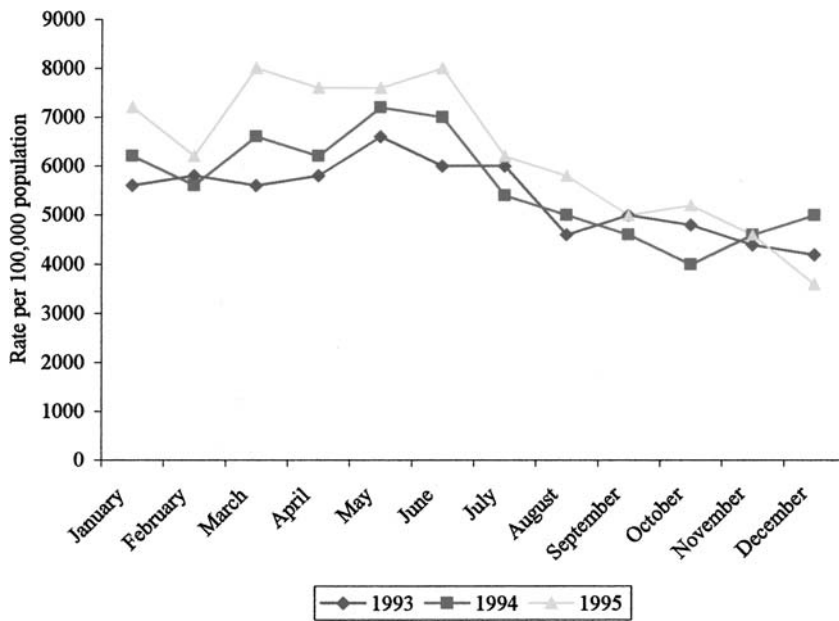
**TABLE 2** Reported Cases of Foodborne Illnesses (per 100,000 Population) by Year, 1990–1995, Thailand

Diseases	1995	1994	1993	1992	1991	1990
Acute diarrhea	1686.05	1690.67	1488.51	1412.99	1398.67	1284.66
Food poisoning	125.67	113.61	113.08	109.03	105.38	98.87
Dysentery—total	129.25	136.17	133.79	137.24	153.31	156.03
Enteric fever—total	24.43	27.14	28.29	24.05	30.17	39.22
Hepatitis—total	17.45	24.94	27.36	31.91	31.38	29.9
A	0.46	0.5	0.5	1.28	0.54	0.52
Non-A, Non-B	0.2	0.11	0.19	0.16	0.12	0.09
Unspecified	13.67	19.87	22.26	25.12	24.73	23.67
Anthrax	0.17	0.01	0.02	0.07	0.14	0.06
Trichinosis	0.41	0.31	0.3	0.69	0.19	0.12
Amebiasis, other organs	0.02	0.03	0.06	0.03	0.05	0.01
Mushroom poisoning	0.87	1.26	1.4	1.3	1.06	1.01
Cassava poisoning	—	—	—	—	—	—
Poisoning by other noxious foodstuff	—	—	—	—	0.03	0.09

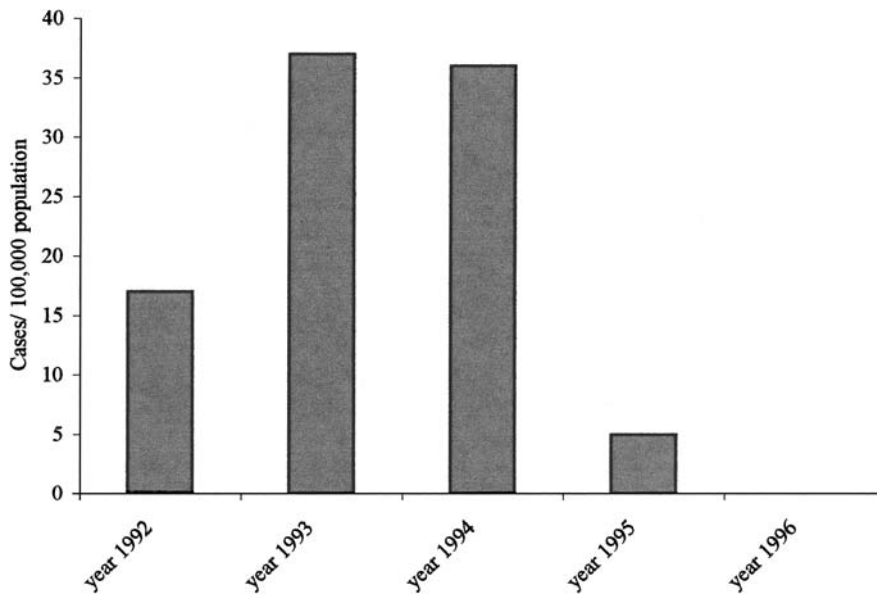


**TABLE 3** Reported Cases and Deaths of Foodborne Illnesses by Year, 1992–1998, Thailand

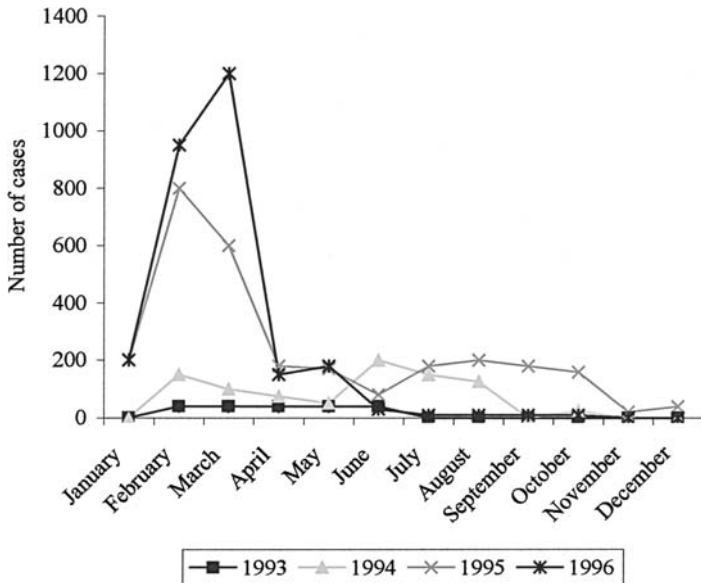
Diseases	Cases (deaths)						
	1998	1997	1996	1995	1994	1993	1992
Acute diarrhea	1,131,595 (322)	1,054,904 (352)	981,072 (397)	1,002,532 (370)	999,106 (506)	868,338 (411)	816,553 (389)
Food poisoning	111,125 (23)	102,454 (26)	82,281 (35)	74,723 (19)	67,141 (12)	65,965 (19)	63,005 (21)
Dysentery—total	58,910 (71)	50,416 (16)	8395 (21)	76,850 (13)	80,469 (18)	78,045 (16)	79,312 (27)
Bacillary	—	47,376 (12)	51,522 (18)	—	—	—	—
Amebic	—	1689 (1)	2048 (3)	—	—	—	—
Unspecified	—	1351 (—)	1225 (—)	—	—	—	—
Enteric fever—total	Pending	8731 (11)	6894 (1)	14,528 (3)	16,041 (5)	16,502 (7)	13,900 (7)
Typhoid	—	5584 (3)	5600 (1)	—	—	—	—
Paratyphoid	—	212 (—)	488 (1)	—	—	—	—
Unspecified	—	—	—	—	—	—	—
Hepatitis—total	—	7932 (37)	—	10,374 (39)	14,759 (59)	15,961 (48)	18,442 (61)
A	Pending	270 (—)	202 (—)	274 (1)	295 (—)	294 (1)	741 (1)
Non-A	—	—	—	166 (—)	67 (—)	108 (—)	93 (—)
Non-B	—	—	—	8130 (33)	11,744 (54)	12,985 (47)	14,515 (57)
Unspecified	—	—	—	102 (—)	7 (3)	12 (—)	39 (6)
Anthrax	—	—	—	242 (—)	183 (—)	174 (1)	397 (1)
Trichinosis	—	—	—	—	—	—	—
Amebiasis, other organs	—	—	—	14 (1)	17 (—)	33 (—)	15 (—)
Mushroom poisoning	—	—	—	515 (9)	742 (13)	817 (10)	754 (7)
Cassava poisoning	—	—	—	—	—	—	—
Poisoning by other noxious foodstuff	—	—	—	—	—	—	—



**FIGURE 1** Reported cases of food poisoning per 100,000 population by year, Thailand, 1993–1995.



**FIGURE 2** Cases of severe diarrhea per 100,000 population in Bangkok, 1992–1996.



**FIGURE 3** Cases of severe diarrhea reported per month in Bangkok, 1993–1996.

to ampicillin and cotrimoxazole; in 1993 only 16.67% were sensitive to ampicillin and 22.22% were sensitive to cotrimoxazole (10). Data from the Infectious Disease Hospital, Nonthaburi, demonstrated that the organism was totally resistant to co-trimoxazole (personal communication).

### 3. Salmonellosis

According to the Ministry of Public Health, Thailand, cases of enteric fever fall into three categories: typhoid fever, paratyphoid fever, and unspecified enteric fever. The incidence of disease peaked in 1987 (50.1 cases per 100,000 population) (Fig. 5) and declined gradually thereafter. Out-

**TABLE 4** Risk Factors in *Vibrio cholerae* O139 Bengal Cases Compared with Asymptomatic Age- and Sex-Matched Controls, Samutsakorn, Thailand, November 1993 to June 1994

Risk factors	Cases (n = 105), No. (%)	Control (n = 206), No. (%)	Crude analysis, OR (95% CI)
History of contact with person who had diarrhea:			
Within household	5 (5)	3 (1)	4.5 (0.83–24.4)
Outside household	7 (7)	2 (1)	13.0 (1.5–110.2)
Consumption of:			
Untreated tap water	28 (27)	27 (13)	2.61 (1.38–4.93)
Untreated wild water	11 (10)	4 (2)	7.0 (1.92–25.51)
Ice	72 (69)	128 (61)	1.46 (0.86–2.48)
Raw or partially cooked seafood	21 (20)	13 (6)	3.42 (1.65–7.07)
Food sold by street vendor	26 (25)	30 (14)	2.05 (1.11–3.78)
Attendance at gathering	9 (9)	4 (2)	8.0 (1.64–38.96)

OR = Odds ratio.

95% CI = 95% confidence interval.

**TABLE 5** Risk Factors in *Vibrio cholerae* O139 Ogawa Cases Compared with Asymptomatic Age- and Sex-Matched Controls, Samutsakorn, Thailand, November 1993 to June 1994

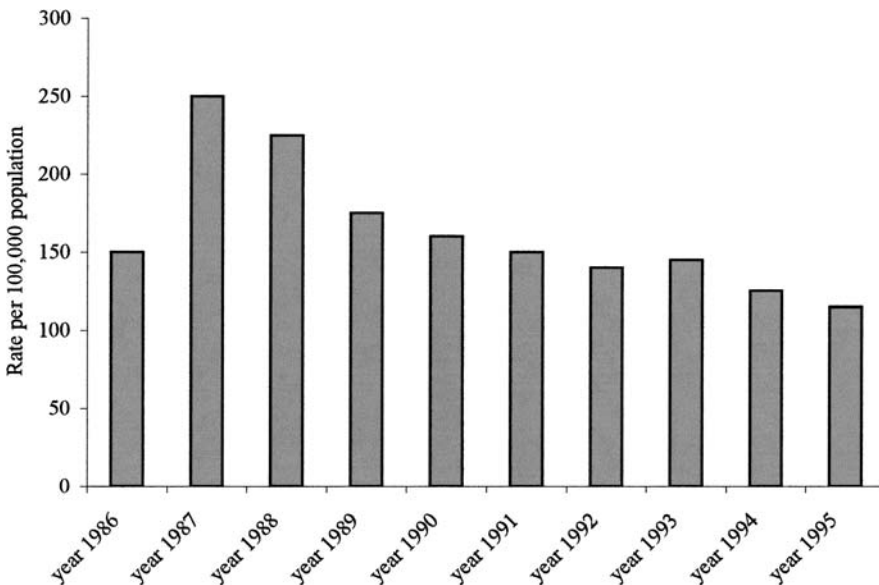
Risk factors	Cases (n = 105), No. (%)	Control (n = 206), No. (%)	Crude analysis, OR (95% CI)
History of contact with person who had diarrhea:			
Within household	10 (10)	2 (1)	19 (2.36–152.6)
Outside household	8 (8)	0 (0)	$p < 0.001$
Consumption of:			
Untreated tap water	27 (26)	30 (15)	2.26 (1.21-4.25)
Untreated wild water	8 (8)	2 (1)	15 (1.81-124.35)
Ice	80 (78)	131 (64)	2.32 (1.25-4.3)
Raw or partially cooked seafood	13 (13)	14 (7)	1.92 (0.87-4.25)
Food sold by street vendor	17 (17)	24 (12)	1.43 (0.76-2.71)
Attendance at gathering	5 (5)	1 (0.5)	10.0 (1.17-85.6)

OR = Odds ratio.

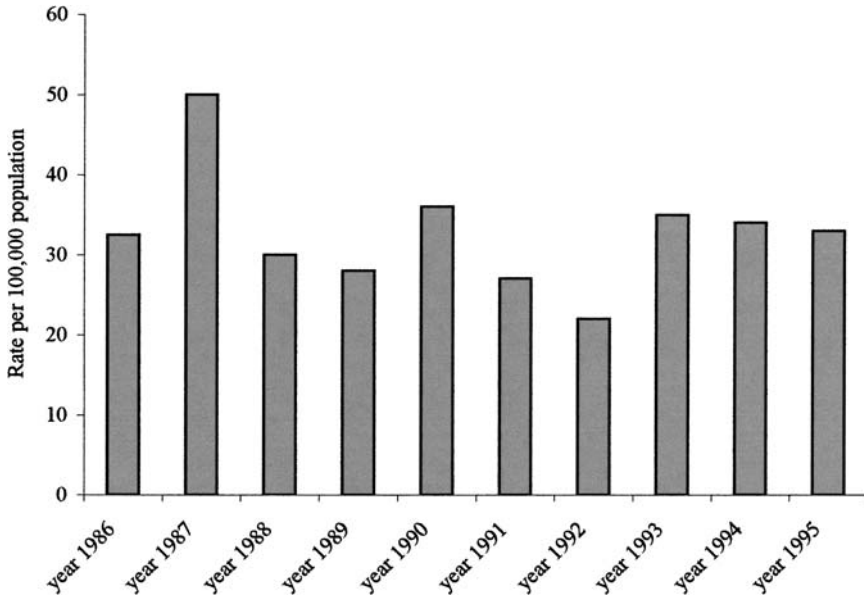
95% CI = 95% confidence interval.

breaks of enteric fever appeared to occur biannually, usually occurred during the rainy season from May to October, and were reported mostly from the northern and southern parts of Thailand (Fig. 6) (6).

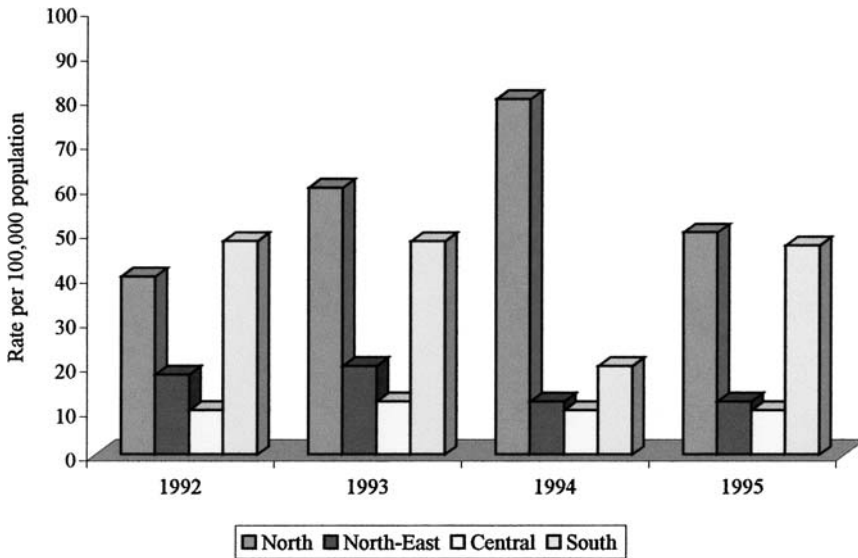
An increase in nontyphoid salmonellosis has been reported in Thailand since 1990 (11,12). Epidemics of certain *Salmonella* serotypes have been observed, such as outbreaks of *S. krefeld* during the years 1976–1978 (13,14). Currently *S. enteritidis* is the most common serotype encountered in



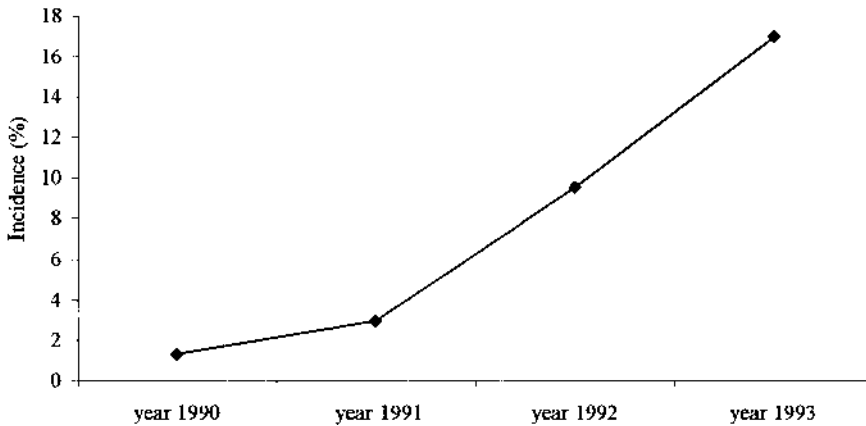
**FIGURE 4** Reported cases of dysentery per 100,000 population by year, Thailand, 1986–1995.



**FIGURE 5** Reported cases of enteric fever per 100,000 population by year, Thailand, 1986–1995.



**FIGURE 6** Reported cases of enteric fever per 100,000 population by region, Thailand, 1992–1995.



**FIGURE 7** Incidence for *S. enteritidis* in Thailand, 1990–1993.

extraintestinal infection in humans (15). Prior to 1990, the isolation of *S. enteritidis* from humans was minimal. Since then, the isolation rate of this serotype has increased, from 1.33% in 1990 to 16.98% in 1993 (Fig. 7) (16). In Thailand, the sudden emergence of this particular serotype causing outbreaks in humans and in chickens strongly suggests that chicken is the source of *S. enteritidis* infection in humans (17). The most frequently isolated *Salmonella* serovars are shown in Table 6. *S. derby* and *S. weltevreden* were most frequently isolated serovars in 1991 and 1992 respectively. This pattern changed in 1993 and 1994, when *S. enteritidis* became one of the most frequently isolated serovars in Thailand. The morbidity markedly increased each year from 1991 to 1995 (Fig. 8), with 105, 307, 471, 659, and 877 cases being identified, respectively (18). Most cases occurred in Bangkok, followed by the southern, northeastern, central, and northern regions, respectively.

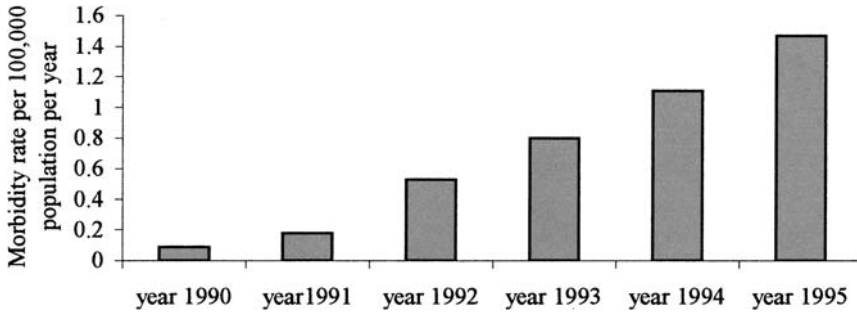
Foods prepared from duck meat and eggs were found to be the source of infection. The common serotypes isolated from duck eggs were *S. typhimurium*, *S. cerro*, *S. tennessee*, *S. amsterdam*, *S. agona*, and *S. infantis*, accounting for 5.5, 4.1, 2.8, 2.1, 1.4, and 1.1% of cases, respectively (19).

#### 4. Hepatitis A Virus

Hepatitis A virus (HAV) infection is a major cause of viral hepatitis in Thailand. It causes 69% of acute viral hepatitis in children less than 15 years of age (20). During the last two decades, a large number of Thai children have tested seropositive for HAV (21). However, in the last 10 years, the seroprevalence of hepatitis A virus infection has changed from hyperendemic into intermediate endemic status (22) because of the improvement of socioeconomic status and personal hygiene. The

**TABLE 6** *Salmonella* Serovars Most Frequently Isolated from Human Specimens, Thailand

Rank	1991	1992	1993	1994
1	<i>S. derby</i>	<i>S. weltevreden</i>	<i>S. enteritidis</i>	<i>S. enteritidis</i>
2	<i>S. weltevreden</i>	<i>S. derby</i>	<i>S. weltevreden</i>	<i>S. derby</i>
3	<i>S. agona</i>	<i>S. enteritidis</i>	<i>S. derby</i>	<i>S. weltevreden</i>
4	<i>S. krefeld</i>	<i>S. typhimurium</i>	<i>S. I, 1,4,5, 12:i:-</i>	<i>S. anatum</i>
5	<i>S. typhimurium</i>	<i>S. I, 1,4,5, 12:i:-</i>	<i>S. typhimurium</i>	<i>S. I, 1,4,5, 12:i:-</i>
6	<i>S. virchow</i>	<i>S. agona</i>	<i>S. krefeld</i>	<i>S. typhimurium</i>
7	<i>S. anatum</i>	<i>S. anatum</i>	<i>S. anatum</i>	<i>S. agona</i>
8	<i>S. enteritidis</i>	<i>S. krefeld</i>	<i>S. agona</i>	<i>S. kedougonu</i>

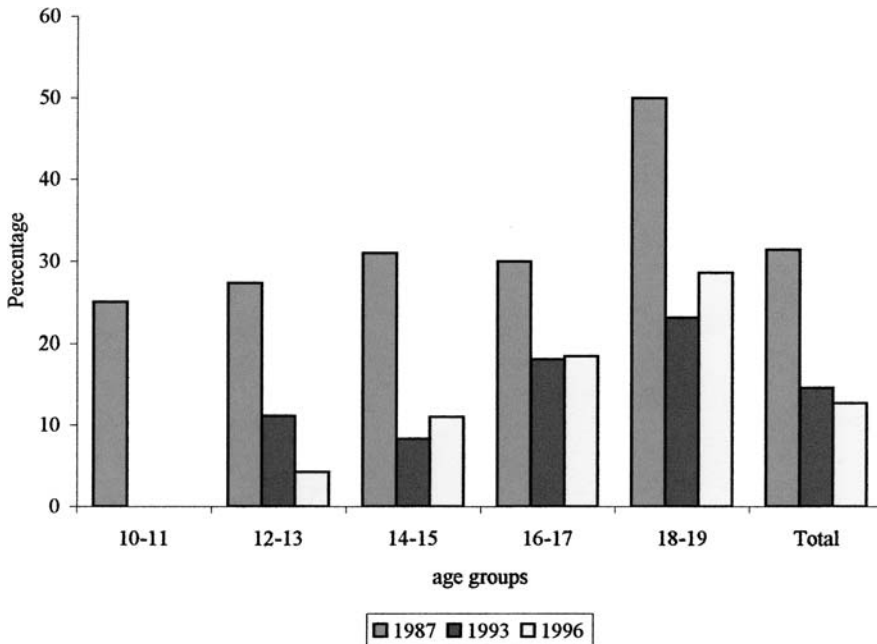


**FIGURE 8** Morbidity rate for *S. enteritidis* in Thailand, 1990–1995.

overall seroprevalence of anti-HAV markedly declined from 31.4% in 1987 to 14.65% in 1993 and 12.75% in 1996. The age-specific prevalence of anti-HAV among secondary school children in Bangkok in 1987, 1993, and 1996 is shown in Figure 9 (23). The decline in disease incidence has resulted in a large proportion of the population that is susceptible to HAV and has led to various outbreaks in Thailand as will be mentioned in the next section.

5. Protozoal Infection

A study carried out between October 1995 and June 1996 in Songkhla, a southern city in Thailand, demonstrated *Cryptosporidium* infection in populations both seropositive and seronegative for the human immunodeficiency virus (HIV). Most of the HIV-seropositive subjects were 20–39 years of age. *Cryptosporidium* oocysts were detected in 10% of HIV-seropositive and in 2% of HIV-seronegative subjects. Immunocompromised individuals, if infected, continue to discharge a



**FIGURE 9** Age-specific prevalence of anti-HAV among secondary school children in Bangkok.

large number of oocysts, unlike immunocompetent persons, in whom the infection is self-limited. This indicates that HIV-infected people or acquired immunodeficiency syndrome (AIDS) patients may play an important role in spreading *Cryptosporidium* infections, possibly in epidemic infections, or in causing secondary infection in the future (24).

## 6. Helminthic Infections

There appears to have been a decline in the prevalence of intestinal helminthic infections over the years, but there have been no updates of nationwide surveys since 1981. A survey between 1980 and 1981 of the whole of Thailand showed that the overall helminthic infection rate was 54.6%, the prevalence in the central region was 36.1%, with a distribution of 27.1, 4.2, 4.5, 0.5, 0.7, and 6.7% for hookworm infection, ascariasis, trichuriasis, strongyloidiasis, taeniasis, and opisthorchiasis, respectively (25).

From a survey conducted in 1989 of 149 Thai adults residing in and around the Bangkok metropolis, 25% were found to have intestinal infection and 23.4% had multiple infection. The distribution of intestinal parasitic infection was 38.3, 17, 4.3, 2, and 2% for strongyloidiasis, opisthorchiasis, trichuriasis, taeniasis, and ascariasis, respectively (26).

A survey conducted in primary school children in Amphoe Muang, Prachin Buri, showed that 28.9% of children had at least one of the following parasite infections: hookworm (28.4%), opisthorchiasis (1.0%), strongyloidiasis (0.9%), trichuriasis (0.5%), and enteriobiasis (0.4%) (27).

Foodborne trematode infections are acquired through ingestion of raw or improperly cooked or processed freshwater fish, shellfish, crabs, or unwashed vegetables containing parasite larvae. Liver infections are also caused by oriental flukes (*Clonorchis sinensis*, *Opisthorchiasis viverrini*, and *O. felineus*). *O. viverrini* is mainly prevalent in Southeast Asia, particularly in northeastern Thailand; more than 7 million persons are estimated to be infected in Thailand. Cholangiocarcinoma, a form of liver cancer, is frequent in areas where *Clonorchis* and *Opisthorchis* are endemic. The annual incidence rate of cholangiocarcinoma in this region is no less than 2 per 100,000 population. In some areas of Thailand, the incidence is at least 135 per 100,000 population. The exact prevalence of intestinal trematode infection caused by *Fasciolopsis buski* is not known, but it is also found in Thailand, especially in the northern area (28).

Wide regional assessment indicated a declining trend in the prevalence of opisthorchiasis, from 34.6% in 1981 to 30.19 and 24.01% in 1991 and 1992, respectively (29). Many species of freshwater fish, particularly those of the family Cyprinidae, have been reported as second intermediate hosts of *O. viverrini*. Examination for metacercariae in freshwater fish carried out during 1992–1996 in 14 provinces revealed that *O. viverrini* were most frequently found in fish from Udon Thani, Sa Kaeo, and Prachin Buri provinces and the Aranyaprathet (30).

Data obtained from liver fluke control operations conducted countrywide in 1996 showed that the prevalence of opisthorchiasis decreased to 21.5% but the incidence varied among the different age groups. Infection is lowest among the 0–14 years age group, while the highest prevalence rate is among the 40 to 49-year-old population group, which can be as high as 27.2%. The increase in prevalence among at-risk population groups has a direct positive relationship with increase in age. This could be explained by the fact that once the liver fluke has infected an individual, it remains viable and reproductive for several years as long as the infected person remains untreated. Infections vary among the different geographical regions. A large percentage of infections is mild. Comparing the numbers between 1981 and 1991, there has been a considerable increase in the central and northern regions, while there was a significant decrease in the northeastern region. The highest prevalence (32.6%) was found in the northern region, while the prevalence rate of the central region is 16.7%. The lowest prevalence (15.3%) occurs in the northeastern region (31,32).

Human trichinosis was first reported in Thailand in 1962 (33). Since then more than 100 well-recognized outbreaks have been noted (34). Most of them occurred in the northern region of the country. An epidemic of trichinosis occurred in Chiang Bann District, Amphoe Chiang Kham, Changwat Phayao (northern part of Thailand) in February 1993. The source meat was a hill-tribe



fig. The most common clinical features included myalgia (100%), fever (93.88%), and facial edema (87.71%) (35).

## B. Indonesia

### 1. Typhoid Fever

Typhoid fever is a frequently occurring disease in Indonesia, especially on the island of Sulawesi. In 1989, 8804 cases of typhoid fever were diagnosed in Ujung Pandang of Southern Sulawesi, suggesting an annual incidence of 3.1 per 1000 inhabitants. Incidence in the city was likely to be higher than in rural areas. Mortality rate was estimated to be 5.1%. A steady rise in the number of cases per month was observed after the rainy season, with a peak in May and June. This suggested that the fecal-oral transmission of *S. typhi* is facilitated by a reduction in the availability of water and the deterioration of the quality of stagnant surface water through the combined effects of rising temperature, evaporation, and absence of fresh water supplies. A case-control study showed a strong association in the risk of typhoid fever with consumption of food from warungs (food stalls in the street). Both cases and controls washed their hands after use of the toilet and before meals, but cases used soap significantly less often (36).

### 2. Hepatitis A

In contrast to Singapore, Thailand, and Malaysia, Indonesia showed marked regional variations of age-related and overall seroprevalence of HAV. The overall seroprevalence varied markedly from 40, to 98%, depending upon the population and the region (high endemicity was reported in Bali). Infection occurred predominantly in individuals under 30 years of age, with a few cases in adults over 30 years of age (37).

### 3. Parasitic Infections

a. *Entamoeba*. A seroepidemiological survey was conducted in East Java, Indonesia, between May 1992 and October 1993 for the prevalence of *E. histolytica*. The prevalence of *E. histolytica* varied from 2 to 15% depending on the test. With the indirect hemagglutination test 23, 31, and 18% of samples obtained in south Sulawesi, Sumatra, and Central Java, respectively, were positive (38).

b. *Cryptosporidiosis*. The prevalence of *Cryptosporidium* infection is higher among children than among adults and is more common in developing countries than in developed countries (39,40). Despite wide distribution and obvious relevance to public health, *Cryptosporidium* remains a little-studied protozoan in Indonesia.

Hospital- and community-based studies were conducted in Surabaya, Indonesia, during 1992 and 1993. The hospital-based study revealed *Cryptosporidium* oocysts in 2.8% of patients with diarrhea. The most susceptible age was less than 2 years old. There was a higher prevalence in the rainy season. In contrast, a community-based study showed that *Cryptosporidium* oocysts were more frequently detected in diarrheal samples (8.2%), exclusively during the rainy season. Contact with cats, rain, flood and crowded living conditions were considered as risk factors for *Cryptosporidium* infection (41).

### 4. Helminthic Infection

Infestation of intestinal parasites remains a major public health problem in many rural areas of Indonesia. Prevalence of helminthiasis is highest in children aged 5–10 years (42,43). Parasitological studies conducted in Indonesia during the last two decades showed prevalence rates of *Ascaris lumbricoides* ranging between 14 and 90% and of *Trichuris trichura* between 1 and 91%. These differed markedly because of geographical and socio-environmental conditions, as well as age of the population (44–46). West Java has been the site of many surveys of human intestinal parasitic

infections. A parasitological survey of children aged 8–10 years from 10 schools in December 1995 showed a prevalence of 76% for *T. trichura* and 44% for *A. lumbricoides* (47).

### C. Lao PDR

#### 1. Cholera

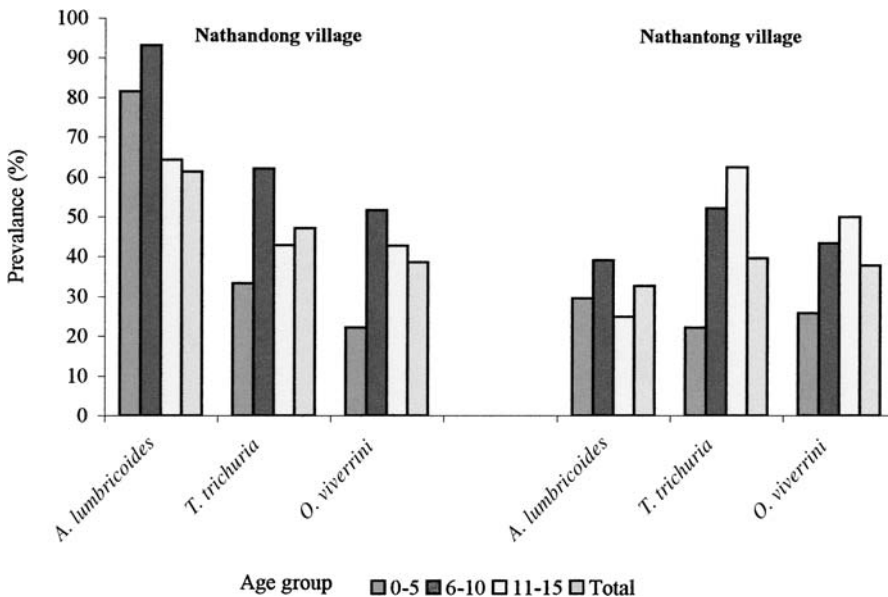
Cholera outbreaks in Lao PDR have been reported since 1993, when two provinces reported cholera cases. However, affected areas increased to 7 provinces through 1994. Bacterial surveys conducted by Midorikawa et al. at Mahosot Hospital, Don Chan Island, and Sok Nyai village revealed 1 *Vibrio* species, 5 enteropathogenic *E. coli* (EPEC), and 3 *Shigella* spp. among 39 isolates at Mahosot Hospital. At Don Chan island, 4 *Vibrio* and 4 *Salmonella* spp. were identified from a total of 15 isolates (48).

#### 2. Hepatitis A

In a study conducted in Vientiane, Lao PDR, during 1995 and 1996, HAV was found to be the most common cause of acute hepatitis. Evidence of acute infection due to hepatitis A virus was found in 14% of cases (49).

#### 3. Helminthiasis

There have been few reports about the prevalence of intestinal parasitic infections in Lao PDR. A study conducted among 128 children under 15 years old in two villages in southern Lao PDR showed that overall prevalence of helminthic infection was 77.3%; individual rates were 64.8% in children under 6 years, 88.5% in those aged 6–10 years, and 81.8% in those above 1 year. The most frequently isolated helminths included *A. lumbricoides* (48.4%), *T. trichura* (43.8%), and *O. viverrini* (37.5%) (Fig. 10) (50).



**FIGURE 10** Prevalence of helminths among children in rural southern Lao PDR.

## D. Malaysia

### 1. Salmonellosis

*Salmonella typhi* is endemic in Malaysia, and outbreaks have occurred in various parts of the country periodically. A study conducted in Malaysia during 1989–1994 described the incidence, extent of infection, and antibiotic susceptibility pattern of nontyphoid salmonellosis. *S. weltevreden* and *S. enteritidis* accounted for nearly half the total number of nontyphoid salmonellosis: 31.06% and 16.06%, respectively. There has been a substantial increase in infection due to *S. enteritidis* since 1993, with more than 30% of salmonellosis from human cases due to *S. enteritidis* with an increasing percentage of drug resistance. Strains resistant to ampicillin, co-trimoxazole, and tetracycline were found to be in the range of 0.8–2.1%, 1.2–7.7%, and 1.7–9.7%, respectively (51).

### 2. Hepatitis A

Over the past 10 years there has been a change in the pattern of infection in the younger age groups of the Malaysian population, similar to those seen in Singapore and Thailand. A marked decrease was seen in the seroprevalence of HAV: in the 0–10 year age group from 39% in 1985 to 15% in 1993, and in the 11–20 year age group from 51% in 1985 to 29% in 1993 (52,53).

### 3. Protozoal Infection

A study conducted at the Tampin Drug Rehabilitation Center in Malaysia established a high prevalence (23%) of asymptomatic carriers of *Cryptosporidium* among exposed HIV-positive intravenous drug users. The majority of them were young adults, and among the ethnic groups, the Malay HIV-positive inmates had the highest prevalence of *Cryptosporidium* infection (54).

### 4. Parasitic Infection

Parasite surveys and published reports from Malaysia have indicated that helminthic infections caused by *A. lumbricoides* and *T. trichura* are common in the country (55–57). A study conducted in five villages in Penang, Malaysia, revealed that the overall prevalence of *A. lumbricoides*, hookworms, and *T. trichura* was not significantly different, with the highest prevalence rate being 46.2%, 41.5%, and 38.7, respectively (Fig. 11). The highest intensity was observed for *A. lumbricoides* (58). It was noted that age was an important factor with regard to both prevalence and intensity. Generally, the prevalence and intensity of infection were low in the extreme lower and higher age groups (1–10 years and >50 years) when compared with other age groups.

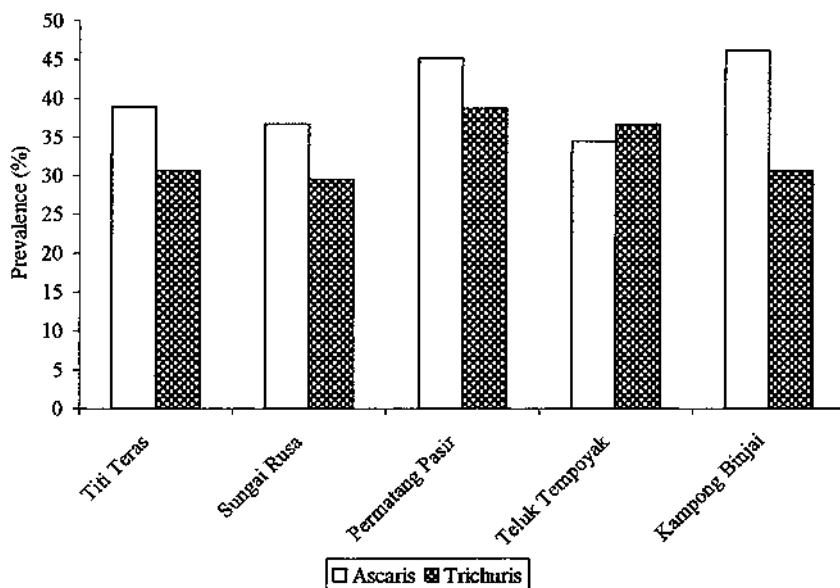
## E. Myanmar

There have been very few reports of foodborne illnesses in Myanmar. Only cholera cases were reported by WHO, as shown in Table 1.

## F. Philippines

In the Philippines, a 1992–1993 study demonstrated an overall HAV seroprevalence of 62% in metropolitan Manila, with age-related seroprevalence that increased progressively with age (59). This may indirectly reflect that some outbreaks of hepatitis A had occurred in this period. An early 1980 study of rural populations demonstrated almost universal endemicity in those 5 years of age or older, while in 1986 Philippine dentists demonstrated 86% HAV seroprevalence and adult expatriate Philippine workers in Saudi Arabia demonstrated 91–95% seroprevalence (60).

The distribution of intestinal protozoa infections is described in a study conducted during 1995, 1996, and 1997 in the community within and near by Baguio City, the Philippines. The prevalence of *E. histolytica* and *Giardia* spp. were 8.11% and 0.26%, respectively. Prevalence of *E. histolytica* was the highest in the 5–14 year age group. The geographic distribution of *E. histolytica* ranged from 0 to 3.52%, with total prevalence of 0.96% (Table 7) (61).



**FIGURE 11** Prevalence of helminths in five villages in northern peninsular Malaysia.

**TABLE 7** Geographic Distribution of *Entamoeba histolytica* in the Philippines

Community	Prevalence (%)
North	
Sapid	2.43
Pico, La Trinidad	1.65
Ambiong	3.38
Dreamland	1.52
Slaughter house	3.52
Fairview Road	0.00
Central	
City camp lagoon	0.00
Lower/Upper QM	0.00
Maridor hill	0.00
San Luis	0.00
Campo Sioco	0.00
South	
Smokey Mountain	0.74
Camp 7	0.00
Donotogan	0.00
Total	0.96

**TABLE 8** Cases of Foodborne diseases, Republic of Singapore, November 1997

Disease	Month ending			Cumulative, first 48 weeks		
	29.11.97	30.11.96	Median 92–96	1997	1996	Median 92–96
Cholera	0	0	0	10	19	19
Enteric fever						
Typhoid fever	3	6	9	79	105	105
Paratyphoid fever	1	2	2	19	207	48
Viral hepatitis						
Hepatitis A	14	12	13	138	142	142
Hepatitis B	18	13	13	167	134	127
Others	2	0	0	11	3	11

## G. Singapore

Table 8 shows the number of cases of specified foodborne notifiable diseases in the Republic of Singapore from 1992 to 1996 (62). The incidence of foodborne diseases in Singapore is low, mainly due to the high standards of environmental and food hygiene (63). However, outbreaks of food poisoning such as shigellosis, typhoid, and cholera have occurred in institutions where there were lapses in the personal and food hygiene of the food handlers in the kitchen (64).

In Singapore, *S. typhimurium* was the prominent nontyphoid *Salmonella* serotype isolated from 1972 to 1993. It was replaced by *S. enteritidis* in 1994 and 1995, accounting for between 32.8 and 36.9% of all nontyphoid *Salmonella* isolates (65). *S. enteritidis* was isolated in a large outbreak of food poisoning, which occurred in an institution in March 1995. All *S. enteritidis* isolates were of the serotype profile 0:1, 9, 12, H:g,m (antigen phase I). All *S. enteritidis* isolates were sensitive to ampicillin, co-trimoxazole and ciprofloxacin (66).

### 1. Hepatitis A

In Singapore, age-related seroprevalence has changed significantly since 1975. The seroprevalence of HAV has decreased from 60% in 10- to 19-year-olds in 1975 to 5.7% in 10- to 14-year-olds in 1985 (67) to less than 1% in 10- to 19-year-olds in 1991 (68). Overall population seroprevalence of HAV declined from 31.8% in 1985 (67) to 21.4% in 1991 (68).

### 2. Parasitic Infections

Parasitic infections adopt a rather low profile in the highly urbanized setting of Singapore. Very few foodborne parasitic infections are encountered. Apart from a few reports of infections with *Clonorchis/Opisthorchis*, *Taenia* spp., and hydatid diseases, there are no other citations of such helminthic infections. Seroprevalence surveys have shown the presence of toxoplasmosis in local meat animals (sheep, pigs, and cattle), and *Toxoplasma* strains have been isolated from pig, tree shrew, slow loris, and guinea pig (69).

In trematode infection, clonorchiasis/opisthorchiasis infections have been seen in local patients in Singapore (70). Most of the patients acquired the infection traveling in endemic countries. The few patients with no history of visit to endemic areas probably acquired the infections locally through eating uncooked Chinese carp, which is imported as fish fry from endemic regions (71). *Opisthorchis* may be important in Thai workers in the republic, because the Thai labor force in Singapore comes mainly from the northeastern province of Thailand, where *Opisthorchis* infection frequently occurs. *Taenia* infections are encountered rather infrequently. Hydatid disease has also been reported by physicians. These diseases occur in patients who have acquired the infection from endemic areas (69).

## H. Vietnam

In Vietnam, foodborne diseases have been considered to be the most widespread public health problem and the second leading cause of illnesses and mortality, even though they have been highly underreported (72).

Cholera has been a major cause of diarrhea in Vietnam for several decades. In 1885, an outbreak of cholera accounted for 50% mortality among French soldiers. The number of cholera cases reported annually from 1910 to 1930 ranged from 5,000 to 30,000 (73). In southern Vietnam, the El Tor biotype first appeared in 1964 with a total of 20,000 cases and a 4.1% mortality rate. Cholera is apparently endemic in the southern region of Vietnam. Outbreaks of cholera due to *V. cholerae* O1 El Tor biotype have been reported in northern Vietnam since 1976. However, cholera is not recognized as endemic in northern Vietnam (74).

*V. cholerae* isolates resistant to a number of antimicrobial agents have been reported around the world (75) and in Vietnam. Tetracycline and trimethoprim/sulfamethazole-resistant isolates and tetracycline, chloramphenicol, and trimethoprim/sulfamethazole-resistant isolates have been found in Vietnam since 1992 (76).

### 1. Hepatitis A

In Vietnam, HAV infection was found to be the principal cause of acute hepatitis in children in Hanoi (7.4%) and Ho Chi Minh City (>50%). A study conducted in Hanoi, Vietnam, during 1993–1995 showed that 29% of the study population had serology evidence of recent HAV infection. Younger cases (<20 years) were more likely to have recent HAV infection (41%) than those of over 20 years (21%) (77). Another study conducted in rural Vietnam showed 93% HAV-Ig positivity in the study population, with the median age of 6.7 years (78).

### 2. Helminthic Infection

A survey conducted in Ha Nam province, Vietnam, showed that 83% of individuals were infected with *A. lumbricoides* and 94% with *T. trichura*. Age-dependent patterns of infection, prevalence, and intensity were similar for *A. lumbricoides* and *T. trichura*. There was a direct relationship between *A. lumbricoides* and *T. trichura* infections: high-intensity *A. lumbricoides* was associated with high-intensity *T. trichura* infection (79).

## II. REPORTED OUTBREAKS BY COUNTRIES

### A. Thailand

Fourteen outbreaks of food poisoning with a total morbidity and mortality rate of 1190 were reported in 1995. The organisms reported as the cause of these outbreaks were *S. aureus*, *B. anthracis*, *Shigella*, *Salmonella*, and EHEC. Chemical poisoning, horseshoe crab poisoning, and mushroom poisoning have also been implicated in foodborne outbreaks (6).

Food-poisoning outbreaks in 1996 reported by the Epidemiological Surveillance, Department of Communicable Diseases Control, Ministry of Public Health, Thailand, are shown in Table 9. A total of 15 outbreaks affecting 1668 cases with 3 deaths due to food poisoning occurred in this year. Out of 8 outbreaks in which the etiology was known, 5 outbreaks were due to bacterial causes, specifically *V. parahaemolyticus* (80).

There were two outbreaks of paratyphoid fever in Bangkok in 1996. The first outbreak occurred between January and February, with a total number of cases of 347. The second outbreak occurred between August and September, with a total number of cases of 474, as shown in Figure 12. All cases were confirmed by hemoculture, and the organism responsible was *S. paratyphi* group A. Most of the cases were reported from the eastern part of Bangkok. The source of the outbreak could not be identified, perhaps due to the prolonged incubation period of the disease (1–3 weeks); thus, the patients could not recall all the foods or food shops where they ate. Preventive measures were

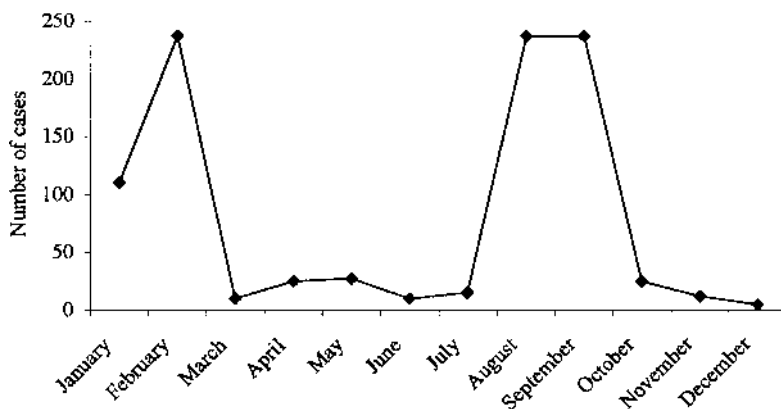
**TABLE 9** Food Poisoning Outbreak, 1996, Thailand

Etiology	No of outbreaks	Cases (deaths)	Type of population	Source of food
Bacteria	8	1305(0)		
<i>V. parahaemolyticus</i>	5	773(0)	1. Teachers and pupils, 525 cases 2. Villagers, 248 cases	Seafood (from hotel) or crabmeat Fried rice
<i>Shigella</i>	1	140(0)	Students	Lunch at school
<i>S. aureus</i>	1	45(0)	Students	Bread with sweet egg paste
<i>S. paratyphi A</i>	1	347(1)	Working age group	No source was found
Insecticide	1	12(0)	Carpenters	Drinking water
Chloroform	1	51(2)	Villagers	
Unknown	5	300(1)		
Total	15	1668(3)		

instituted through public relations and through various media, including strict control of food preparation and cleanliness of various markets in Bangkok (81).

An outbreak of hepatitis A occurred among schoolchildren at Nakhon Si Thammarat in southern Thailand during 1992. During that outbreak 79% of the children were increasingly positive for anti-HAV, increasing to 5 times of normal prevalence when there was no outbreak. The risk of getting HAV infection in this outbreak was increased by the occurrence of symptomatic hepatitis patients in the family and absence of a latrine (82). Another outbreak of hepatitis A occurred in a college at Saraburi province (central Thailand) during 1992, where 40 out of 588 students who were HAV positive had hepatitis A with jaundice. The source of infection was contaminated water in an overflow reservoir, from which the students drank (83).

There was another outbreak of hepatitis A in a secondary school in Suphanburi province (central Thailand) in August 1993. Thirteen cases were reported, and the source of the outbreak was found to be ice water sold in the school (84). A recently reported outbreak occurred in the area of the Department of Ministry of Science and Technology in Bangkok in November 1994. Seven cases were reported to be hepatitis A positive during the last 2 weeks of November. Unclean filtered

**FIGURE 12** Reported cases of paratyphoid in Bangkok by month in 1996.

**TABLE 10** Cholera Cases Reported in Vietnam by the Provinces, 1985–1995

Year	North	Center	South	High plateau
1985	381	3271	702	0
1986	1622	3147	832	0
1987	1018	218	833	0
1988	1389	916	224	12
1989	1	0	129	0
1990	0	798	1161	0
1991	3	142	0	0
1992	12	1849	649	0
1993	0	2684	776	0
1994	216	1822	626	1459
1995	814	3494	1327	453

drinking water was found to be a source of the outbreak. Various measures were taken to improve both drinking water quality and health education to improve personal hygiene (85).

Although *Trichinella spiralis* has been reported to be the most common cause of human trichinellosis, other species can also infect humans. An epidemic of human infection with *T. pseudospiralis* occurred in southern Thailand during 1994–1995. One outbreak affected 59 individuals, with one death. The source of this epidemic was raw pork from a wild pig. The most striking clinical features among patients who could be followed were muscular swelling, myalgia and asthenia persisting for more than 4 months (86).

## **B. Lao PDR**

*V. cholerae* O1 (Ogawa) and non-O1 *V. cholerae* isolates were identified in an outbreak affecting 30 patients in 18 families in the village of Khammuane Province. The outbreak occurred between August 28 and September 5, 1994 (48).

## **C. Malaysia**

An outbreak of cholera occurred during May 1996. The first case of cholera was confirmed on May 11. The biotype causing the outbreak was *V. cholerae* EL Tor serotype Ogawa. Nearly 1000 cases were confirmed in the whole country, most of them in Peneng (87).

## **D. Vietnam**

Cholera outbreaks have also occurred in several cities of central Vietnam during the last decade, as shown in Table 10. The first outbreak with 1459 cases was reported in the high plateau area of Vietnam in 1994. This reflected the widespread incidence of cholera in Vietnam (74).

## **III. FOOD SURVEY AND INTERVENTION**

Many food surveys were conducted in Thailand, especially in Bangkok. Various interventions were also implemented to improve the quality of food, especially in this capital city. A food survey conducted by sampling street food (from all over Bangkok) and ready-to-eat and cooked food (from various supermarkets in Bangkok) was conducted by the Department of Health, Bangkok Metropolitan Administration (BMA), prior to and after intervention in 1997 and 1998 (Table 11) (88).



**TABLE 11** Food Contaminated with Foodborne Spoilage and Pathogen in Bangkok Metropolitan Area Under Clean Shop Campaign Program

Type of contaminant	Street food															
	Street food						Ready-to-cook food				Cooked food					
	Pre-intervention, 1–25 Apr 97 (269 samples)		Postintervention				Pre-intervention, 6 May– 18 Jul 97 (256 samples)		Post-intervention, 3 months after (85 samples)		Pre-intervention, 6 May– 18 Jul 97 (449 samples)		Post-intervention, 3 months after (56 samples)		Post-intervention, 9 months after (563 samples)	
			6 Nov.– 23 Dec 97, 6 months after (219 samples)		14 Oct– 24 Nov 98, 1 year after (142 samples)											
N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Within standard	152	56.5	120	58.9	114	80.3	79	30.9	48	56.5	372	82.9	39	69.6	507	90.1
> standard	117	43.5	90	41.1	28	19.7	177	69.1	37	43.5	77	17.1	17	30.4	56	9.9
Total plate count > std	55	20.5	32	14.6	7	4.9	NR	NR	8	9.4	46	10.2	13	23.2	34	6.0
MPN coliform > std	81	30.1	65	29.7	25	17.6	NR	NR	NR	NR	43	9.6	4	7.1	10	1.8
MPN <i>E. coli</i> > std	47	17.5	46	21.0	10	7.0	57	22.3	16	18.8	20	4.5	10	17.9	17	3.0
Pathogen found in sample:																
<i>Salmonella</i> spp	15	5.5	10	4.6	3	2.1	149	58.2	22	25.9	6	1.3	7	7.1	9	1.6
<i>S. aureus</i>	7	2.6	1	0.5	1	0.7	5	1.95	1	1.2	1	0.2	—	—	1	0.2
<i>C. perfringens</i>	12	4.5	19	8.7	4	2.8	26	10.2	6	7.1	1	0.2	1	1.8	9	1.6
<i>V. cholerae</i>	6	2.2	7	3.2	1	0.7	10	3.9	3	3.5	4	0.9	—	—	2	0.4
non-O1/nonO139																
<i>V. parahaemolyticus</i>	—	—	1	0.5	—	—	3	1.2	3	3.5	—	—	—	—	—	—
<i>B. cereus</i>	—	—	—	—	—	—	3	1.2	—	—	2	0.5	—	—	—	—

NR = Not recorded.

**TABLE 12** Type and Number of Samples Contaminated with *S. aureus*, 1993–1996

Type of sample	No. samples	No. samples contaminated		Amount of <i>S. aureus</i> (MPN per gram)				
		<i>S. aureus</i>	%	3	3.6	7.3	9.1	>15
Pasteurized frozen shrimp	614	20	3	1	9	2	4	4
Pasteurized frozen fish	192	6	3	—	1	—	2	3
Pasteurized frozen squid	185	3	2	—	1	—	—	2
Pasteurized frozen seafood	131	10	8	—	3	—	2	5

There was marked improvement in street food over 1 year after intervention (from 56.5 to 80.3% of food that met the standards recommended by the Ministry of Public Health). Only 2.1, 0.7, 2.8, and 0.7% of street food samples were found to have *Salmonella* spp., *S. aureus*, *C. perfringens*, and *V. cholerae*, respectively. *V. parahaemolyticus* and *B. cereus* were not isolated. A similar trend occurred with the percentage of contaminated ready-to-eat food and cooked food after 3 months of intervention. The Department of Health, Bangkok Metropolitan Administration, within the past 2–3 years undertook various types of campaigns; one was the “star token for a clean shop campaign,” announcing 10 principles for street food sellers to follow:

1. To improve street food hygiene, improve personal hygiene of food handlers in preparing raw foodstuffs, handling, wearing caps and aprons and also train all food handlers about food hygiene.
2. Cover all foodstuffs to prevent dust, insects, and animals from coming into contact with the food.
3. Wash dishes with dish detergent and rinse with clean water.
4. Keep all clean dishes, spoon, etc. in clean airy containers.
5. Choose appropriate containers or dishes for a particular food.
6. Use the standard ketchup, fish sauces, vinegar recommended by the government.
7. All dishes containing food should be covered.
8. Drinking water and ice should be clean and kept in a clean container separate from the food.
9. The area for setting food should be at least 60 cm above the floor.
10. Use a garbage container with a cover and use separate containers for different kinds of garbage.

All stores that met these criteria were given a star token and recognized by food buyers as a standard food hygiene shop. The BMA is now expanding this campaign throughout Bangkok. It is recommended that travelers to Bangkok look for this sign in any food shop in which they eat.

**TABLE 13** Percentage of Frozen Cooked Food Contaminated with *S. aureus*, 1994–1996

Year	No. samples	<i>S. aureus</i>	% of samples
1994	275	13	4.73
1995	329	12	3.65
1996	508	14	2.76
Total	1112	39	3.51

Another type of survey was conducted from 1993 to 1996 to assess the contamination rate of *S. aureus* in frozen cooked seafood using the most probable number method. As shown in [Table 12](#), of 1112 samples of different kinds of exported frozen cooked seafood from factories located in southern Thailand, 3.51% were found to be contaminated with *S. aureus*. There has been a reduction of contaminants of frozen cooked food over the years, as shown in [Table 13](#) (89). There were no data available on food surveys and intervention from other countries in Southeast Asia.

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## Foodborne Diseases in Southern South America

**Bernadette D. G. M. Franco, Mariza Landgraf, and Maria Teresa Destro**

*Universidade de São Paulo, São Paulo, Brazil*

**Dilma S. Gelli**

*Instituto Adolfo Lutz, Secretaria do Estado da Saúde de São Paulo, São Paulo, Brazil*

The systematic reporting of foodborne diseases in Latin America and the Caribbean was launched in 1994, when the Pan American Health Organization (PAHO) and the World Health Organization (WHO) implemented a regional information system for the surveillance of outbreaks, called SIRVE-ETA (*Sistema Regional de Información para la Vigilancia de las Enfermedades Transmitedas por los Alimentos*) (1). Until then, data on outbreaks, when existing, were scattered in a number of hardly approachable local documents, journals, magazines, and proceedings of meetings or congresses.

The main objectives of SIRVE-ETA are the collection and the dissemination of data on foodborne disease episodes in Latin America and the Caribbean. This regional information system is part of the Plan of Action of the Regional Program on Technical Cooperation for Food Protection, established by PAHO/WHO in 1986, whose main goals are to provide safe foods, to reduce morbidity and mortality in the region, and to reduce the economic impacts of these illnesses. The Plan of Action and SIRVE-ETA are coordinated by the Pan American Institute for Food Protection and Zoonosis—INPPAZ (*Instituto Panamericano de Protección de Alimentos y Zoonosis*), located in Buenos Aires, Argentina (2).

This chapter was prepared using the data on foodborne disease outbreaks available at INPPAZ for the period between 1995 and 2001 (2). Among 19 countries that report regularly to this Institution, eight can be considered as part of southern South America: Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Peru, and Uruguay. Since no data from Bolivia are available at INPPAZ, this country was not included in this review.

The terms used in this text were the ones adopted by PAHO/WHO (3). *Food* corresponds to any substance destined for human consumption, including water, beverages, and any other component used for food preparation. Cosmetics, tobacco, or substances used as medicine are excluded. A *foodborne disease outbreak* is defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food. A *vehicle* is the food associated with an outbreak through strong epidemiological evidence or laboratory diagnosis.

### I. ESTIMATED RATE OF ILLNESS

Considering that SIRVE-ETA is in its early stages and that is a high percentage of nonreported outbreaks, the exact size of the problem related to foodborne diseases in the region remains unknown.

According to INPPAZ (Table 1), 5283 outbreaks occurred in Latin America and the Caribbean between 1995 and 2001, affecting 174,976 people and causing 275 deaths. Among these, 1358 outbreaks, 35,924 cases, and 123 deaths occurred in the southern countries included in this review

**TABLE 1** Foodborne Disease Outbreaks, Cases, and Deaths in Latin American Countries, 1995–2001

	1995			1996			1997			1998			1999			2000			2001			Total		
	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D
Argentina	1	19	0	5	143	0	33	355	0	1	50	0	32	1893	0	77	822	4	2	23	0	151	3305	4
Brazil	0	0	0	86	6564	1	0	0	0	1	32	0	203	4495	1	228	6174	3	0	0	0	518	17,265	5
Chile	78	939	0	256	3099	2	3	46	0	0	0	0	0	0	0	0	0	0	0	0	0	337	4084	2
Ecuador	0	0	0	17	545	10	8	428	1	13	436	11	1	77	0	4	251	0	0	0	0	43	1737	22
Paraguay	13	178	0	11	44	0	13	418	0	16	103	0	14	139	0	13	274	0	6	47	0	86	1203	0
Peru	0	0	0	29	1525	58	6	118	0	15	615	2	36	1956	27	26	1160	2	0	0	0	112	5374	89
Uruguay	5	443	0	12	201	0	17	424	0	18	303	0	41	736	1	12	186	0	6	663	0	111	2956	1
Subtotal	97	1579	0	416	12,121	71	80	1789	1	64	1539	13	327	9296	29	360	8867	9	14	733	0	1358	35,924	123
Other Latin American countries	<u>554</u>	<u>20,748</u>	<u>51</u>	<u>787</u>	<u>27,323</u>	<u>25</u>	<u>774</u>	<u>25,606</u>	<u>34</u>	<u>620</u>	<u>20,023</u>	<u>12</u>	<u>694</u>	<u>26,181</u>	<u>8</u>	<u>496</u>	<u>19,171</u>	<u>22</u>	<u>0</u>	<u>0</u>	<u>0</u>	<u>3925</u>	<u>139,052</u>	<u>152</u>
Total	651	22,327	51	1203	39,444	96	854	27,395	35	684	21,562	25	1021	35,477	37	856	28,038	31	14	733	0	5283	174,976	275

FBDO = Foodborne disease outbreaks; C = cases; D = deaths.

Source: Ref. 3.

(3). These numbers indicate that the average number of outbreaks per year in Latin America and the Caribbean was 754.7 and in the southern countries, 194.0. The average number of cases per year in the region was 24,997 and 5132 in the southern countries. The average number of deaths per year in the region was 39.3 and in the southern countries, 17.6.

The estimated population in Latin America and the Caribbean in 2000 was 520 million (4), so the detected rate of illness in these countries in 1995–2001 can be calculated as 1.45 outbreaks/million/year, 48.18 cases/million/year, and 0.078 deaths/million/year. Considering only the southern countries, where the estimated population in 2000 was 270 million (4), the rate of illness in the same period can be assumed to have been 0.72 outbreaks/million/year, 19.0 cases/million/year, and 0.034 deaths/million/year.

However, it has to be considered that the number of foodborne disease outbreaks reported by INPPAZ represents only part of those that really occur. Foodborne illnesses are underreported because ill persons do not seek medical care when the symptoms are mild or the illnesses or laboratory findings are not regularly communicated to public health officials. In some countries, like Brazil, only some foodborne diseases require mandatory notification (5). Usually, only episodes affecting a large number of people are spontaneously notified. In addition, specimens for laboratory diagnosis are not always available or laboratories do not perform the necessary diagnostic tests. Many foodborne diseases are caused by pathogens that have not yet been identified and thus cannot be diagnosed.

In the United States, foodborne diseases have been estimated to cause 6–81 million illnesses each year (6). During outbreaks in the United States between 1988 and 1992, an average of 15,475 cases was reported each year (7), which corresponds to only 0.26% of the lowest estimate. If a similar percentage is applied to the average number of reported cases of foodborne disease outbreaks in Latin America and the Caribbean between 1995 and 2001 (24,997), the real number of cases per year can be estimated to be at least 10 million. Considering only the southern South American countries, where the average number of cases per year was 5132, this estimated number would be around 2 million.

## II. REPORTED ILLNESSES

In Latin America and the Caribbean, a total of 5283 foodborne outbreaks, 174,976 cases, and 275 deaths were reported to INPPAZ between 1995 and 2001 (Table 1). Southern South American countries contributed with 25.7% of these outbreaks, 20.5% of the cases, and 44.7% of the deaths. Among the outbreaks, 38.1% occurred in Brazil, 24.8% in Chile, 11.1% in Argentina, 8.3% in Peru, 8.2% in Uruguay, 6.3% in Paraguay, and 3.2% in Ecuador.

These data must be interpreted very carefully because epidemiological surveillance in some of these countries may not exist or, if so, may not reflect reality. For example, according to INPPAZ, there were no outbreaks in Brazil in 1995, 1997, and/or 2001. However, data from the Health Department of the State of Paraná, Brazil, indicate the occurrence of 200, 156, and 164 foodborne disease outbreaks in that state in the years 1995, 1996, and 1997, respectively, with a total of 15,203 estimated cases (8).

The data about etiological agents, types of food associated with the outbreaks, and location of food mishandling correspond to the latest information available at INPPAZ when this chapter was prepared (3). Table 2 indicates the etiology of the foodborne disease outbreaks that occurred in southern South America between 1995 and 2001. The causative agent could not be determined in 38.5% of the episodes. The number of cases linked to them represents 26.3% of the total cases and 14.6% of the individuals that died.

As shown in Table 2, bacteria were the most frequent cause of outbreaks (86.2%) and cases (94.8%) in episodes where the etiological agent could be determined. Toxins caused the second highest number of outbreaks, followed by chemicals, parasites, and viruses. However, the highest number of deaths was due to intoxications with chemicals (2.2 deaths/outbreak), especially methanol

**TABLE 2** Foodborne Disease Outbreaks, Cases, and Deaths by Etiological Agent, Southern South America, 1995–2001

Agents	1995			1996			1997			1998			1999			2000			2001			Total		
	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D
<b>Bacteria</b>																								
<i>Salmonella</i> spp.	13	635	0	104	6925	1	25	345	0	13	287	0	92	2922	0	101	2787	7	3	8	0	351	13,909	8
<i>Staphylococcus</i> spp.	16	248	0	51	556	0	5	91	0	3	98	0	40	1043	1	32	765	0	4	53	0	151	2854	1
<i>Salmonella</i> Enteritidis	1	26	0	2	38	0	5	259	0	9	130	0	23	925	0	14	367	0	1	650	0	55	2395	0
<i>C. perfringens</i>	7	232	0	7	238	0	0	0	0	1	15	0	6	292	0	9	249	0	0	0	0	30	1026	0
<i>Vibrio cholerae</i>	0	0	0	0	0	0	1	19	1	12	354	11	6	165	0	2	167	0	0	0	0	21	705	12
<i>Shigella</i> spp.	0	0	0	1	19	0	1	230	0	1	32	0	5	819	0	7	830	0	2	8	0	17	1938	0
<i>Salmonella</i> Typhi	0	0	0	2	24	0	0	0	0	2	106	0	7	173	0	1	19	0	0	0	0	12	322	0
<i>Bacillus cereus</i>	0	0	0	3	182	0	0	0	0	0	0	0	1	13	0	7	36	0	1	3	0	12	234	0
<i>Cl. botulinum</i>	0	0	0	0	0	0	1	3	0	0	0	0	2	9	1	0	0	0	0	0	0	3	12	1
Others	4	69	0	14	168	0	6	115	0	3	95	0	19	470	0	20	779	0	2	8	0	68	1704	0
Total	28	575	0	80	1225	0	19	717	1	31	830	11	201	6831	2	92	3212	0	10	722	0	720	25,099	22
<b>Toxins</b>																								
Saxitoxin	0	0	0	5	15	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	15	1
Micotoxins	0	0	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4	0
Other toxins	17	87	0	35	206	0	0	0	0	1	2	0	1	2	0	0	0	0	1	3	0	55	300	0
Total	17	87	0	42	225	1	0	0	0	1	2	0	1	2	0	0	0	0	1	3	0	62	319	1
<b>Chemicals</b>																								
Methanol	0	0	0	16	136	52	0	0	0	1	6	1	0	0	0	0	0	0	0	0	0	17	142	53
Pesticides	1	6	0	1	7	0	5	175	0	0	0	0	4	60	24	2	115	1	0	0	0	13	363	25
Potassium bromate	0	0	0	0	0	0	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	1	15	0
Nitrites	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0
Others	0	0	0	2	40	0	0	0	0	0	0	0	1	11	1	1	6	0	0	0	0	4	57	1
Total	1	6	0	3	47	0	7	193	0	0	0	0	5	71	25	3	121	1	0	0	0	36	580	79
<b>Parasites</b>																								
<i>Trichinella spiralis</i>	0	0	0	4	104	1	9	154	0	0	0	0	1	168	0	0	0	0	0	0	0	14	426	1
<i>E. histolytica</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	13	2	0	0	0	0	0	0	1	13	2
Total	0	0	0	4	104	1	9	154	0	0	0	0	2	181	2	0	0	0	0	0	0	15	439	3
<b>Viruses</b>																								
Rotavirus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	40	0	0	0	0	2	40	0
Total	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	40	0	0	0	0	2	40	0
Subtotal	46	668	0	129	1601	2	35	1064	1	32	832	11	209	7085	29	97	3373	1	11	725	0	559	15,348	44
Unknown	51	911	0	287	10,520	69	45	725	0	32	707	2	118	2211	0	263	5494	8	3	8	0	799	20,576	79
Total	97	1579	0	416	12,121	71	80	1789	1	64	1539	13	327	9296	29	360	8867	9	14	733	0	1358	35,924	123

FBDO = Foodborne disease outbreaks; C = cases; D = deaths.

Source: Ref. 3.



and pesticides. The number of affected people in foodborne disease outbreaks caused by bacteria was higher than by the other agents (34.9 cases/outbreak), followed by parasites (29.3 cases/outbreak), while viruses presented 20 cases per episode.

Table 2 indicates that *Salmonella* spp. (including *S. Enteritidis* and *S. Typhi*) was the most frequent agent, being responsible for 58.1% of the outbreaks due to bacteria, and 66.2% of the cases. *Staphylococcus* spp. ranked second, with 21.6% of the outbreaks and 11.4% of the cases. *Clostridium perfringens* was linked to 4.2% of the outbreaks and 4.1% of the cases. Other microorganisms were involved in 16.8% of the episodes. It is interesting to note that the case per outbreak rates in episodes caused by *Shigella* spp. and *Salmonella* spp. were significantly higher (114.0 and 39.8 persons, respectively) than those linked to other pathogens. As expected, *Clostridium botulinum* was the one causing the highest case fatality ratio (8.3%), followed by *Vibrio cholerae* (1.7%) and *Salmonella* sp. (0.06%).

The official data available at INPPAZ about cholera in southern South America in 1995–2001 (3) (Table 2) must be viewed with caution. In January 1991, the Seventh Pandemia of cholera arrived in Latin America, coming from a Peruvian coastal village. From its explosive onset in Peru, the epidemic has marched across Central and South America, affecting almost all countries. From 1991 to 1995, Latin American nations reported over one million cases of cholera, including more than 11,000 deaths. In 1995, total reports of cholera cases declined, continuing a trend that has been observed each year since 1991 (9,10). However, in 1998 WHO reported a large increase in cholera cases in Peru. In the first 4 weeks of 1998 a total of 2863 cases with 16 deaths were reported compared with only 174 cases with one death in the corresponding period of 1997. These cases occurred in areas where no or very few cases had previously been reported (11). Momen reported that the disease resurged in the Brazilian Amazon region in 1997, causing more than 5,000 cases (2,600 confirmed) (12). WHO also reported several outbreaks in the period 1996–1999: one in Ecuador in 1996, with 416 cases and 4 deaths (13), two in Bolivia in 1997, with a total of 723 cases (half confirmed) and an unknown number of deaths (14), one in Chile in 1998, with 33 cases (12 confirmed) (15); and two in Brazil, in 1998 and 1999. The Brazilian outbreak in 1998 occurred in the northeastern part of the country, with 376 cases in one week. The source of contamination was thought to be the river that supplies the water to the population (16). The outbreak of 1999 occurred in the south, with 235 cases (205 confirmed) and 3 deaths, caused by seafood (17).

In 1995–2001, toxins were responsible for the highest number of outbreaks (40.0%) caused by other sources than bacteria (Table 2). On the other hand, the case-per-outbreak rate was low (5.2 persons/outbreak).

Chemicals were involved in 31.3% of the foodborne disease outbreaks caused by sources other than bacteria. Methanol was linked to 47.2% of the outbreaks of chemical origin, 24.5% of the cases, and 67.1% of the deaths. Methanol also presented the highest fatality rate (37.3%) among all causative agents. Pesticides were linked to 36.1% of the outbreaks due to chemicals and to 62.6% of the cases of this group. They were responsible for 25 deaths.

Parasites and viruses caused a low number of outbreaks (15 and 2, respectively), but the morbidity indexes were high: 29.3 and 20.0 cases/outbreak, respectively.

### III. FREQUENCY, RELATED FOODS, AND PLACE OF MISHANDLING

As presented in Table 3, the major vehicles associated with foodborne disease outbreaks reported by INPPAZ in 1995–2001 were eggs and mayonnaise (17.8%). However, the number of outbreaks without a known source was similar (17.3%).

Eggs and mayonnaise were responsible for 21.5% of the outbreaks with a known vehicle and for the highest percentage of cases (31.8%). The fatality rate linked to the consumption of this group of food was extremely low, however.

Outbreaks caused by more than one type of food (miscellaneous foods) were the second most frequent (14.5%). They were responsible for 17.5% of the cases with identified vehicle.

**TABLE 3** Foodborne Disease Outbreaks, Cases, and Deaths by Vehicle of Transmission, Southern South America, 1995–2001

	1995			1996			1997			1998			1999			2000			2001			Total		
	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D
Eggs/																								
mayonnaise	9	486	0	82	6449	0	11	317	0	10	112	0	54	1269	0	74	1707	1	1	12	0	241	10,352	1
Miscellaneous	19	353	0	65	1187	2	14	274	0	12	315	1	33	1169	0	50	1727	5	4	661	0	197	5686	8
Dairy	9	72	0	69	1620	0	3	42	0	1	50	0	28	378	24	24	291	0	0	0	0	134	2453	24
Red meat	13	308	0	28	530	1	11	193	0	7	61	0	39	1533	0	31	588	0	2	8	0	131	3221	1
Seafood	20	101	0	53	340	1	2	20	0	2	169	0	6	46	2	10	240	0	0	0	0	93	916	3
Poultry	6	54	0	7	175	0	5	34	0	6	313	0	24	1027	0	17	218	0	0	0	0	65	1821	0
Water	2	15	0	4	41	0	3	150	1	6	83	7	8	976	0	24	806	0	0	0	0	47	2071	8
Flours	2	85	0	6	62	0	8	203	0	2	44	0	8	131	1	16	1725	0	0	0	0	42	2250	1
Desserts	4	35	0	20	351	1	1	19	0	0	0	0	7	89	0	5	67	0	2	38	0	39	599	1
Beverages	2	27	0	19	161	53	1	2	0	1	6	1	5	208	1	2	8	0	0	0	0	30	412	55
Vegetables	0	0	0	4	100	4	2	6	0	3	18	0	3	51	0	5	335	0	0	0	0	17	510	4
Fruits	0	0	0	1	3	0	1	15	0	0	0	0	1	5	0	3	15	0	0	0	0	6	38	0
Mushrooms	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	1	3	0
Others	4	11	0	14	177	0	2	43	0	4	55	4	21	914	1	34	978	2	1	3	0	80	2181	7
Subtotal	40	328	0	128	1410	59	25	492	1	24	688	12	237	7796	29	296	8708	8	10	722	0	1123	32,513	113
Unknown	<u>57</u>	<u>1251</u>	<u>0</u>	<u>288</u>	<u>10,711</u>	<u>12</u>	<u>55</u>	<u>1297</u>	<u>0</u>	<u>40</u>	<u>851</u>	<u>1</u>	<u>90</u>	<u>1500</u>	<u>0</u>	<u>64</u>	<u>159</u>	<u>1</u>	<u>4</u>	<u>11</u>	<u>0</u>	<u>235</u>	<u>3411</u>	<u>10</u>
Total	97	1579	0	416	12,121	71	80	1789	1	64	1539	13	327	9296	29	360	8867	9	14	733	0	1358	35,924	123

FBDO = Foodborne disease outbreaks; C = cases; D = deaths.

Source: Ref. 3.

Flour accounted for 42 outbreaks, with 2250 cases. The average number of cases/outbreak (53.6) for this group of food was 20% higher than for water (44.1 cases/outbreak).

Foods of animal origin (seafood, red meat and dairy products, miscellaneous foods, poultry, and eggs/mayonnaise) were the main cause of foodborne disease outbreaks reported by INPPAZ (861/1358; 63.4%).

Beverages presented the highest fatality rate among all identified sources (13.35%). As shown in [Table 3](#), consumption of methanol is probably the main reason for this index, which is significantly higher than the one found for the second most frequent cause of death, represented by dairy products (0.99%).

The association between vehicle and etiological agents would be an interesting source of information. Nevertheless, these data are not available at INPPAZ for southern South America countries.

[Table 4](#) shows that 31.2% of foodborne disease outbreaks occurred due to mishandling of foods in households, followed by dining halls (21.0%) and hotels and restaurants (9.1%). Episodes due to consumption of foods in dining halls, however, accounted for the largest number of cases (38.8%), followed by households (18.6%) and hotels and restaurants (12.5%). On the other hand, the greatest number of deaths (46.3%) occurred due to mishandling of foods in places different from the listed ones. Outbreaks due to mishandling of food in the aforementioned places were also responsible for the highest morbidity rate.

#### IV. RISK ASSESSMENT

According to the Codex Alimentarius Commission, risk assessment is a scientifically based process consisting of four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (18). For an appropriate risk assessment, hazard identification, based on data on incidence of outbreaks and prevalent agents, is an important step. Despite the underestimation of the number of outbreaks in Latin American countries, the prevalent hazards are well known. Among bacteria, the most common are *Salmonella* and *Staphylococcus* species ([Table 2](#)), but there are a significant number of unknown microorganisms and other agents associated with foodborne outbreaks. Toxins play an important role, and so do chemicals, parasites, and viruses. Considering the outbreaks with known etiology, bacterial hazards were responsible for 86.2% of them (94.8% of cases and 21.0% fatal cases), toxins for 7.4% (1.2 and 1.0%, respectively), chemicals for 4.3% (2.2 and 75.2%, respectively), parasites for 1.8% (1.7 and 2.9%, respectively), and viruses for 0.2% (0.2 and 0%, respectively).

Hazard characterization and exposure assessment rely upon information about the size of the susceptible population, the distribution of hazards in the food, the conditions of production, storage, and usage of foods, and the eating habits of the consumers. In Latin America, many of these questions do not have clear answers. There are no quantitative data about the hazards in a given food or about their distribution in the food. [Table 3](#) indicates that eggs and mayonnaise are significant vehicles in foodborne disease outbreaks for southern South America. Red meat, dairy products, poultry, water, and seafood are also vehicles to be considered in management priorities. [Table 4](#) shows that mishandling of foods appears as the main cause of outbreaks that occurred in southern South America from 1995 to 2001. Inappropriate storage and/or usage of foods accounted for 38.3% of the outbreaks and 18.6% of the cases. However, dining halls, schools, hotel/restaurants, and street vendors were responsible for 45.5% of the outbreaks and 58.5% of cases in this period. These results indicate that the persons involved in outbreaks are those who eat out. There are no data about age and/or health conditions of the affected people.

Although prevalent, bacteria caused less fatalities than chemical agents. Methanol caused the highest number of deaths per outbreak in Peru, so this chemical should be a priority for public health authorities and risk managers in this country.

There are not enough data on the number and distribution of etiological agents in foods or consumer eating habits in each subpopulation in the region. The consumption of seafood is more

**TABLE 4** Foodborne Disease Outbreaks, Cases, and Deaths by Place of Food Mishandling, Southern South America, 1995–2001

	1995			1996			1997			1998			1999			2000			2001			Total		
	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D	FBDO	C	D
Households	27	352	0	82	782	4	19	275	0	16	203	2	133	1499	0	143	1525	1	5	48	0	424	4884	7
Dining halls	40	515	0	76	2607	2	7	44	0	10	458	0	59	3064	0	78	2590	2	2	7	0	272	9285	4
Hotels/restaurants	9	563	0	26	269	1	6	124	0	12	238	0	38	749	0	30	556	1	2	652	0	123	3151	2
Street vendors	10	90	0	51	344	1	1	27	0	6	152	0	5	118	0	1	58	0	1	4	0	75	793	1
Schools	0	0	0	6	184	2	3	140	0	0	0	0	5	94	0	20	1127	0	0	0	0	34	1545	2
Hospitals	0	0	0	1	12	0	3	154	0	0	0	0	2	23	0	4	214	0	0	0	0	10	403	0
Others	1	24	0	64	876	46	7	197	0	7	340	7	24	1805	2	63	2121	2	3	20	0	169	5383	57
Subtotal	87	1544	0	306	5074	56	46	961	0	50	1391	9	266	7352	2	339	8191	6	13	731	0	1107	25244	73
Unknown	<u>10</u>	<u>35</u>	<u>0</u>	<u>110</u>	<u>7047</u>	<u>15</u>	<u>34</u>	<u>828</u>	<u>1</u>	<u>14</u>	<u>148</u>	<u>4</u>	<u>61</u>	<u>1944</u>	<u>27</u>	<u>21</u>	<u>676</u>	<u>3</u>	<u>1</u>	<u>2</u>	<u>0</u>	<u>251</u>	<u>10680</u>	<u>50</u>
Total	97	1579	0	416	12121	71	80	1789	1	64	1539	13	327	9296	29	360	8867	9	14	733	0	1368	35924	123

FBDO = Foodborne disease outbreaks; C = cases; D = deaths.

Source: Ref. 3.

common in the Latin American Pacific Coast area than in others. PAHO reported that the odds ratio to develop diarrhea by consuming street food in La Paz (Bolivia), Bogotá (Venezuela), Quito (Ecuador), Lima (Peru), Santo Domingo (Dominican Republic), Guatemala (Nicaragua), Mexico City, and Culiacan (Mexico) varies from 1.00 to 1.17, depending on the number of meals per day (19). So far, there is not enough specific information on pathogen-food-consumer interactions to properly estimate the risks of foodborne disease outbreaks in southern South America.

Thus, microbiological risk assessment remains a big challenge for food processors, risk managers, and public health authorities in southern South American nations. Many attempts toward better comprehension of these concepts have been made recently.

## V. ONGOING AND PROPOSED MEASURES TO REDUCE FOODBORNE ILLNESS

The first effective measure toward the reduction of foodborne illnesses in Latin America and the Caribbean was the creation of the Plan of Action of the Regional Program on Technical Cooperation for Food Protection, established in 1986 by PAHO/WHO. The functioning of this regional program depends on the implementation of an effective surveillance system because of the need for reliable data on the occurring outbreaks. To contribute to the development of surveillance in these countries, PAHO and epidemiologists from some member countries prepared in 1993, and reedited in 2001, the *Guide for the Establishment of Surveillance Systems on Foodborne Diseases and Investigation of Foodborne Outbreaks (GUIA VETA: Guia para el Establecimiento de Sistemas de Vigilancia Epidemiologica de Enfermedades Transmitidas por Alimentos y la Investigación de Brotes de Toxi-infecciones Alimentarias)*. At this moment, this is the most significant tool for the investigation of foodborne disease outbreaks in the region (2). The main objectives of this guide are:

- To collect and organize information about the outbreaks
- To gather and interpret data on frequency, distribution and severity of cases, etiological agents, vehicles, sources of contamination, target population, critical control points, and other important factors
- To disseminate the collected information
- To stimulate the investigation and notification of foodborne disease outbreaks
- To provide guidance for control and prevention measures
- To investigate new problems and to foresee the new tendencies in the occurrence of foodborne disease outbreaks

Based on GUIA-VETA, PAHO/WHO started in 1994 a regional information system for the surveillance of outbreaks (SIRVE-ETA). The responsibilities of PAHO/WHO and member countries for the implementation of SIRVE-ETA were set in the document “Guidelines for the Implementation of Regional Surveillance System on the Occurrence of Foodborne Disease Outbreaks” (*Orientaciones para la Implementación del Sistema de Vigilancia Regional sobre la Ocurrencia de Enfermedades Transmitidas por los Alimentos*). This document provides all the forms needed for quarterly reporting of foodborne disease outbreaks.

The foodborne disease outbreak surveillance system in each country is part of the general disease surveillance system of PAHO/WHO (1). In each country, trained people report quarterly to INPPAZ data relating to number of outbreaks, etiological agents, foods involved, locations, age of affected persons, and so on. The information coming from the participating countries is collected and organized by INPPAZ, which gives feedback through a bulletin (*Boletín INPPAZ en las Américas*) and through its website ([www.inppaz.org.ar](http://www.inppaz.org.ar)). This is a passive surveillance system, which relies on reporting of outbreaks to local, state, provincial, and national health departments, which in turn report to INPPAZ.

Although foodborne diseases are very common, only a fraction of them are routinely reported. The results of a survey run by PAHO in 14 Latin American countries, including 7 out of 8 countries belonging to the southern region, indicate that the main reasons for this underreporting are:

In many countries, the report of foodborne disease outbreaks or cases to the health authorities is not mandatory.

The outbreaks reported to the national health authorities are not forwarded to INPPAZ.

In the majority of countries, health departments act at three levels (local, state or province, and national), and any break in communication among them results in cases not being reported.

Insufficiently trained personnel, shortage of financial resources, and lack of an effective net of public health laboratories to run food analysis using appropriate methodologies hamper the complete implementation of SIRVE-ETA (1).

## VI. ESTIMATED EFFECTIVENESS OF THESE MEASURES

The Regional Program on Technical Cooperation for Food Protection, implemented by PAHO/WHO, is helping the countries of the region to rebuild their national surveillance systems in spite of the large number of nonreported outbreaks. The information collected by INPPAZ is very useful for national food safety programs, since it indicates that foodborne diseases are an important public health problem. It also indicates that microbial pathogens are the main cause of foodborne diseases in Latin America, with infections caused by *Salmonella* and *Staphylococcus* spp. occurring most frequently. Foods of animal origin are the main vehicles in outbreaks, so strategies for food safety should focus on these pathogens and these types of food.

The knowledge accumulated from the national systems and from SIRVE-ETA is very useful for the establishment of control and prevention measures by the local health authorities, which can promote the application of effective technologies in food preparation. This knowledge is also necessary for the correct application of GMP and HACCP programs.

Considering that the majority of the reported foodborne disease outbreaks in the region occurred due to mishandling, educational programs directed at communities are mandatory. The public should be alerted to hazardous conditions that affect them and should be stimulated to become concerned about its food supply. The data collected by SIRVE-ETA is useful for preparing educational materials about foodborne disease hazards and preventive measures to be used routinely in home food preparation. Special educational programs can be targeted at high-risk groups such as children, elderly, pregnant women, immunocompromised, etc.

Because of the lack of data from previous years and because SIRVE-ETA is still being implemented, it cannot be established if the number of outbreaks is diminishing in southern South America. In spite of the increasing implementation of GMP and HACCP in many countries in recent years, it is too early to evaluate their benefits to food safety. Further refinements of foodborne disease estimates will require continued and improved active surveillance.

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# The WTO Agreement on the Application of Sanitary and Phytosanitary Measures

## An International Trade Agreement with Implications for National and International Food Safety Standards

**Kazuaki Miyagishima**

*University of Kyoto, Kyoto, Japan*

**Fritz Käferstein**

*International Food Safety Consultant, Nyon, Switzerland*

Centuries ago people realized that there was a link between health and trade. In the fourteenth century, Italian city states like Venice began to develop quarantine systems to guard against the importation of bubonic plague, which they believed came to them through trade (1).

When the major international organizations were set up some 50 years ago after the end of World War II, the founders recognized this linkage. The original General Agreement on Tariffs and Trade (GATT), which was instituted in 1947 and still remains an integral part of GATT 1994, includes provisions for countries to apply measures “necessary to protect human, animal or plant life or health” if they do not unjustifiably discriminate between countries where similar conditions prevail or act as a disguised restriction on international trade (2). The World Health Assembly in 1949 called attention “to the need for eliminating quarantine restrictions of doubtful medical value which interfere with international trade and travel” (3). Therefore, there is a reciprocal understanding that health must be protected over and above business interests, but that health protection measures should not intrude on commerce without justifications (4).

### I. FOOD PRODUCTION—SAFETY FIRST

The possible use of health protection measures to restrict trade has been a concern since the beginning of multilateral trade negotiations. However, it was nearly 50 years before trading partners concluded the Agreement on the Application of Sanitary and Phytosanitary Measures in 1994 (4). By that time, international trade in food had reached an unprecedented volume, representing some \$294 billion. As the scale of international trade in food further increases (\$458 billion in 1997), there is a growing threat from foodborne pathogens (5). In addition, hazards to human health also occur in the form of mycotoxins, pesticide residues, and other substances, the presence of which may lead to rejection or destruction of consignments by importing countries (6).

Despite the risk to health related to the international food trade, this international trade is essential as it has at least a twofold benefit: (a) it introduces a wider variety of foods into the diet by providing consumers with a bigger and better choice of products, thus contributing to better nutrition,



and (b) it provides food-exporting countries with foreign exchange, which is indispensable for the economic development of many countries, and thus for an improvement in the standard of living for many peoples.

International trade in foods, however, can be impeded by tariff and nontariff barriers at national borders. Some of them may be required to protect the health of consumers or to protect the domestic economy; others are simply detrimental to international trade. To address this concern, the Joint FAO/WHO Codex Alimentarius Commission (Codex in short) was established in 1962 to protect the health of the consumers and, at the same time, to ensure fair practices in food trade. Codex has since elaborated a number of food standards, guidelines, and recommendations. However, while member governments of Codex have been asked to formally accept these standards, it has been left to governments to decide whether they should or should not implement them, given that Codex texts have not been directly linked to an international trade scheme such as GATT. (See [Chapter 48](#).)

## II. ESTABLISHMENT OF THE WORLD TRADE ORGANIZATION

The Uruguay Round of Multilateral Trade Negotiations was concluded in April 1994 with the signing of the Marrakesh Agreement, and it gave birth to a number of multilateral trade agreements to which all Members of the World Trade Organization (WTO), established in January 1995, are committed (7).

One important outcome of the Uruguay Round was that countries agreed to reduce tariff barriers for many agricultural commodities so as to further encourage free trade. As a result, nontariff barriers became a real concern because they could undermine the promotion of international trade if put into practice in an arbitrary or discriminatory way.

To address some of these concerns, the WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) was drawn up to ensure that countries apply measures to protect human and animal health (sanitary measures) and plant health (phytosanitary measures) based on the assessment of risk, or, in other words, based on science. The SPS Agreement incorporates, therefore, safety aspects of foods in trade.

It is important to mention another WTO agreement here—the Agreement on Technical Barriers to Trade (TBT Agreement). This agreement, which had been in existence as a plurilateral agreement since the Tokyo Round, was revised and converted into a multilateral agreement through the Uruguay Round. It covers all technical requirements and standards (applied to all commodities), such as labeling, that are not covered by the SPS Agreement. Therefore, the SPS and TBT Agreements can be seen as complementing each other.

One of the main objectives of the SPS Agreement is to improve human and animal health as well as the phytosanitary situation in all WTO Member countries. This is to be addressed through the establishment of a multilateral framework of rules and disciplines that will guide the development, adoption, and enforcement of sanitary and phytosanitary measures and minimize their negative effects on trade. As a natural consequence, the SPS Agreement recognized the standards and related texts of the Codex Alimentarius Commission as international points of reference. Today the SPS Agreement is regarded as being a strong instrument that will further the goal of the Codex Alimentarius Commission, that is, to harmonize food standards worldwide.

Likewise, international standards established by the International Office of Epizootics (OIE) (see [Chapters 49, 50](#)) and the relevant international and regional organizations operating within the framework of the International Plant Protection Convention (IPPC) have been recognized in the SPS Agreement as providing references with regard to animal and plant life or health.

In order to comply with the provisions of the SPS Agreement, it may often be necessary to strengthen national food control systems. This may require both manpower and financial investment. To address these particular difficulties, which may be encountered by any developing country, the SPS Agreement also includes provisions for technical assistance to be provided by other countries

or through international organizations. The SPS Agreement thus provides an ideal opportunity for developing countries to build modern food control and safety schemes or to upgrade existing ones.

### **III. BENEFITS AND CHALLENGES OF THE SPS AGREEMENT**

#### **A. Rights and Obligations**

As with most agreements, the SPS Agreement provides both rights and obligations to contracting parties, i.e., WTO Members.

#### **B. Information Exchange**

Many trade problems can be prevented if trading partners share information with each other about sanitary measures before they are put into force. This can best be achieved when notifications are made before such measures are enacted by regulatory authorities and other countries are afforded an opportunity to comment. This is one of the most important concepts of the SPS Agreement, and it is called *transparency* (Article 7 of the SPS Agreement).

Notifications from WTO Members about their proposed sanitary/phytosanitary measures are regularly made available to other WTO Members, either by direct mailing or via the Internet, and any WTO Member country can address enquiries to another Member country regarding its sanitary requirements, practices, and agreements. To facilitate this, each WTO Member country establishes one *enquiry point*, which is responsible for providing answers to all reasonable questions from interested Members, as well as relevant documentation. Also, all sanitary and phytosanitary regulations that have been adopted need to be published promptly to allow interested Members to become acquainted with them.

To implement the transparency provision, each country should establish well-organized procedures with regard to the setting of national regulations that allow other countries to comment on any proposed new measure before it is finalized. In addition, coordination between competent governmental departments should be established in order to ensure the effective operation of enquiry points.

#### **C. International Harmonization**

No matter what side of the globe they live on, consumers should enjoy adequate protection against the risks of foodborne diseases. This can be achieved, without restricting international trade, if all countries harmonize their regulations by using international standards as a basis for their sanitary measures.

For food safety, the SPS Agreement recognizes, as the international reference, the standards, guidelines, and recommendations established by the Codex Alimentarius Commission relating to food additives, veterinary drug and pesticide residues, contaminants, methods of analysis and sampling, and codes and guidelines of hygienic practices.

Codex standards and related texts are deemed necessary to protect human health. *As long as a country employs these standards, its measures are presumed to be consistent with the provisions of the SPS Agreement.* Harmonization with Codex will eliminate the necessity of one country having to provide other countries with justifiable reasons as to why the measures they are applying are necessary in order to protect human health.

Many new ideas are being integrated into Codex texts. These include the recommendation to adopt a *risk-based* approach in achieving food safety objectives. One such approach is the Hazard Analysis and Critical Control Point (HACCP) system [Codex Alimentarius, Supplement to Volume 1B General requirements (Food Hygiene), 1997]. This risk-oriented approach, recommended by the Codex General Principles of Food Hygiene, allows the food industry and governments to target limited resources to the most critical steps of food production and distribution, rather than having

to comply with a long list of product and procedure specifications as has been traditionally prescribed. The application of the HACCP principles, be it voluntary or mandatory, presupposes that the national food regulatory authority should also adapt itself to this approach, and this puts more emphasis on the authority's audit and training functions than on the physical inspection and laboratory analysis of consignments. Although this does not completely eliminate the necessity for inspection of the final product, the concept of *process control* needs to be stressed in any national food safety program.

Another important trend in Codex is its *horizontal approach*. Codex is in the process of elaborating general standards covering food additives, contaminants, and toxins to provide a wider basis for protecting consumers' health. Countries can better adapt themselves to this approach by implementing a *generic regulation* applicable to a wide range of products rather than maintaining an inventory of registered foods with specifications.

## D. Risk Assessment

WTO Members retain the right to take sanitary measures necessary for the protection of human life or health. In so doing, countries can determine the appropriate level of sanitary protection, which may be higher than that achieved by adhering to stipulated international standards. The sanitary measures, however, must be based on *risk assessment* (Article 5 of the SPS Agreement). WTO Members have to ensure that these measures are nondiscriminatory, not more trade-restrictive than necessary, and are not maintained without sufficient *scientific evidence*.

International standards do not yet cover all aspects of food safety, and it may be necessary for a country to introduce sanitary measures in those areas where international standards are nonexistent. In such a case, efforts should be made to expedite the work of the Codex Alimentarius Commission to establish a new text covering the field in question. If a country has to employ a sanitary measure before an international standard is established, then it should apply risk assessment when determining the content of such measure.

Risk assessment techniques used by the World Health Organization (WHO) have been published and are made widely available for reference. These techniques include methodologies in toxicological evaluation, exposure assessment, and other related methods used by expert bodies such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meetings on Pesticide Residues (JMPR). A joint FAO/WHO expert body charged with assessing the risks related to biological hazards in food began working in 2000. Sound knowledge of the functioning of these risk assessment bodies not only assists countries in better understanding the scientific background of international standards based on evaluations made by these bodies, but is useful in building national risk assessment procedures modeled on those used by the international bodies (Article 5:1 of the SPS Agreement).

When conducting risk assessment at the national level, it is desirable that authorities have epidemiological data on the incidence of foodborne diseases in their country and information about the level of exposure of the country's population, particularly vulnerable groups, to foodborne hazards. Countries are therefore encouraged to participate in the Global Environment Monitoring System—Food Contamination Monitoring and Assessment Programme (GEMS/Food) coordinated by WHO to either establish or improve their capacity to conduct monitoring of pesticide and contaminant intakes. At the international level, the monitoring data obtained from countries will provide important indicators in establishing international standards for certain chemicals in foods. WHO also maintains databases on the incidence of foodborne diseases of biological origin.

Another important element in the SPS Agreement is the concept of a *disease-free area* (Article 6 of the SPS Agreement). Recognizing the sanitary and phytosanitary characteristics of the geographical area from which the product originates and those of the area where the product is being received will help remove unnecessary measures and economize valuable resources. This consideration constitutes an integral part of the risk assessment exercise.

## E. Equivalence

In applying a particular sanitary measure, it is important to recognize what level of health protection will actually be achieved through its implementation. Where more than one measure is equally effective in achieving a given appropriate level of health protection, WTO Members are committed to accepting the sanitary measures used by other Members as being *equivalent measures*, even if they are different from their own requirements. The exporting country needs, of course, to demonstrate that its measures will achieve the appropriate level of sanitary protection laid down by the importing country (Article 4:1 of the SPS Agreement).

To reinforce the recognition of equivalency in the area of food in trade, it is useful to adhere to the Codex Principles for Food Import/Export Inspection and Certification and the Codex Guidelines for the Design, Operation, Assessment and Accreditation of Food Import and Export Inspection and Certification Systems (Codex Alimentarius Volume 1A).

It is, however, to be noted that while recognizing equivalence, the SPS Agreement requires WTO Members to ensure that the sanitary or phytosanitary measures they establish or maintain are not more trade-restrictive than required to achieve their appropriate level of sanitary or phytosanitary protection, taking into account technical and economic feasibility (Article 5:6 of the SPS Agreement).

## IV. THE ROLE OF THE HEALTH SECTOR

Because the food safety provisions in Codex standards, guidelines, and recommendations are recognized as international references to be used for harmonizing national regulations, it is of paramount importance that the health sector of each country fully participates in and contributes to the work of the Codex Alimentarius Commission. Otherwise there is a risk that the Codex Alimentarius Commission could adopt standards, guidelines, or recommendations that might not be fully adequate from a health protection point of view.

To coordinate work of different ministries/departments responsible for food control, countries are encouraged to establish a national Codex Committee, with the participation of all government and other key players, which would serve as a focal point for information exchange and the development of a national Codex policy. In addition to sending delegations to Codex meetings, countries can provide written comments in reply to Codex Circular Letters, which is a cost-effective means of participating in the work of Codex.

In order to overcome the delay in mailing Codex documents, an increasing number of Codex working papers are made available on the Internet (<http://www.codexalimentarius.net/>) for easy access by government agencies and other interested parties. If representation by the health sector in Codex meetings is not possible, diplomatic representatives stationed in those countries where Codex meetings are held could be mobilized so that their countries' positions are better reflected in the deliberations and decisions of Codex.

Another important forum in which the health sector should take full part is the WTO Committee on Sanitary and Phytosanitary Measures (SPS Committee), which holds sessions in Geneva, Switzerland. The SPS Committee carries out the functions necessary to implement the provisions of the SPS Agreement. It provides a regular forum for consultations between countries, and questions can be asked of Member delegations regarding the specific notifications they have made.

WTO Members, especially those who encounter difficulty in meeting the requirements of the SPS Agreement, are encouraged to request technical assistance from other Members during the sessions of the SPS Committee, or at any other time. Technical advice, credits, donations and grants, including training opportunities, can be provided to Members in need, either bilaterally or through international organizations (Article 9 of the SPS Agreement). Representatives of relevant international organizations are often present as observers at meetings of the SPS Committee, and requests for technical information can also be discussed with them directly.

## **V. FREQUENTLY ASKED QUESTIONS**

### ***Question 1: Do developing countries benefit from the SPS Agreement?***

*Answer:* Yes. Adherence to the Agreement provides an opportunity to upgrade a national food safety program, with assistance provided by international or bilateral cooperation organizations or agencies. It will result in better protection of the health of consumers domestically, and in improved confidence with regard to exported products on world markets. Moreover, if exporting developing countries base their sanitary and phytosanitary measures on international standards, they will be less exposed to arbitrary restrictions from importing countries.

### ***Question 2: What is the difference between SPS and TBT?***

*Answer:* As far as foods are concerned, the SPS Agreement covers those aspects related to health, i.e., food additives, residues of veterinary drugs and pesticides, codes and guidelines of hygienic practice, etc. Other aspects such as labeling are covered by the TBT Agreement.

### ***Question 3: How can one obtain a Codex text?***

*Answer:* All Codex Member countries have a national Codex Contact Point. It serves as a documentation center for Codex standards, guidelines, and recommendations, as well as for all Codex working papers. Also, a CD-ROM containing all final Codex texts in three languages (English, French, and Spanish) is available from the Codex Secretariat.

### ***Question 4: What is Risk Assessment as it relates to food safety?***

*Answer:* Risk Assessment is defined in the SPS Agreement as “the evaluation of the likelihood of entry, establishment or spread of a pest or disease within the territory of an importing Member according to the sanitary or phytosanitary measures which might be applied, and of the associated potential biological and economic consequences; or the evaluation of the potential for adverse effects on human or animal health arising from the presence of additives, contaminants, toxins or disease-causing organisms in food, beverages or feedstuffs.” As it relates to food safety, the Codex interim definition for Risk Assessment is “a scientifically based process consisting of the following steps: (i) hazard identification; (ii) hazard characterization; (iii) exposure assessment; and (iv) risk characterization.”

### ***Question 5: Does each WTO member always have to tackle risk assessment in order to fulfill the obligations of the SPS Agreement?***

*Answer:* No. As long as countries base their food regulations and import requirements on Codex standards, guidelines, and recommendations, they are fulfilling their obligations in regard to the SPS Agreement. However, when setting regulations in areas where no international standards exist, when applying more stringent requirements to imported products than to domestic products, or when applying import requirements which are stricter than Codex standards, guidelines, and recommendations, countries ought to ensure that those measures are based on risk assessment.

## **VI. FUTURE PERSPECTIVES**

As outlined above, the SPS Agreement provides today a powerful tool to advance international harmonization of food standards. As of January 2000, 5 years after the entry into force of the Agreement, its entire provisions became applicable to all WTO Members, including least-developed countries. Given that much is still to be done to fully put into effect the rights and duties the Agreement

incurs at the national level and that more experiences be gained to ensure the unambiguous implementation of the Agreement at the international level, the Agreement is expected to live through forthcoming trade negotiations and remain, for years to come, a key for reconciling health and food trade.

Over and above the areas identified in the preceding sections as fields for further work and efforts, there exist two additional domains that would possibly constitute substantial challenges for governments in both conceptual and technical terms. The one relates to the means to define the “appropriate level of health protection” in a concrete manner. Not only is it difficult to adequately formulate or express (mathematically or else) such a level, but also any attempt to apply it uniformly to a range of different categories of foodborne risks (e.g., microbial or chemical, short-term or long-term) may encounter various problems. The other touches upon the issue of so-called precautionary measures or approaches. While the SPS Agreement recognizes explicitly the right of WTO Members to provisionally adopt a sanitary measure in cases where scientific evidence is insufficient, there has so far been no single and common understanding as to how and in which conditions this right should be exercised by regulatory authorities.

To deal with these unavoidable but most important issues, a participatory process of consensus building between all interested parties appears to be indispensable at both national and international levels, however laborious and lengthy an exercise it might be.

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# The International Organization for Standardization

**Martha Petró-Turza**

*Hungarian Standards Institution (MSZT), Budapest, Hungary*

**Catherine Guissé**

*Association Française de Normalisation (AFNOR), Saint-Denis La Plaine, France*

## I. GENERAL INFORMATION

International standardization began in the electrotechnical field when the International Electrotechnical Commission (IEC) was established in 1906. Pioneering work in other fields was carried out by the International Federation of the National Standardizing Associations (ISA), which was set up in 1926. The emphasis within ISA was on mechanical engineering. Its activities came to an end in 1942.

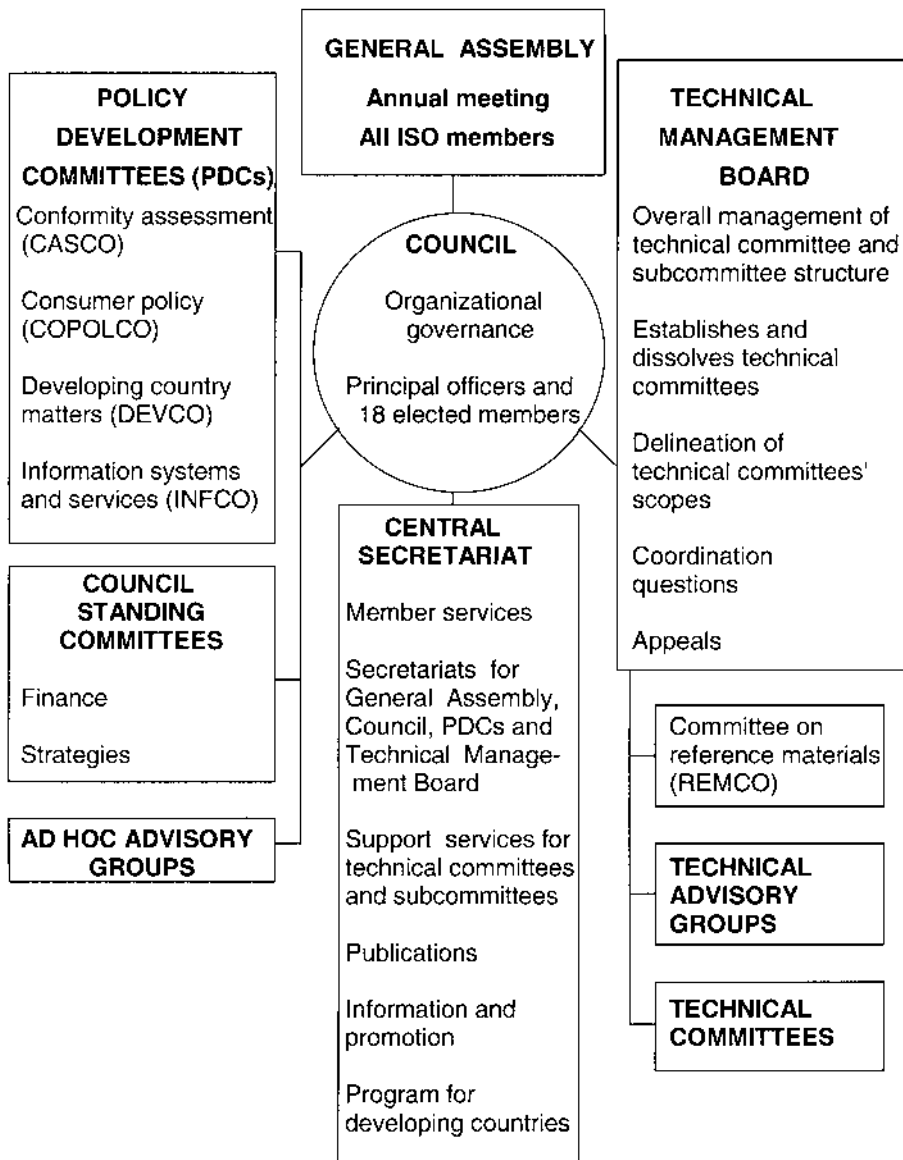
After the Second World War, in 1946, delegates from 25 countries met in London and decided to create a new international organization, the objective of which would be “to facilitate the international coordination and unification of industrial standards.” The new organization, the International Organization for Standardization (ISO), officially began operating in 1947.

Because the name of the International Organization for Standardization would have different abbreviations in different languages (IOS in English, OIN in French), it was decided to use a word derived from the Greek *isos* meaning “equal.” Therefore, the short form of the Organization’s name is ISO in all languages (1).

### A. The Purpose and Characteristics of ISO

The purpose of ISO is to promote the development of standardization and related activities in the world with a view to facilitating international exchange of goods and services and developing cooperation in the spheres of intellectual, scientific, technological, and economic activity. ISO deals with the full spectrum of human activity; its work program ranges from standards for traditional activities, such as agriculture and construction, through mechanical engineering to the newest information technology developments, such as digital coding of audiovisual signals for multimedia applications. In cooperation with its partners in United Nations specialized agencies, and via IEC and ITU (International Telecommunication Union), which deal with standardization for the electrical and electronic engineering and telecommunication sectors, ISO provides easy access to a coherent and consistent portfolio of standards covering all sectors (5).

Since the mid-1980s, ISO has undertaken to help developing countries benefit from the use of International Standards and to help them in setting up and developing standardization infrastructures suited to the specific needs of their own economies (7). ISO is a nongovernmental organization and its members are not, therefore, national governments, but the national bodies “most representative of standardization” in their respective countries (1). By the end of 1999 ISO had 133 member organizations from countries large and small, industrialized and developing, in all regions of the world, on the basis of one member per country (5). The structure of ISO is shown in [Figure 1](#).



**FIGURE 1** ISO's structure. (From Ref. 2.)

The technical work of ISO is regulated by a stringent procedure developed and continuously improved since its inception—more than 50 years. The overall coordination of this technical work is made by the ISO Technical Management Board, with the assistance of the ISO Central Secretariat located in Geneva.

ISO standards are technical agreements that provide a framework for compatible technologies worldwide. Developing technical consensus on an international scale is a major operation. The standards-development work is actually carried out in technical bodies comprising technical committees (TC), subcommittees (SC), working groups (WG), and ad hoc study groups. At the end of 1999



ISO had 186 active technical committees, 576 subcommittees, 2057 working groups, and 40 ad hoc study groups (3). It is estimated that each working day of the year, 15 ISO technical meetings are in progress, on average, somewhere in the world, in which some 30,000 experts participate annually (1). ISO has a decentralized management: secretariats of the technical committees and subcommittees are provided by ISO member bodies located in 35 countries (3).

ISO members can freely choose whether or not they would like to participate in a given ISO technical committee or subcommittee, and, if yes, with what kind of membership status—as a P- or O-member. P-members participate actively in the work, with an obligation to vote and, whenever possible, participate in meetings. O-members follow the work as observers; they receive committee documents and have the right to submit comments and to attend meetings (8).

Every participating member has the right to take part in the development of any standard it judges important for its country's economy. No matter what the size or strength of its economy, each participating member in ISO has one vote. ISO's activities are thus carried out in a democratic framework where each country is on an equal footing to influence the direction of ISO's work at the strategic level, as well as the technical content of its individual standards (1).

ISO standards are voluntary. ISO does not enforce their implementation. A certain percentage of ISO standards—mainly those concerned with health, safety, and environment—has been adopted in some countries as part of their regulatory framework or is referred to in legislation for which it serves as the technical basis. However, such adoptions are sovereign decisions by the regulatory authorities or governments of the countries concerned. ISO itself does not regulate or legislate (1).

ISO standards are market-driven. They are developed by international consensus among experts drawn from the industrial, technical, and business sectors that have expressed the need for a particular standard. Experts from government, regulatory authorities, testing bodies, academia, consumer groups, or other relevant bodies may also participate and contribute. Increasing ISO's market relevance is among its major strategies. Although ISO standards are voluntary, the fact that they are developed in response to market demand and are based on consensus among the interested parties ensures their widespread use (1).

## **B. Contact of ISO with Other Organizations**

The ISO system could not function without the strong national infrastructures provided by ISO members. They produce thousands of national consensus positions on ISO proposals each year and select and instruct tens of thousands of individuals, who serve in delegations that represent the national views in the relevant committees of ISO.

Many ISO members also belong to regional organizations having cooperative programs in standardization and standards-related fields. These members ensure cooperative relationships with ISO as a whole, and, with respect to developing standards, commit themselves to the concept of thinking nationally and regionally and acting internationally. ISO has recognized regional standards organizations representing Africa, the Arab countries, the area covered by the Commonwealth of Independent States, Europe, Latin America, the Pacific area, and the South-East Asia nations (1).

These recognitions are based on a commitment by the regional bodies to adopt ISO standards, whenever possible without change, as national standards of their members, and not to initiate the development of divergent standards if appropriate ISO standards are available for direct adoption (1). The Vienna Agreement on technical cooperation between ISO and CEN (European Committee for Standardization), which was approved by both organizations in 1991, is a useful working model, the principles of which are worthy of emulation in other regions (6).

ISO is well respected throughout the world. It has a reputation for integrity and neutrality and also enjoys a high status among international organizations. ISO, together with IEC and ITU, is building a strategic partnership with the World Trade Organization (WTO) with the common goal of promoting a free and fair global trading system. WTO, the World Bank, the International Chamber of Commerce, etc. agree on the importance of International Standards to facilitate trade, and this can be seen by their policies and programs in many contexts (1,5).

ISO's work is of interest to many other international organizations. Some of these make a direct technical contribution to the preparation of ISO standards, others, particularly intergovernmental organizations, contribute to the implementation of ISO standards, for example, by utilizing them in the framework of intergovernmental agreements. ISO has adopted arrangements for associating these organizations closely with all stages of the work. At present, ISO liaises with some 550 international and regional organizations interested in some aspects of ISO's standardization work. These include the 28 or so international standards-developing bodies outside the ISO/IEC system. Each of these bodies works in a specific area, usually with a United Nations mandate, for example, the Codex Alimentarius Commission. ISO and IEC together produce about 85% of all International Standards, and the other specialized bodies account for the rest (1).

## **II. INTERNATIONAL STANDARDIZATION OF FOOD PRODUCTS**

### **A. Structure, Scope, and Standards of ISO/TC 34, Agricultural Food Products**

ISO Technical Committee 34, Agricultural Food Products (ISO/TC 34), was established in the same year as ISO—1947. Since the beginning, its secretariat has been provided by the Hungarian standardization body, which was a government institute until 1995. As a result of the political changes that took place in 1989 in Hungary, a new standardization body was established in 1995. The Hungarian Standards Institution (MSZT) is an independent self-governing body of public interest, with registered membership. The current structure of ISO/TC 34, unchanged for more than 20 years, is given in [Table 1](#).

Subcommittee 1, Propagation Materials, was established in the 1950s and dissolved in 1983. As [Table 1](#) shows, fields of activity of TC 34 subcommittees cover practically all the important products of agriculture and the food industry: oilseeds and grains; fruits, vegetables, milk, meat, and derived products; edible fats and oils; stimulant foods such as tea and coffee; and even products that increase the hedonic value of foods, such as spices and condiments. Besides products for human consumption, there is a TC 34 subcommittee (SC 10) to deal with the standardization of animal feeds as well. Most subcommittees of ISO/TC 34 are product-oriented; only two of them (and the Technical Committee itself) deal with the development of so-called horizontal standards, namely SC 9, Microbiology, and SC 12, Sensory Analysis (10).

ISO members have traditionally followed the work of TC 34 with great interest. In 1961, out of a total of 44 ISO members, 84% were members of this technical committee (19 P-members and 18 O-members). At the end of 1999, with a total membership of 76 (38 P-members and 38 O-members), ISO/TC 34 was still the second largest technical committee of ISO. The largest technical committee was ISO/TC 176, Quality Management and Quality Assurance, with 77 members (8).

ISO/TC 34 has a Steady Editorial Committee (SEDCO). Its main task is the final technical, grammatical, and structural control of the Draft International Standards (DIS), Final DISs (FDIS), and International Standards (IS) before publication. In addition SEDCO ensures the uniform features of food standards as well (8).

Initially, a greater part of the standardization work was undertaken by the TC, while the task of the SCs was rather limited. In the course of time, parallel with the increase in number of standards and subcommittees, more and more parts of the standardization work have been transferred to the subcommittees, and the role of the TC has changed. At present there are only a few standards under the direct responsibility of the TC, but it has a wide range of other duties. These are the coordination and harmonization of the work of its subcommittees, cooperation with the international organizations in liaison, operation of SEDCO, publication of the annual report of ISO/TC 34, and the development, continuous updating, and implementation of the Strategic Policy and Business Plan of the Technical Committee.

**TABLE 1** Current Structure of ISO/TC 34

Subcommittee	Working group	Title	Country providing secretariat	Number of member bodies
	AG	Advisory group	Hungary	
SC 2		Oleaginous seeds and fruits	France	38
SC 3		Fruit and vegetable products	Poland	48
SC 4		Cereals and pulses	Hungary	45
SC 5		Milk and milk products	Netherlands	43
SC 6		Meat and meat products	Netherlands	42
SC 7		Spices and condiments	India	41
SC 8		Tea	United Kingdom	44
SC 9		Microbiology	France	46
	WG 1	Microbiological analysis of meat and meat products	Netherlands	
SC 10		Animal feeding stuffs	Netherlands	44
SC 11		Animal and vegetable fats and oils	United Kingdom	45
SC 12		Sensory analysis	France	43
SC 13		Dry and dried fruits and vegetables	Turkey	37
SC 14		Fresh fruits and vegetables	Turkey	41
	WG 1	Terminology	Poland	
SC 15		Coffee	Brazil	47
	WG 1	Test methods concerning green coffee	United Kingdom	
	WG 2	Determination of caffeine content	Netherlands	
	WG 3	Test methods concerning roasted and soluble coffee	Switzerland	
	WG 4	Vocabulary	United Kingdom	

The scope of ISO/TC 34 is worded as follows: “Standardization for products of agricultural origin used for human and animal feeding purposes as well as for animal and vegetable propagation materials with particular reference to terminology, sampling, methods of test and analysis, product specifications and requirements for packaging, storage and transportation” (8). Within this scope, presently 528 International Standards exist, under the responsibility of ISO/TC 34. The current work program of the Technical Committee contains 326 work items; of these, 189 deal with the revision of existing standards and 137 are new projects. This means that 36% of the valid International Standards are under revision, which consists mainly of updating. This has been a rather constant proportion for years, but we endeavor to decrease it by shortening the revision period. The distribution of International Standards and work items between subcommittees is given in [Table 2](#) (9).

The numbers speak for themselves and show the activity of the whole technical committee. Percentage distribution of our standards according to their content is as follows:

- Analytical or test methods: 70%
- Product specifications: 12%
- Storage conditions and transport: 9%
- Sampling: 4%
- Nomenclatures and vocabularies: 3%
- Others: 2%

**TABLE 2** Distribution of International Standards and Work Items of the Work Program of ISO/TC 34 Between Subcommittees

Subcommittee	Title	Number of existing ISs	Number of work items	
			Under development	Under revision
SC 2	Oleaginous seeds and fruits	27	3	6
SC 3	Fruit and vegetable products	43	0	28
SC 4	Cereals and pulses	53	6	21
SC 5	Milk and milk products	92	58	32
SC 6	Meat and meat products	31	3	8
SC 7	Spices and condiments	66	1	13
SC 8	Tea	19	5	5
SC 9	Microbiology	21	11	13
SC 10	Animal feeding stuffs	19	20	7
SC 11	Animal and vegetable fats and oils	40	17	17
SC 12	Sensory analysis	19	8	9
SC 13	Dry and dried fruits and vegetables	14	0	5
SC 14	Fresh fruits and vegetables	52	0	15
SC 15	Coffee	23	3	9
TC 34	Agricultural food products	<u>9</u>	<u>2</u>	<u>1</u>
	Total	528	137	189

Initially, ISO/TC 34 standardized test methods, which gave basic information about products, could be carried out under simple laboratory conditions. With the increase in customers' demands, standard methods follow more and more the development of analytical chemistry. At present, almost all modern instrumental analytical methods can be found among the standardized methods of food analysis. However, we must not forget the existing great differences in the technical development and financial possibilities of our member bodies. Therefore, in many cases we standardize not only one but also two or more different methods for the determination of the same component or characteristic. This makes it possible for technical personnel to choose the method that is the most suitable for their purposes and can be carried out under the given conditions. This is why we confirm at regular intervals many time-honored methods, although they could be replaced by more modern ones. However, to settle doubts or disputes, in cases when alternative methods are standardized, one of them is marked as the reference method.

The second group of standards deals with product specifications. Most of them describe the minimum requirements of the given food or food product.

Among the others, the group of standards containing vocabularies or nomenclatures is worthy of mention. These International Standards, translated trilingually or bilingually, are comprehensively used in the world trade and accepted by other international organizations as well.

## **B. Contact of ISO/TC 34 with Other ISO Technical Committees and Other Organizations**

With the goal of ensuring the continuous and mutual exchange of information between ISO technical committees active in related fields, ISO/TC 34 is in permanent contact with four other ISO Technical Committees (Table 3). As regards the regional standardization organizations, only CEN, the European Committee for Standardization, is in direct connection with ISO. As previously mentioned, this cooperation is carried out within the frame of the Vienna Agreement (6), which, among others, includes:

**TABLE 3** Other ISO Technical Committees in Permanent Contact with ISO/TC 34

Technical committee	Title
ISO/TC 47	Chemistry
ISO/TC 54	Essential oils
ISO/TC 93	Starch (including derivatives and by-products)
ISO/TC 176/SC 2	Quality management and quality assurance— Quality systems

Source: Ref. 9.

- Regular exchange of general information (mementos, catalogues, work programs, etc.)
- Cooperation on standards drafting
- Adoption of existing International Standards as European Standards
- Parallel approval of Standards
- Monitoring
- Follow-up of comments from ISO member bodies

Considering the fact that CEN was established 14 years later (1961) than ISO (1947) and the CEN standardization work in food areas started only 27 years after its establishment (in 1988), it is not surprising that the number of relevant CEN standards (100) is far smaller than that of ISO standards (528), and 35% of these European Standards are accounted for by the adoption of the relevant ISO standards.

ISO/TC 34 is in liaison with 11 other International Organizations, a list of which is shown in Table 4. AOAC International is also in direct liaison with each subcommittee of ISO/TC 34, and CAC, EC, and UN/ECE with almost all of them. On the other hand, NMKL is in liaison with the subcommittees only through the technical committee.

In addition, 34 other international organizations are in liaison only with subcommittees of ISO/TC 34. Among them, 14 are in liaison with more than one SC and 20 with only one of them. This is understandable, as organizations specialized in certain fields are interested only in the work of the relevant subcommittee. For example, ESA (European Spice Association) is interested only in SC 7, and the ICO (International Coffee Organization) is interested only in SC 15. On the other hand, for example, WCO (World Customs Organization) and FAO (Food and Agriculture Organization of the United Nations) are interested in the work of more than one SC, mainly those that develop

**TABLE 4** International Organizations in Liaison with ISO/TC 34

Abbreviation	Name
AOAC	AOAC International
CAC	Codex Alimentarius Commission
CEA	European Confederation of Agriculture
EAAP	European Association for Animal Production
EC	European Commission
IUPAC	International Union of Pure and Applied Chemistry
NMKL	Nordic Committee on Food Analysis
OIML	International Organization of Legal Metrology
OIV	International Vine and Wine Office
UN/ECE	United Nations Economic Commission for Europe
WHO	World Health Organization

Source: Ref. 9.

product specifications standards. Of course, as everywhere, here we can also find exceptions. For example, ICC (International Association for Cereal Science and Technology) is in liaison with more than one subcommittee (SC 2, SC 4, SC 7, SC 9, and SC 10). This shows that ICC has a wide range of interest, which covers not only cereals but almost all seeds.

Representatives of liaison organizations meet regularly to exchange their ideas and harmonize their activities. The secretariat of this so-called interagency meeting was held by ISO for a long time, and it was taken over by AOAC International a few years ago.

### **C. Activity of ISO/TC 34 Related to Microbiology**

Microorganisms are present in many foods and food products, some of which are very useful. For example, microorganisms are used to produce food products such as yogurt, cheese, salami, wine, etc. by fermentation. However, other microorganisms represent a significant danger for human health by contamination of food products.

While food safety is one of the most obvious basic requirements, microbiological contamination, a very common kind of impurity, can be present in practically any food if the hygienic conditions of their production and/or storage conditions are not appropriate. Therefore, it is essential to develop suitable methods for controlling the microbiological state of foods. It is therefore not surprising that elaboration of microbiological standards is an important task of ISO/TC 34 (10). Although the Technical Committee has a special Subcommittee (SC 9) for this purpose, three others (SC 4, SC 5, and SC 6) also discuss standards on this topic. This can be attributed to historical and other reasons as well. It is a fact that the above-mentioned three subcommittees were established more than 10 years earlier than SC 9, the subcommittee that initially developed only general standards, mainly guidelines that could be used horizontally, for almost all types of food products.

Taking into account that the risk of foodborne illness has dramatically increased in parallel with the globalization of trade, the importance of microbiological control of food products is also enhanced. This has brought forth the demand for reliable international standard methods.

In this situation the concentration of knowledge and resources seemed to be the most adequate way to meet the requirements. This procedure has already started within ISO/TC 34. Subcommittee 4 has one microbiological standard, ISO 7698:1990, Cereals, Pulses and Derived Products—Enumeration of Bacteria, Yeasts and Molds. At the last systematic review of this standard, the Subcommittee decided to ask SC 9 to carry out its revision and take over its responsibility.

Table 5 shows a list of microbiological standards that are under the responsibility of SC 5, and Table 6 contains those standards that belong to SC 6. (For changes in the latter, see below.)

### **D. Purpose of ISO Technical Committee 34 Subcommittee 9, Microbiology**

Several methods of analysis have been described for the detection and/or enumeration of pathogenic microorganisms present in food products. But method diversity presents a potential trade barrier, and the blocking of imported products, even for just a few days, can lead to considerable market share losses. In addition, a multiplicity of methods often results in duplication of analysis and an overall increase in cost.

Furthermore, the authorities, at both regional and international levels, require harmonized microbiological analyses, and standardization is a privileged harmonization tool for these methods. National and international authorities define, within the Codex Alimentarius on Food Hygiene, “criteria” (absence of a microorganism or maximum tolerated number of microorganisms per quantity of product) that allow ensuring the commercialization and the exchange of food products without endangering the health of the consumers.

It is easy to recognize that to define a “criterion” without defining a method for detecting or enumerating the given microorganism represents an obvious cause of a trade barrier, as the results of an analysis method depend greatly on the technique employed and on the recommended media.

**TABLE 5** Existing Specific Microbiological Standards for Dairy Products Under the Responsibility of ISO/TC 34/SC 5

Standard	Year of publication	Title
ISO 5541-1	1986	Enumeration of coliforms—Part 1: Colony-count technique at 30 degrees C (Under revision)
ISO 5541-2	1986	Enumeration of coliforms—Part 2: Most probable number technique at 30 degrees C (Under revision)
ISO 6610	1992	Enumeration of colony-forming units of microorganisms—Colony-count technique at 30 degrees C (Under revision)
ISO 6611	1992	Enumeration of colony-forming units of yeasts and/or molds—Colony-count technique at 25 degrees C
ISO 6730	1992	Enumeration of colony-forming units of psychrotrophic microorganisms—Colony-count technique at 6.5 degrees C
ISO 6785	1985	Detection of <i>Salmonella</i> (Under revision)
ISO 8261	1989	Preparation of test samples and dilutions for microbiological examination (Under revision)
ISO 10560	1993	Detection of <i>Listeria monocytogenes</i>
ISO 11866-1	1997	Enumeration of presumptive <i>Escherichia coli</i> —Part 1: Most probable number technique
ISO 11866-2	1997	Enumeration of presumptive <i>Escherichia coli</i> —Part 2: Most probable number technique using 4-methylumbelliferyl-beta-D-glucuronide (MUG)
ISO 11866-3	1997	Enumeration of presumptive <i>Escherichia coli</i> —Part 3: Colony-count technique at 44 degrees C using membranes

Source: Ref. 1.

**TABLE 6** Existing Specific Microbiological Standards for Meat Products Under the Responsibility of ISO/TC 34/SC 6

Standard	Year of publication	Title
ISO 3100-2	1988	Sampling and preparation of test samples—Part 2: Preparation of test samples for microbiological examination
ISO 5552	1997	Detection and enumeration of Enterobacteriaceae without resuscitation—MPN technique and colony-count technique
ISO 6391	1997	Enumeration of <i>Escherichia coli</i> —Colony-count technique at 44 degrees C using membranes
ISO 13681	1995	Enumeration of yeasts and molds—Colony-count technique
ISO 13720	1995	Enumeration of <i>Pseudomonas</i> spp.
ISO 13721	1995	Enumeration of lactic acid bacteria—Colony-count technique at 30 degrees C
ISO 13722	1996	Enumeration of <i>Brochothrix thermosphacta</i> —Colony-count technique

Source: Ref. 1.

**TABLE 7** Participation Table of ISO/TC 34/SC 9

P-members	O-members	Liaison with other organizations
Australia (SAI)	Argentina (IRAM)	AIIBP: International Association of the Manufacturers of Stocks and Soups
Austria (ON)	Belgium (IBM)	AOAC: AOAC International
Canada (SCC)	Croatia (DZNM)	CAC: Codex Alimentarius Commission
China (CSBTS)	Cuba (NC)	EC: European Commission
Colombia (ICONTEC)	Estonia (EST)	ICC: International Association for Cereal Science and Technology
Czech Republic (CSNI)	Ethiopia (QSAE)	ICMSF: International Commission on Microbiological Specifications for Foods of the IUMS
Denmark (DS)	Finland (SFS)	ICUMSA: International Commission for Uniform Methods of Sugar Analysis
Egypt (EOS)	India (BIS)	IDF: International Dairy Federation
France (AFNOR)	Indonesia (BSN)	IOCCC: International Office for Cocoa, Chocolate and Sugar Confectionery
Germany (DIN)	Ireland (NSAI)	IUMS: International Union of Microbiological Societies
Hungary (MSZT)	Mexico (DGN)	WHO: World Health Organisation
Iran (ISIRI)	Saudi Arabia (SASO)	
Italy (UNI)	Slovakia (UNMS)	
Netherlands (NNI)	Slovenia (SMIS)	
Norway (NSF)	South Africa (SABS)	
Poland (PKN)	Sri Lanka (SLSI)	
Portugal (IPQ)	Trinidad and Tobago (TTBS)	
Romania (ASRO)	USA (ANSI)	
Russian Federation (GOST R)	Viet Nam (TCVN)	
Spain (AENOR)	Yugoslavia (SZS)	
Sweden (SIS)		
Switzerland (SNV)		
Tanzania (TBS)		
Thailand (TISI)		
Turkey (TSE)		
United Kingdom (BSI)		

Source: Ref. 1.

Therefore it is essential to draw up reference methods for each criterion. It is here that standardization plays a major role.

## E. Structure of ISO/TC 34/SC 9 and Development of Its Standards

Subcommittee 9 (SC 9) of ISO/TC 34 was established in the middle of the 1970s. Since then, AFNOR (the national body of standardization for France) has provided its secretariat, within which horizontal reference methods are developed.

The objective of the work of SC 9 is the harmonization of standards by integrating as far as possible sector-based particularities. It is thus that sectorial standards are only drawn up in the case of absolute necessity, in any case taking into account the general directives of the SC 9.

A working group (WG1) devoted to microbiological analysis of meat and meat products, which was set up earlier within the framework of ISO/TC 34/SC 6, was transferred to SC 9 in 1998 but maintained its Dutch convenorship.

At present ISO/TC 34/SC 9 has 46 member bodies and is in liaison with 11 other organizations. Their list is shown in [Table 7](#). ISO/TC 34/SC 9 works in close relation with the mirror working group of the European Committee for Standardization (CEN/TC 275 Working Group 6—AFNOR secretariat). As the scope of this CEN Working Group concerns only pathogenic microorganisms, all ISO standards are not elaborated under the Vienna Agreement. Therefore, most of microbiological standards are elaborated under ISO structure.



Once a year ISO/TC 34/SC 9 organizes a meeting to discuss its main projects. Otherwise, all work is done by correspondence. Experts designated by their national bodies of standardization prepare the technical content of the working documents of the Subcommittee. These are circulated among member bodies for comments, redrafted according to the comments, and circulated again as a committee draft (CD). This procedure is repeated twice but always at a higher level [Draft International Standards (DIS), then Final Draft International Standards (FDIS)]. When a document reaches a higher level, the criteria for its acceptance become stricter. This procedure ensures reaching as wide a consensus as possible before an International Standard is published (4).

## F. Standards and Work Program of ISO/TC 34/SC 9

The work of SC 9 focuses on methods of analysis for the detection and/or counting of microorganisms such as *Listeria monocytogenes*, coagulase-positive *Staphylococci*, *Escherichia coli*, *Salmonella*, *Shigella*, pathogenic *Vibrio*, *Clostridium perfringens*, and others. However, SC 9 also works on general standards concerning good practices of laboratories or preparation of test samples.

Methods of analysis elaborated as reference documents include in most cases two plating-out media as well as two plates per dilution to be inoculated. For certain applications, industry scientists have developed more rapid or more practical methods than the reference methods. These are the so-called “alternative” methods. ISO/TC 34/SC 9 is currently preparing a standard which will allow validation of these alternative commercial methods with respect to the reference methods.

The following subclauses report on the scope and/or principle of work items of the current work program of SC 9, arranged into main groups. The existing International Standards are only listed in the relevant tables as they are available from the ISO Member Bodies or from the ISO Central Secretariat.

### 1. General Standards and Work Items under the Responsibility of ISO/TC 34/SC 9

General standards and work items under the responsibility of SC 9 are listed in Table 8.

*ISO 6887-1: Preparation of test samples, initial suspension and decimal dilutions for microbiological examination—Part 1: General rules for the preparation of the initial suspension and decimal dilutions (Parts 2, 3, 4, and 5 are specific parts; see Table 9):* This standard defines the general rules for the preparation of the initial suspension and of decimal dilutions for microbiological examination. Taking into account the variety of food and feed products to which this Standard is applied, SC 9

**TABLE 8** General Standards and Work Items Under the Responsibility of ISO/TC 34/SC 9

Number	Year of publication	Title
ISO 6887-1	1999	Preparation of test samples, initial suspension and decimal dilutions for microbiological examination—Part 1: General rules for the preparation of the initial suspension and decimal dilutions
ISO 7218 <sup>a</sup>	1996	General rules for microbiological examinations (an amendment of this standard is under preparation)
ISO 11133-1 <sup>a</sup>		Guidelines on preparation and production of culture media—Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory
ISO 11133-2 <sup>a</sup>		Guidelines on preparation and production of culture media—Part 2: Practical guidelines on performance testing of culture media
ISO 13369 <sup>a</sup>		Guidance on the determination of water activity
ISO 16140 <sup>a</sup>		Protocol of validation for alternative methods

<sup>a</sup>Work program items for 2000.

Source: Refs. 1 and 9.

**TABLE 9** Methods of Analysis for Detection of Microorganisms, Standards and Work Items Under the Responsibility of ISO/TC 34/SC 9

Number	Year of publication	Title
ISO 6579 <sup>a</sup>	1993	General guidance on methods for the detection of <i>Salmonella</i> (Under revision)
ISO 8523	1991	General guidance for the detection of Enterobacteriaceae with pre-enrichment
ISO 8914	1990	General guidance for the detection of <i>Vibrio parahaemolyticus</i>
ISO 10272	1995	Horizontal method for detection of thermotolerant <i>Campylobacter</i>
ISO 10273	1994	General guidance for the detection of presumptive <i>Yersinia enterocolitica</i>
ISO 11290-1 <sup>a</sup>	1996	Horizontal method for the detection and enumeration of <i>Listeria monocytogenes</i> —Part 1: Detection method (Under revision)
ISO 16654 <sup>a</sup>		Horizontal method for the detection of <i>Escherichia coli</i> O157
ISO XXXX <sup>a</sup>		In preparation (trials)—Pathogenic <i>Vibrio</i>

<sup>a</sup>Items for work program 2000.

Source: Refs. 1 and 9.

has decided to expand Parts 2, 3, 4, and 5 of ISO 6887 for specifying rules for the preparation of the test sample and of the initial suspension. As a matter of fact, for a number of products it is necessary to take special precautions especially when preparing the initial suspension because of the physical state of the product (such as a dry product, a highly viscous product) or due to the presence of inhibitory substances (such as spices, salted fishes, etc.) or because of the acidity. Part 2 will focus on meat products, Part 3 on dairy products, Part 4 on fish products, and Part 5 on other products.

*ISO 7218/A1: General rules for microbiological examinations—Amendment 1:* This standard gives general instructions for carrying out microbiological examinations in accordance with specific standards. The purpose of this standard is to help ensure the validity of the examinations, to ascertain that the general techniques used for conducting these examinations are the same in all laboratories, to help achieve homogeneous results in different laboratories, and to contribute to the protection of the health of the laboratory personnel by preventing risks of infection. This standard may be used wholly or partly for the accreditation of a laboratory by national organizations. Amendment 1, which is under preparation, focuses on the expression of results.

*ISO 11133-1: Guidelines on preparation and production of culture media—Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory:* This standard will provide the general terminology related to quality assurance of the preparation of culture media and specifies the minimum requirements to be used for the microbiological analysis of products intended for human consumption or animal feeding. These requirements are applicable to three categories of culture media used in laboratories that prepare and/or use culture media for performing microbiological analyses:

Commercially manufactured ready-to-use media

Media prepared from commercially available dehydrated formulations (either complete, e.g., plate count agar, or basal media to which supplements are added, e.g., Baird-Parker agar)

Media prepared from its individual components

*ISO 11133-2: Guidelines on preparation and production of culture media—Part 2: Practical guidelines on performance testing of culture media:* This standard will specify criteria and methods for the performance testing of culture media and will apply to organizations producing and/or distrib-

uting media to microbiological laboratories, to laboratories that prepare culture media for their own use as well as commercial bodies that supply microbiological laboratories with ready-to-use or semi-finished reconstituted or dehydrated media, noncommercial bodies that supply media to third parties, and microbiological laboratories that need to evaluate the performance of culture media.

*ISO 13369: Guidance on the determination of water activity:* This standard sets out the principles of physical methods for determining the water activity of foodstuffs. Microorganisms require water for their metabolic activities, but only a certain fraction, the so-called free water, of the total water present in any foodstuff is available for this purpose. The amount of free water depends on the nature and quantity of the particles dissolved in the aqueous phase of the product. This is termed “water activity” ( $a_w$ ). The various species of microorganisms only tolerate water activities within certain levels. Water activity can therefore be used to predict microbial growth and determine the microbiological stability of a food product, and it also provides an important, quantitatively determinable criterion for estimating the times for which foodstuffs can be kept.

*ISO 16140: Protocol of validation for alternative methods:* This standard defines the general principle and the technical protocol for the validation of alternative methods in the field of microbiological analysis of food, animal feeding stuff, and related areas for (a) the validation of alternative methods to be used for official control and (b) the international acceptance of the results obtained by the alternative method. It also gives the general principles of certification of these alternative methods, based on the validation protocol defined in this standard.

Where an alternative method is used on a routine basis for internal laboratory use without the requirement to meet (higher) external criteria of quality assurance, a less stringent comparative validation of the alternative method than that set in this standard may be appropriate.

## 2. *Methods of Analysis for Detection of Microorganisms, Standards, and Work Items Under the Responsibility of ISO/TC 34/SC 9*

Standards and work items specify methods for the detection of microorganisms and under the responsibility of SC 9 are listed in [Table 9](#).

*ISO 6579: Horizontal method for the detection of Salmonella (Revision of ISO 6579:1993):* This International Standard specifies a horizontal method for the detection of *Salmonella*, including *Salmonella* Typhi and *Salmonella* Paratyphi. The test sample is enriched in two selective liquid media: RVS broth and MKTTn broth. The plating out is made on two different media: XLD agar and any other solid selective medium. Appropriate biochemical and serological tests confirm the strain of *Salmonella* (TSI agar, urea agar).

*ISO 11290-1: Horizontal method for the detection of Listeria monocytogenes—Part 1: Detection method (Part 2 specifies an enumeration method, see [Table 10](#)):* Although systematic review of this standard is due only in 2001, Member Bodies has decided to start its revision as some European trials showed that the fidelity results could be improved. This revision will concern only the isolation media (actually Oxford and Palcam agar).

*ISO 16654: Horizontal method for the detection of Escherichia coli O157:* The principle of the detection of *E. coli* O157 is based on the separation and concentration of microorganisms by means of immunomagnetic beads coated with antibodies to *E. coli* O157. Immunomagnetic particles with adhering bacteria are then isolated by subculture onto Cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and the user’s choice of a second selective isolation agar. This is then confirmed by indole production and agglutination with *E. coli* O157.

## 3. *Methods of Analysis for the Enumeration of Microorganisms, Standards, and Work Items Under the Responsibility of ISO/TC 34/SC 9*

Standards and work items specify methods for the enumeration of microorganisms, which are under the responsibility of SC 9, listed in [Table 10](#).

*ISO 4833: General guidance for the enumeration of microorganisms—colony-count technique at 30°C:* This International Standard specifies a horizontal method for the enumeration of microor-

**TABLE 10** Methods of Analysis for Enumeration of Microorganisms, Standards and Work Items Under the Responsibility of ISO/TC 34/SC 9

Number	Year of publication	Title
ISO 4831	1991	General guidance for the enumeration of coliforms—MPN technique
ISO 4832	1991	General guidance for the enumeration of coliforms—Colony-count technique
ISO 4833 <sup>a</sup>	1991	General guidance for the enumeration of microorganisms—Colony-count technique at 30°C (Under revision)
ISO 6888-1	1999	Horizontal method for the enumeration of coagulase-positive staphylococci ( <i>Staphylococcus aureus</i> and other species)—Part 1: Technique using Baird-Parker agar medium
ISO 6888-2	1999	Horizontal method for the enumeration of coagulase-positive staphylococci ( <i>Staphylococcus aureus</i> and other species)—Part 2: Technique using rabbit plasma fibrinogen agar medium
ISO 6888-3 <sup>a</sup>		Horizontal method for the enumeration of coagulase-positive staphylococci ( <i>Staphylococcus aureus</i> and other species)—Part 3: MPN for low number (Under preparation)
ISO 7251 <sup>a</sup>	1993	General guidance for the enumeration of presumptive <i>Escherichia coli</i> —MPN technique (Under revision)
ISO 7402 <sup>a</sup>	1993	General guidance for the enumeration of Enterobacteriaceae without resuscitation—MPN technique and colony-count technique (Under revision)
ISO 7932 <sup>a</sup>	1993	General guidance for the enumeration of <i>Bacillus cereus</i> —Colony-count technique at 30°C (Under revision)
ISO 7937	1997	Horizontal method for the enumeration of <i>Clostridium perfringens</i> —Colony-count technique
ISO 11290-2 <sup>a</sup>	1998	Horizontal method for the detection and enumeration of <i>Listeria monocytogenes</i> —Part 2: Enumeration method (Under revision)
ISO 15213 <sup>a</sup>		Horizontal method for the enumeration of sulfite-reducing bacteria, growing under anaerobic conditions
ISO 15214	1998	Horizontal method for the enumeration of mesophilic lactic acid bacteria—Colony-count technique at 30°C
ISO 16649-1 <sup>a</sup>		Horizontal method for the enumeration of presumptive <i>Escherichia coli</i> —Part 1: Colony-count technique at 44°C using membranes and 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronic acid
ISO 16649-2 <sup>a</sup>		Horizontal method for the enumeration of presumptive <i>Escherichia coli</i> —Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronic acid
ISO 17410 <sup>a</sup>		Horizontal method for the enumeration of psychrotrophic microorganisms
ISO 18593 <sup>a</sup>		Horizontal method for the enumeration of aerobic bacteria from surfaces using contact plates and swab methods
ISO XXXX <sup>a</sup>		Horizontal method for the enumeration of low number of <i>Bacillus cereus</i> (in preparation)
ISO XXXX <sup>a</sup>		In preparation (trials)— <i>Shigella</i>

<sup>a</sup>Work program items for 2000.

ganisms by counting the colonies growing in a solid medium after aerobic incubation at 30°C. The revised version of the document includes plate count medium for the examination of dairy products as well. Among other modifications, the new version gives more detailed instructions for counting of the colonies.

*ISO 6888-3: Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species)—Part 3: MPN for low numbers:* This part of this International Standard specifies a horizontal method for the enumeration of coagulase-positive staphylococci in products intended for human consumption or feeding of animals obtained by most probable number technique. It is recommended for products where staphylococci are expected to be stressed and in low numbers, for example, dried products. Coagulase-positive staphylococci will primarily be *S. aureus*, but *S. intermedius* and some strains of *S. hyicus* also produce free coagulase. The medium used in this standard is Baird-Parker agar.

*ISO 7251: General guidance for the enumeration of presumptive Escherichia coli—MPN technique; ISO 7402: General guidance for the enumeration of Enterobacteriaceae without resuscitation—MPN technique and colony-count technique:* The revision of these two standards was decided during their systematic review in 1998, and the first working documents are under preparation.

*ISO 7932/A1: Horizontal method for the enumeration of presumptive Bacillus cereus—colony-count technique at 30°C:* The revision of this standard was made for the inclusion of precision parameters and limitations of confirmatory tests. The confirmation tests are replaced by the hemolysis test on blood agar, and as the VP test described gives discrepant results it was deleted from the first edition of the standard.

*ISO 11290-2: Horizontal method for the enumeration of Listeria monocytogenes—Part 2: Enumeration method:* This standard was published in 1998. However taking into account that some European trials have shown that the fidelity of the results could be improved, SC 9's members decided to revise the two parts of this standard. This revision will concern only the isolation media (actually Oxford and Palcam agar).

*ISO 15213: Horizontal method for the enumeration of sulfite-reducing bacteria, growing under anaerobic conditions:* This standard specifies a method of analysis by inoculation of iron sulfite medium and anaerobic incubation at 37°C ± 1°C for 24–28 hours.

*ISO 16649-1 and ISO 16649-2: Horizontal method for the enumeration of presumptive Escherichia coli—Part 1: Colony-count technique at 44°C using membranes and 5-bromo-4-chloro-3-indolyl-β-glucuronic acid—Part 2: Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl-β-glucuronic acid:* Part 1 of this standard describes a horizontal method for the enumeration of *E. coli* in products intended for human consumption or feeding of animals, by the colony-count technique at 44°C on a solid medium containing a chromogenic ingredient for detection of the enzyme β-glucuronidase. The principle is based on the resuscitation of the test sample onto cellulose membranes overlaid on minerals in modified glutamate agar. *E. coli* is isolated after incubation on TBX agar. The second part of this standard excludes the stage of resuscitation on membranes.

*ISO 17410: Horizontal method for the enumeration of psychrotrophic microorganisms:* This International Standard specifies a method for the enumeration of psychrotrophic microorganisms by means of the colony-count technique at 6.5°C. The inoculation is made on a solid nonselective culture medium, and the plates are incubated at 6.5°C for 10 days.

*ISO 18593: Horizontal method for the enumeration of aerobic bacteria from surfaces using contact plates and swab methods:* It is important to determine the number of viable microbes on the surfaces of utensils, working tables, and other equipment in contact with food to know the level of contamination during production, or the effectiveness of cleaning and disinfection of the surfaces. This horizontal method describes both a surface contact method using contact plates (e.g., RODAC plates) or dipslides and a swab (wipe) technique. With destructive techniques (e.g., scraping off surfaces), more accurate data about the presence of microorganisms will be obtained. However, the alternative methods are very useful to obtain an estimate of the microbial contamination of surfaces. Results are often presented as hygiene scores based on the number of colony-forming units per cm<sup>2</sup> present on a test surface.

**TABLE 11** Specific Standards and Work Items Under the Responsibility of ISO/TC 34/SC 9

Number	Year of publication	Title
ISO 6887-2 <sup>a</sup>		Preparation of test samples, initial suspension and decimal dilution for microbiological examination—Part 2: Specific rules for the preparation of the test samples and initial suspension of meat and meat products (New part of the standard under preparation)
SO 6887-3 <sup>a</sup>		Preparation of test samples, initial suspension and decimal dilution for microbiological examination—Part 3: Specific rules for the preparation of the test samples and initial suspension of milk and milk products (New part of the standard under preparation)
ISO 6887-4 <sup>a</sup>		Preparation of test samples, initial suspension and decimal dilution for microbiological examination—Part 4: Specific rules for the preparation of the test samples and initial suspension of fish products (New part of the standard under preparation)
ISO 6887-5 <sup>a</sup>		Preparation of test samples, initial suspension and decimal dilution for microbiological examination—Part 5: Specific rules for the preparation of the test samples and initial suspension of products other than milk and milk products, meat and meat products and fish products (New part of the standard under preparation)
ISO 7954	1987	General guidance for enumeration of yeasts and molds—Colony-count technique at 25°C
ISO 11289	1993	Heat-processed foods in hermetically sealed containers—Determination of pH
ISO 17604 <sup>a</sup>		Carcasses sampling for microbiological analysis

<sup>a</sup>Work program items for 2000.

#### 4. Specific Standards Under the Responsibility of ISO/TC 34/SC 9

Specific standards and work items under the responsibility of SC 9 are listed in Table 11. With regards to ISO 6887-2, ISO 6887-3, ISO 6887-4, and ISO 6887-5, the development of these standards as new parts of the ISO 6887-1 was justified previously.

*ISO 17604: Carcasses sampling for microbiological analysis:* This international standard specifies sampling methods for the determination of aerobic colony counts and pathogenic bacteria on the surface of freshly (less than 24 hours) slaughtered (red) meat animals. The bacteriological sampling can be carried out as part of (a) the process control (and to verify process control) in cattle, horse, swine, sheep, goat and game raised in captivity slaughter establishments; (b) the safety assurance system of the product; (c) surveillance programs for the prevalence of enteropathogenic microorganisms. This standard includes the use of destructive and nondestructive techniques depending on the purpose of the sample collection.

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# The Role of Codex Alimentarius in International Standards Setting

H. Michael Wehr

*U.S. Food and Drug Administration, Washington, D.C., U.S.A.*

## I. INTRODUCTION

The Codex Alimentarius (meaning food code) Commission, or Codex, as the organization is usually termed, is an international food standards organization whose importance has increased substantially since the signing of the GATT Uruguay Round Trade Agreements and the implementation of the World Trade Organization (WTO). With the formal recognition given to Codex in the WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), Codex has moved to the forefront of international science-based standards-setting bodies associated with food safety and quality. Its standards, guidelines, and recommendations have substantial impact on food products that are internationally traded. The efforts of Codex in the areas of sound science and risk analysis will substantially affect the manner in which international food standards setting is undertaken. Codex additionally has the potential to significantly affect domestic food regulation because of the linkage of Codex to international trade agreements and because of its impact on food regulatory bodies in individual member countries.

This chapter describes the history and organization of Codex, why it has become increasingly important, and how it develops and adopts food safety and quality standards. The policies Codex has established to undertake its scientific standards-setting activities are described, as are the proposed Codex principles for risk analysis and its component parts: risk assessment, risk management, and communication. The chapter also outlines the work of Codex in the field of food hygiene, focusing on the Codex Committee on Food Hygiene (CCFH). The types of food hygiene codes of practice and other food hygiene texts that are developed by Codex are reviewed, and the work of CCFH in the developing fields of microbiological risk assessment and microbiological risk management is discussed.

## II. WHAT IS CODEX?

The Codex Alimentarius Commission (CAC) is an international intergovernmental body that develops science-based food safety and commodity standards, guidelines, and recommendations to promote consumer protection and facilitate world trade (1,2). Codex is a subsidiary body of two United Nations organizations, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). The Codex Secretariat's offices are located within the FAO offices in Rome, Italy. The work of the Codex Secretariat is funded through the FAO and WHO, while much of the cost of operating the various Committees of Codex is paid for by those countries that serve as hosts for the meetings of the Committees (see below). Currently Codex has 165 member countries.

Codex was established in 1962 when FAO and WHO recognized the need for international



food standards to protect the health of consumers and to guide the world's growing food industry in producing quality food. Under the General Principles of the Codex Alimentarius (1), Codex is charged with developing food standards for adoption and use by member countries. The Codex Alimentarius itself is a collection (14 volumes) of international food standards adopted by the Codex Alimentarius Commission and presented in a uniform manner. The purpose of these standards is to protect the health of consumers and facilitate fair practices in food trade (1). Codex texts are in the form of specific food standards, codes of practice, guidelines and recommendations. The scope of the Codex Alimentarius includes standards for all principle foods, whether processed, semi-processed, or raw, for distribution to the consumer. Materials to be used for further processing into foods are included in a standard to the extent necessary to achieve the purpose of the standard. The Codex Alimentarius includes provisions for food hygiene, food additives, pesticide residues, contaminants, food irradiation, labeling, and methods of analysis and sampling. It also includes provisions of an advisory nature in the form of codes of practice, guidelines, and recommended measures. Since its inception, Codex has adopted more than 235 commodity standards, 3200 Maximum Residue Limits (MRLs) for pesticides, 50 MRLs for veterinary drugs, and 25 guidelines for contaminants (3). Codex has established standards for over 1000 food additives and has established over 40 hygienic and technological codes of practice. The work and activities of Codex are available on the Internet at [www.codexalimentarius.net](http://www.codexalimentarius.net).

### **III. WHY IS CODEX IMPORTANT?**

While Codex has been recognized within the international food scientific and regulatory communities since its inception, only limited adoption of Codex standards and texts by countries has occurred. This historical situation can be expected to change because of recent multilateral trade agreements.

In 1994, the GATT Uruguay Round of International Trade Negotiations established the WTO and several trade agreements including the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) and the Agreement on Technical Barriers to Trade (TBT Agreement) (4). Article 3.1 of the SPS Agreement requires countries to base their sanitary and phytosanitary measures on international standards, guidelines, and recommendations unless they can scientifically justify a more stringent standard. Section 3 (a) of Annex A of the SPS Agreement states that, for food safety, certain standards, guidelines, and recommendations established by the Codex Alimentarius Commission shall be recognized as international standards under the Agreement. These include those relating to food additives, veterinary drug and pesticide residues, contaminants, methods of analysis and sampling, and codes and guidelines of hygienic practice. Article 3.2 of the SPS Agreement states that a country's sanitary and phytosanitary measures which conform to international standards, guidelines, or recommendations shall be presumed to be consistent with the relevant provisions of the SPS Agreement and of General Agreements on Tariff and Trade established in 1994. Thus, while Codex standards are voluntary, countries have an obligation to base their own food safety standards on the work of Codex. Additionally, products produced in accordance with Codex standards essentially have safe harborage in international trade.

Codex is also important within the context of the TBT Agreement, which deals with product specifications not related to safety including commodity composition, packaging, and certain labeling standards. The TBT Agreement also requires countries to use international standards. While Codex is not specifically referenced in the TBT Agreement, the WTO has recognized Codex standards as those to be used preferentially in resolving technical trade disputes.

Codex thus becomes a true international focal point for food safety and quality with major

impact on international trade and, potentially, on domestic food regulations. Its importance is therefore significantly greater than it was previously.

#### IV. THE STRUCTURE OF CODEX

Codex undertakes its work through the Codex Alimentarius Commission and a series of horizontal general subject committees, vertical commodity committees, task forces, and regional coordinating committees. Horizontal committees are those committees whose work applies to multiple commodities, such as the Codex Committee on Food Hygiene. Vertical committees are those that deal with a single commodity type or single commodity grouping (e.g., fish and fishery products) and whose work encompasses all aspects associated with that commodity (e.g., composition, food additives, pesticide residues, food hygiene, product labeling, methods of analysis). An Executive Committee with international geographic representation acts for the Commission between its meetings; the Commission meets once every 2 years. The structure of Codex is shown in [Figure 1](#).

Nine worldwide general subject or horizontal committees establish Codex standards, codes of practice, or guidelines that apply across all commodities. Included in this category is the work of Codex Committees that involve pesticide residue MRLs, veterinary drug MRLs, and maximum permitted use levels for food additives. Also included in this category is the work of the Codex Committees on Food Labeling, Food Import and Export Inspection and Certification Systems, General Principles (the initial forum for Codex policy and procedural matters), and Nutrition and Foods for Special Dietary Uses. The work of the final general subject committee, the Codex Committee on Food Hygiene, is discussed later in the chapter. Readers are referred to the Codex Procedures Manual (1) for specific terms of reference for all Codex Committees.

A series of Codex commodity committees develops standards specific for various food products. These standards provide essential composition information for product types as well as information relating to residues and contaminants (pesticides and other compounds as appropriate), food additives, food hygiene, labeling, packaging, and methods of analysis. For these latter areas, the commodity standards reference general standards developed by the horizontal Committees. Among the most active of commodity committees are those involved with fish and fishery products, milk and milk products, nutrition and foods for special dietary uses (which, as noted above, is also considered a horizontal committee), fresh fruits, and vegetables, and processed fruits and vegetables.

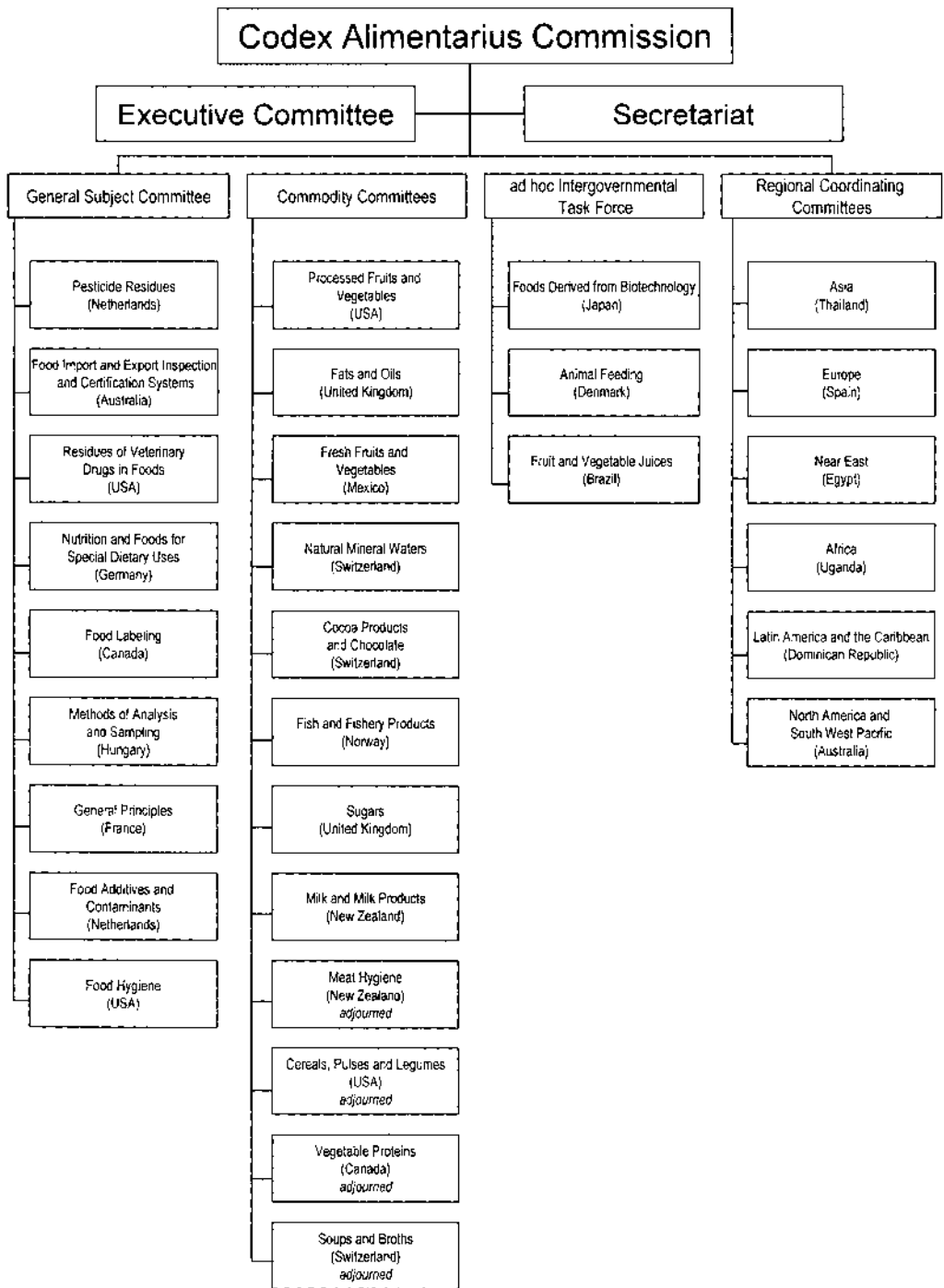
Each of these horizontal and vertical Committees is hosted by a country (see Fig. 1). The host country is responsible for arranging and securing the meeting site, pays for the meeting, and provides the Chairperson for the Committee.

Codex has recently established a new type of subsidiary body, the Ad-Hoc Intergovernmental Task Force. The intent is to use Task Forces when the work is considered to be essentially of a one-time nature or when the task is limited and occasional. Three Task Forces have been established, associated with the areas of Foods Derived from Biotechnology, Animal Feeding Practices, and Fruit and Vegetable Juices.

A series of Regional Coordinating Committees is also maintained by Codex. These committees at one time were primarily involved with the establishment of standards applicable only to the region. Today they are primarily involved with reviewing and analyzing the broader Codex issues as they may affect the region. Regional Coordinating Committees also serve as a venue for establishing regional positions on proposed international standards.

Codex also utilizes the work and/or input of two other types of organizations.

Joint WHO/FAO Expert Committees provide technical scientific risk assessment expertise for food additives, veterinary drugs, contaminants, and pesticide residues. The Joint Expert Committee on Food Additives (JECFA) provides this assistance for food additives, contaminants, and veterinary drugs, while the Joint Meeting on Pesticide Residues (JMPR) fills this role for pesticides. These committees are organizationally outside of Codex, but, in large measure, their work is determined by the agenda of the Codex Committees responsible for food additives, veterinary drugs, and pesti-



**FIGURE 1** The structure of Codex Alimentarius.

**TABLE 1** Step Procedure of the Codex Alimentarius for Development and Adoption of Standards and Other Texts

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Steps 1–4:	Codex Commission approves proposal for new standard or other text and assigns it to Committee. Secretariat arranges for draft of proposed standard or text. Proposed draft standard or text sent to countries for comments. Draft proposed standard or text and country comments initially reviewed by assigned Codex Committee.
Step 5:	Initial review of proposed draft standard or text by the Codex Alimentarius Commission.
Steps 6–7:	Draft proposed standard or text forwarded to member countries for second review. Second review, considering country comments, carried out by assigned Codex Committee.
Step 8:	Final review of proposed standard or text by Codex Alimentarius Commission. Standard or text adopted, modified and adopted, or rejected by Commission.
Fast Track:	Proposed standard or text adopted at Step 5 when no objection exists.

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cides. Risk assessments are evaluated by these expert committees according to established protocols, and their recommendations are utilized by the appropriate Codex committees to establish MRLs or maximum permitted use levels. A similar expert committee has been proposed for the field of microbiological risk assessment with the likelihood that the group will be established; currently Codex is utilizing a series of Meetings of Experts to assess various microbiological risk assessments that have been done by individual countries.

Also important to the work of Codex are international nongovernmental organizations (INGOs). These organizations have no vote in the adoption of standards (see below) but they can intervene in, and influence the discussion on, issues before the Commission and its subsidiary bodies. There are many INGOs; examples of such organizations include the World Trade Organization, the European Commission, Consumers International, the International Organization for Standardization (ISO), AOAC International, International Council of Grocery Manufacturer Associations (ICGMA), the International Commission on Microbiological Specifications for Foods (ICMSF), and the International Dairy Federation (IDF). Some of the INGOs (e.g., ICMSF, IDF, AOAC International) have served as technical advisors to appropriate Codex Committees (e.g., ICMSF to the Codex Committee on Food Hygiene, IDF to the Codex Committee on Milk and Milk Products). The role of INGOs is substantial in providing technical insight and important perspectives on the various issues under consideration by Codex.

## **V. THE STANDARDS DEVELOPMENT AND ADOPTION PROCEDURE OF CODEX**

Codex utilizes an eight-step procedure to elaborate and adopt standards and other texts (1). The process is a purposefully deliberative one that ensures that all member countries are given opportunity to consider the issues coming before any individual Codex Committee. The eight-step procedure is summarized in Table 1.

New Codex standards, guidelines, codes of practice, and other texts may be proposed by any member government or by individual Codex Committees. New work must be approved by the Commission or by the Executive Committee with subsequent confirmation by the Commission.

Steps 1 and 2 of the Codex procedure involve the initial development processes for the elaboration of Codex texts. Step 1 involves the initial approval by the Commission to proceed with new work. In some cases, especially in such areas as guidelines and codes of practice (including codes of hygienic practice), a discussion paper may be developed by the appropriate Codex Committee on a proposed issue to determine whether the subject warrants further consideration by Codex. Step 1 also involves an assignment by the Executive Committee or the Commission to the appropriate

Codex committee. Step 2 of the elaboration process involves the preparation of the draft proposed text, either by a member country or by the Secretariat. In the case of residues of pesticides or veterinary drugs, the Codex Secretariat distributes the recommendations for MRLs, when available, from JMPR or JECFA.

Step 3 of the Codex step procedure involves the initial submission of the document to member countries for review and comment. Often, formal written comments on proposed Codex standards and texts are prepared by countries for consideration by the assigned Codex Committee.

Step 4 is the first formal review of the proposed standard or text by the assigned Codex Committee. At this stage, standards and texts are normally reviewed in depth and revisions are made as appropriate.

Step 5 is the first review by the full Commission. This review is important since normally only 25–30% of all member countries attend any specific meeting of a horizontal or vertical Codex Committee. Total country participation at Commission meetings is substantially higher, hence this initial review by the Commission may be the first time that a country comments on a specific proposed Codex standard or text.

At Step 6, the now revised standard, including any comments made by the Commission, is sent to all member countries for a second review. Again, as with Step 3, formal written comments are often prepared by a country for review by the assigned Committee.

Step 7 is the second opportunity for the assigned Codex Committee to review the revised proposed Codex standard or text. The Committee has at hand the written country comments at Step 6.

Step 8 is the second and final review by the Codex Commission. The Commission has the option of adopting the standard at this point, amending and adopting the standard, sending the standard back to Committee for further work, or rejecting the proposal. Normally, this review results in the adoption of the standard.

At any time during the process, a Codex Committee or the Commission can retain a document at any specific Step, or can return a document to a previous step. Both of these instances frequently occur, particularly for proposed standards or texts that have difficult technical issues or in situations where consensus on a document cannot be reached.

The desire in Codex is for decisions to be reached by consensus, although provisions exist within the formal Codex operating procedures for voting on proposed standards and texts. Voting on decisions relating to the adoption of Codex texts has occurred very infrequently, and is usually associated with highly controversial issues (e.g., the elaboration of MRLs for growth-promoting animal hormones).

An accelerated or “fast track” procedure is provided for in which Steps 6 and 7 are omitted. Thus, Steps 5 and 8 are combined and the proposed Codex standard or text may be adopted at the first consideration by the Commission. A two-thirds majority vote is required to “fast track” a Codex standard or text. Normally, this process is used for noncontroversial items such as pesticide MRLs.

It is important to note that the Commission meets only once every 2 years. Codex Committees meet, on the average, once every 12–18 months. Hence, it takes some period of time to elaborate and adopt a Codex standard. As previously noted, such deliberations are important to ensure the development of appropriate and correct standards or texts, particularly in light of the impact of Codex under the SPS and TBT Agreements and the ramifications that these interrelationships have on international trade.

## **VI. NATIONAL DELEGATIONS AND DECISION MAKING IN CODEX**

Member countries of Codex maintain a Codex Contact Point; these points are listed on the Codex Internet Website. They are the focal point for Codex operations within a country. Functions of national Codex contact points vary from country to country, but activities include the management of national delegations to Codex Committees, interaction with stakeholders, coordination and/or

development of country positions on Codex documents, and receipt and distribution of Codex texts. Some Codex contact points maintain a library of Codex documents.

Representation at meetings of Codex Committees and the Commission is made through national delegations from each country. Since Codex is an intergovernmental body, the country Delegate (and Alternate Delegate, if one is appointed) to any specific Codex Committee and to meetings of the Commission must be a governmental official(s). Delegations may also contain both governmental and nongovernmental advisors. Advisors are normally selected on the basis of the expertise and the assistance they can provide to the Delegate on the technical and/or policy issues before the Codex Committee or Commission. Selection of nongovernmental advisors may also be based on the constituencies they represent. Countries limit the size of delegations and normally provide balance with respect to issues/needed expertise and constituency representations (e.g., industry, consumers).

In preparation for specific Codex meetings, countries prepare positions on issues (proposed standards and other texts) coming before the Committees. These positions normally form the basis for a country's intervention (that is, verbally presenting a position or point of view) on the issue at the meeting of the Committee. Countries often present draft positions at public meetings to ensure transparency and the broadest representation possible in determining final country positions. Countries may also share their national positions with other countries to develop support for their position on an issue. Countries may involve interested parties through public meetings and other mechanisms in the development, review, and comment activities carried out for proposed Codex standards and texts.

Codex meetings normally proceed through a set agenda, discussing all issues assigned to the Committee on which it is working. Countries intervene in the discussion in an orderly fashion, providing their position. Consensus among the Delegates is normally reached after several rounds of intervention and debate. As noted previously, Codex operates by intent and practice on a consensus basis, although procedures for voting are maintained. Once discussion or debate is concluded on an issue, consensus on the standard or text is reached within the context of the Step procedure. In the early stages of consideration, countries and the Committee may agree to a preliminary consensus position, recognizing that the issue will be revised at a future meeting where the Committee's final consensus decision may change. Ultimately, the Committee either reaches a final consensus on the issue, a vote is taken, or (very rarely) the Committee agrees to cease work on the item. Countries wishing to record their opposition to a decision of a Committee or the Commission may do so. Should voting on an issue occur, each country is allotted one vote.

## **VII. CODEX AND SCIENCE**

From its beginning, the work of Codex has been science-based. Experts and specialists in a wide range of scientific disciplines have contributed to every aspect of Codex activities to ensure that Codex standards withstand scientific scrutiny. It is fair to say that the work of the CAC, and that of FAO and WHO in their supportive roles, has provided a focal point for food-related scientific research and investigation, and the Commission itself has become a most important medium for the exchange of information about food.

The SPS Agreement provides the basis and driving force for the current policy on international food standards.

The SPS agreement, which went into effect in 1995, includes provisions that require the use of science, including risk assessment, in standards setting. The specific provisions are:

Article 2.2. Members shall ensure that any sanitary and phytosanitary measure is based on scientific principles.

Article 5.1. Members shall ensure that their sanitary and phytosanitary measures are based on an assessment, as appropriate to the circumstances, of the risks to human, animal or plant life or health.

**TABLE 2** Codex Alimentarius Statements of Principle Concerning the Role of Science in the Codex Decision-Making Process and the Extent to Which Other Factors Are Taken into Account

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1. The food standards, guidelines, and other recommendations of Codex Alimentarius shall be based on the principle of sound scientific analysis and evidence, involving a thorough review of all relevant information, in order that the standards assure the quality and safety of the food supply.
  2. When elaborating and deciding upon food standards, Codex Alimentarius will have regard, where appropriate, to other legitimate factors relevant for the health protection of consumers and for the promotion of fair practices in food trade.
  3. In this regard it is noted that food labeling plays an important role in furthering both of these objectives.
  4. When the situation arises that members of Codex agree on the necessary level of protection of public health but hold differing views about other considerations, members may abstain from acceptance of the relevant standard without necessarily preventing the decision by Codex.
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Codex, recognizing its role under the SPS Agreement as the reference international organization for food safety standards, initiated a process to strengthen its policies and operational practices with respect to the use of science in standards setting.

In 1995 the CAC established a set of four *Statements of Principle Concerning the Role of Science in the Codex Decision-Making Process and the Extent to Which Other Factors Are Taken into Account* (1) (see Table 2), or Sound Science Principles, as they are commonly referred to by those involved with Codex. These four principles established the fundamental policy by which Codex will undertake its standards-setting work. The first principle clearly articulates that food standards, guidelines, and other recommendations of the Codex Alimentarius shall be based on the principle of sound scientific analysis and evidence. The second principle, relating to what are termed “other legitimate factors,” is more complex and states that Codex, in its standards-setting activities, will have regard, where appropriate, to other legitimate factors relevant for the health protection of consumers and for the promotion of fair practices in food trade. The determination of what comprises “other legitimate factors” (OLFs) is currently being undertaken by the CAC. There are differences of opinion among countries as to what constitutes an OLF. Countries generally accept that OLFs relating to the risk-assessment process (e.g., good veterinary practices, good agricultural practices) are appropriate. Acceptance may be somewhat more difficult with factors such as economic feasibility, environmental impact, impact on health status, and consumer perception of risk. Acceptance will be most difficult with, and will likely be inappropriate for, such factors as animal welfare, consumer right to know, cost increases (or decreases) associated with technology, and relative risk (that is, the risk(s) associated with a product compared to other risks to which a consumer is exposed) that some countries believe ought to be OLFs. Provisions of the SPS Agreement provide guidance as to factors countries can legitimately take into account when establishing sanitary and phytosanitary measures (e.g., economic factors including the relative cost-effectiveness of alternative approaches to limiting risks, relevant processes and production methods, and relevant environmental conditions). These SPS provisions are likely to assist in clarifying the determination of acceptable OLFs for Codex standards setting. WTO jurisprudence in trade dispute settlement is ultimately the probable method by which the most controversial and difficult OLFs will be determined.

At the request of Codex, FAO and WHO have undertaken a series of Joint Expert Consultations (5–7) to elucidate the basic principles of Risk Analysis. Working from the findings of three Joint Expert Consultations dealing with the three components of risk analysis (risk assessment, risk management, risk communication) and from the discussions of several key Codex horizontal committees (Food Hygiene, Pesticide Residues, Residues of Veterinary Drugs in Foods, and Food Additives and Contaminants), Codex is in the process of delineating a comprehensive series of *Working Principles of Risk Analysis* (8) that will lay a further scientific foundation for the elaboration of Codex standards, guidelines, and recommendations.

This Codex effort in establishing Sound Science Principles and *Working Principles of Risk Analysis* should establish a comprehensive scientific framework for the work Codex undertakes in elaborating standards. This effort is also providing a framework for countries to use in their own individual food safety standards setting. As such, the work of Codex extends far beyond the organization itself. This comprehensive sound science/risk analysis approach to standards setting, which is compatible with the provisions of the SPS Agreement, is beginning to be seen in new draft Codex standards. A good example is the proposed draft *Code of Hygienic Practice for Milk and Milk Products* discussed below.

## VIII. CODEX AND FOOD HYGIENE

The Codex Committee on Food Hygiene (CCFH) is primarily responsible for food hygiene matters within Codex. Food hygiene generally encompasses hygiene as it relates to good production, manufacturing, distribution and marketing practices, the Hazard Analysis and Critical Control Point (HACCP) system, microbiological criteria for foods, and microbiological risk assessment and risk management. The terms of reference for CCFH (1) are essentially threefold. The first involves drafting basic provisions on food hygiene applicable to all food. In practice this takes the form of the development (and revision when needed) of Codex Codes of Hygienic Practice for various food commodities. A detailed discussion of these Codes of Hygienic Practice is presented below. The second responsibility is to consider/review food hygiene provisions of Codex commodity standards or other texts. A detailed discussion of this responsibility is also presented below. A third generic responsibility of the CCFH is to consider specific hygiene problems assigned to it by the Commission. Codex committees generally serve as a resource to the Commission in their area of expertise (e.g., Codex Committee on Food Labeling for labeling issues, Codex Committee on Food Import and Export Inspection and Certification Systems for import/export control issues). CCFH plays this role in the field of food hygiene.

### A. Codes of Hygienic Practice

The Codex Codes of Hygienic Practice provide guidance on the hygienic production and processing of foods. The base reference document in this area is the *Recommended International Code of Practice: General Principles of Food Hygiene* (9). In addition to introductory sections on objectives and scope, this general food hygiene code of practice contains detailed recommended food hygiene practices for the following areas:

- Primary production
- Establishment: design and facilities
- Control of operation
- Establishment: maintenance and sanitation
- Establishment: personal hygiene
- Transportation
- Product information and consumer awareness
- Training

This document also contains a HACCP annex, which presents the recommendations of Codex for the application of HACCP to food production. The Codex approach to HACCP utilizes the seven internationally recognized principles of HACCP. The annex presents a detailed discussion on the application of each principle including the establishment of critical control points (CCP) and critical limits for each CCP. Also presented is a flow diagram for the application of HACCP (Logic Sequence for Application of HACCP), an example of a decision tree to identify CCPs and an example of a HACCP worksheet.



The CCFH also undertakes the development of commodity-specific codes of hygienic practice. These codes reference the *Recommended International Code of Practice: General Principles of Food Hygiene* and are constructed so that only hygiene provisions supplemental to those present in the General Principles code and specific for the commodity type covered in the code are given. Examples of codes of hygienic practice that have been or are being developed by Codex include those for the following product areas:

- Canned Fruit and Vegetable Products
- Processed Meat Products
- Milk and Milk Products
- Fresh Fruits and Vegetables
- Packaged/Bottled Water Other Than Natural Mineral Waters
- Refrigerated Packaged Foods with Extended Shelf Life
- Transport of Foodstuffs in Bulk and Semi-Packaged Foods

The current development of a proposed draft *Code of Hygienic Practice for Milk and Milk Products* (10) deserves special mention because the construction of this Code is particularly reflective of the new responsibilities of Codex under the SPS Agreement. The development of this Code arose from prior work of the CCFH dealing with soft cheeses and the difficulty experienced by the Committee in dealing with complex microbiological risk issues associated with the production of soft unripened uncured cheeses made from raw milk. Countries differed as to the microbiological risk associated with this product type. The result to date has been the development of a draft proposed Code that permits countries to manufacture products in multiple ways (in which the microbiological risks may be different). Countries have the right to accept or reject product based on their scientifically established Appropriate Level of Protection (ALOP). This Code, for the first time, employs SPS terminology and concepts relating the construct of a specific Codex document to the provisions of the SPS Agreement and reflects the new and enhanced role of Codex under the SPS Agreement.

## **B. Hygiene Provisions of Commodity Standards**

Codex develops recommended standards for a multitude of specific food commodities. Provisions of these standards include sections on essential composition (a basic standard of identity), packaging, and labeling. These standards also contain sections on maximum permitted residue levels for pesticides, veterinary drugs, contaminants, and maximum permitted use levels for food additives; each of these sections refers to general broad Codex standards for pesticide residue MRLs, veterinary drug MRLs, and food additive permitted use levels.

These commodity standards also contain a general section on food hygiene. The current recommended wording for the hygiene section of all commodity standards is (1):

It is recommended that the products covered by the provisions of this standard be prepared and handled in accordance with the appropriate sections of the *Recommended International Code of Practice—General Principles of Food Hygiene* (CAC/RCP 1-1969, Rev. 3-1997) and other relevant Codex texts such as Codes of Hygienic Practice and Codes of Practice.

The products should comply with any microbiological criteria established in accordance with the *Principles for the Establishment and Application of Microbiological Criteria for Foods* (CAC/GL 21-1997).

As the above-mentioned Codex commodity committees develop individual commodity standards, the CCFH has the responsibility to review the food hygiene section to ensure that the Section's provisions are adequate for the product. This is normally a routine activity, and the recommended wording noted above is approved. CCFH has the capability to add specific additional

provisions to the food hygiene section of commodity standards should the committee deem them necessary to ensure the safe production and/or processing of the product(s) covered by the specific commodity standard. The review of eight generic dairy product standards, including that for cheese, is a case in point (11). Because of concern about potential microbiological risk issues associated with some product types (e.g., soft unripened uncured cheeses made from raw milk), the CCFH approved the following additional provision for the hygiene section of the eight dairy product standards.

From raw material production to the point of consumption, the products covered by this standard should be subject to a combination of control measures, which may include, for example, pasteurization, and these should be shown to achieve the appropriate level of public health protection.

### **C. Other Food Hygiene Activities**

In the context of its broad food hygiene responsibilities, the work of CCFH extends beyond the confines of specific Codex codes of hygienic practice and the hygiene provisions of specific Codex commodity standards. The work of CCFH is wide-ranging and significant, providing guidance to both Codex and countries, and affects the field of food microbiology generally. Work of CCFH in this broader context includes efforts in microbiological risk assessment and microbiological risk management (see below), the establishment of guidance for the use of microbiological criteria in foods (see below), the consideration of the application of HACCP to small business, the broader application of HACCP generally (including such areas as the application of HACCP to various categories of foods and third-party certification), and the development of the concept of Food Safety Objectives (FSOs).

### **D. Microbiological Criteria**

Recognizing (a) the current and historical interest in the area of microbiological criteria for foods, (b) the changing nature of food regulatory systems to incorporate HACCP, (c) the potential for misuse of specific microbiological criteria, and (d) the developing science of risk analysis as applied to the field of food microbiology, the CCFH developed the document *Principles for the Establishment and Application of Microbiological Criteria for Foods* (12) to provide guidance to both Codex and its member countries on the establishment and application of microbiological criteria for foods at any point in the food chain from primary production to final consumption. The document defines a microbiological criterion to be an entity that defines the acceptability of a product or a food lot, based on the absence, presence, or number of, microorganisms (including parasites), and/or the quantity of their toxins/metabolites, per unit(s) of mass, volume, area, or lot. The components of a criterion are specified to be:

A statement of the microorganism of concern and/or their toxins/metabolites and the reason for that concern

The food to which the criterion applies

The point(s) in the food chain where the criterion applies

The analytical methods for detection and/or quantification

A plan defining the number of field samples to be taken and the size of the analytical unit

The number of analytical units that should confirm to these limits

The action to be taken when the criterion is not met

The guidance in the *Principles for the Establishment and Application of Microbiological Criteria for Foods* describes the purposes for which microbiological criteria can be applied, specifically:

- To formulate design requirements
- To indicate the required microbiological status of raw materials, ingredients, and end-products at any stage of the food chain as appropriate
- To examine foods (including raw materials and ingredients) of unknown origin or when other means of verifying the efficacy of HACCP-based systems and good manufacturing practices are not available
- To determine that processes are consistent with the *Codex General Principles of Food Hygiene*

The guidance notes that microbiological criteria can be applied by regulatory authorities to define and check compliance with microbiological requirements. Food business operators may use the microbiological criteria to ensure compliance with regulatory provisions, to formulate design requirements, and to examine end-products as one of the measures to verify and/or validate the efficacy of the HACCP plan.

The document outlines general considerations for establishing and applying microbiological criteria; e.g., evidence of an actual or potential public health hazard, the microbiological status of the raw material, the effect of processing, the category of consumers utilizing the product, the cost/benefit of implementing a criterion, and the intended use of the food. Also discussed is the rationale for establishing microbiological limits, sampling plans, and analytical methods.

Within CCFH, consideration has been given to the development of recommendations for specific microbiological criteria for foods, particularly for certain foodborne pathogens. While this activity is related to the development of the field of microbiological risk assessment (see below) and will take some period of time to accomplish, it is likely that CCFH will provide such recommendations in the future. It is likely, for example, that a recommendation for a microbiological criterion for *Listeria monocytogenes* in selected sensitive food products may be developed by CCFH.

## **IX. MICROBIOLOGICAL RISK ASSESSMENT AND RISK MANAGEMENT**

Codex, through the work of the CCFH, has developed a fundamental guidance document on microbiological risk assessment and is in the process of developing a companion document on microbiological risk management.

### **A. Microbiological Risk Assessment Guidance Document**

Codex has established the first international intergovernmental guidance document for microbiological risk assessment, *Principles and Guidelines for the Conduct of Microbiological Risk Assessment* (13). The scope of this document generally applies to risk assessment of microbiological hazards in food and is designed to ensure that sound science principles and practices are applied uniformly in evaluating microbiological hazards in foods. The document recognizes that microbiological risk assessment is a developing science, and that while the process should include quantitative information to the greatest extent possible, qualitative judgments will have to be made.

Eleven general principles applicable to the conduct of microbiological risk assessment (Table 3) are presented in the document. These principles state that microbiological risk assessment should be conducted according to the structured four-step approach that is common to risk assessment generally (hazard identification, hazard characterization, exposure assessment, and risk characterization). The principles also incorporate provisions relating to performing a microbiological risk assessment, including the need for:

- Functional separation between risk assessment and risk management
- A requirement for transparency in what is being done
- Identification of the limitation of data and resource constraints
- Consideration of the dynamics of microbial growth

**TABLE 3** Codex Alimentarius General Principles of Microbiological Risk Assessment

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1. Microbiological Risk Assessment should be soundly based on science.
  2. There should be a functional separation between Risk Assessment and Risk Management.
  3. Microbiological Risk Assessment should be conducted according to a structured approach that includes Hazard Identification, Hazard Characterization, Exposure Assessment, Risk Characterization.
  4. A Microbiological Risk Assessment should clearly state the purpose of the exercise, including the form of Risk Estimate that will be the output.
  5. The conduct of a Microbiological Risk Assessment should be transparent.
  6. Any constraints that impact the Risk Assessment such as costs, resources, or time, should be identified and their possible consequences described.
  7. The Risk Estimate should contain a description of uncertainty and where the uncertainty arose during the Risk Assessment process.
  8. Data should be such that uncertainty in the Risk Estimate can be determined; data collection systems should, as far as possible, be of sufficient quality and precision that uncertainty in the Risk Estimate is minimized.
  9. A Microbiological Risk Assessment should explicitly consider the dynamics of microbiological growth, survival, and death in foods and the complexity of the interaction (including sequelae) between human and agent following consumption as well as the potential for further spread.
  10. Wherever possible, Risk Estimates should be reassessed over time by comparison with independent human illness data.
  11. A Microbiological Risk Assessment may need reevaluation as new relevant information becomes available.
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Specifying the uncertainties associated with each step of the risk assessment process

Reassessment as new data (e.g., new quantitative information on the occurrence of microorganisms in food, new dietary intake information) are received

Guidance is provided on the steps required to carry out a microbiological risk assessment. Each of the four structured steps of risk assessment is discussed in detail. Helpful information specific to the application of microbiological risk assessment is given. For example, influences that may affect the hazard characterization are detailed (e.g., replication rates of microorganisms, virulence and infectivity, delay of onset of clinical symptoms, effect of food attributes such as fat content). Similar information is described for the exposure assessment step (e.g., characteristics of the pathogenic agent, the microbial ecology of the food, the methods of packaging, processing, distribution, and storage).

The need for proper documentation and reassessment as new data develop (an important consideration in the comparatively new field of microbiological risk assessment) is stressed in the guidance document.

## **B. Proposed Joint Expert Committee on Microbiological Risk Assessment**

As noted above, FAO and WHO maintain two primary expert groups, the Joint Expert Committee for Food Additives and Contaminants and the Joint Meeting on Pesticide Residues, that provide scientific technical risk assessment assistance and guidance to several Codex Committees. Using recognized chemical risk assessment procedures, these expert committees review the technical acceptability of risk assessments carried out for pesticides, veterinary drugs, other contaminants, and food additives. Additionally, they may provide guidance on the establishment of MRLs for pesticide and veterinary drug residues, maximum permitted levels for certain contaminants, and maximum permitted use levels for food additives.

A similar joint expert committee has been proposed by the CCFH for the field of microbiological risk assessment and has the support of the CAC. Recognizing the developing nature of microbiological risk assessment, the precise responsibilities of the proposed expert committee are under consideration and very likely will expand and change as the field develops and full quantitative microbiological risk assessments become more common.

## C. Microbiological Risk Management

The CCFH is developing a new document on *Principles and Guidelines for the Conduct of Microbiological Risk Management* (14). This document, when adopted by the CAC, will provide the first international guidance to both Codex committees and to countries on how to undertake and implement microbiological risk management activities.

The document builds on a series of risk management principles developed by the FAO/WHO Joint Expert Consultation on Risk Management and Food Safety (6) and provides a framework for undertaking microbiological risk management. In this document, microbiological risk evaluation is outlined as involving several steps, including the following:

- Identification of a microbiological food safety problem
- Establishment of risk profile
- Identification of the microbiological risk management goals and risk managers
- Ranking of hazards for microbiological risk management
- Establishment of microbiological risk assessment policy for the conduct of risk assessment
- Commissioning of the microbiological risk assessment
- Consideration of the results of the microbiological risk assessment

The document separately discusses each of these steps in microbiological risk evaluation.

The document reviews the area of microbiological option assessment, including the identification of available microbiological risk management options, the selection of the preferred option, and the final management decision. Sections are also presented on implementation, monitoring, and review.

An important aspect of this paper is the incorporation of a new entity in risk analysis that can be used in risk management programs—the concept of Food Safety Objectives (FSOs). FSOs have been developed to describe the link between a sanitary measure(s) and the appropriate level of protection that a country chooses for its consumers for a specific foodborne risk. FSOs are likely to become an important operational element in future food safety programs.

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## The Office International des Epizooties Part I

**Alfonso Torres**

*Cornell University, Ithaca, New York, U.S.A.*

**Anne Goodman**

*U.S. Department of Agriculture, Riverdale, Maryland, U.S.A.*

### **I. DESCRIPTION OF THE WORLD ORGANIZATION FOR ANIMAL HEALTH**

The World Organization for Animal Health, or the Office International des Epizooties (OIE), is the international standard-setting organization that deals with animal health issues, including zoonoses. Diseases of concern are primarily transmissible diseases. The organization headquarters are in Paris. It was formed in 1924 in response to a worldwide spread of the cattle disease, rinderpest.

The OIE is an intergovernmental organization. Each Member Country has one delegate and one vote on an issue. The Delegate is usually the Chief Veterinary Officer (CVO) of the Member Country. As of July 2002, the Organization had 162 Member Countries.

### **II. PURPOSE OF THE ORGANIZATION**

The OIE's primary function is to inform governmental veterinary services of the occurrence and course of epizootics that could endanger animal or human health. The OIE issues animal disease warnings through its on-line international disease reporting system on the Internet (<http://www.oie.int/>). This system is updated weekly, and summary tables are presented for diseases of particular interest. For example, the web site contains a summary table reporting the occurrence of bovine spongiform encephalopathy worldwide. Other information provided on-line includes recommended surveillance and control mechanisms for animal diseases, a list of international reference reagents, press releases of international interest, a description of the Organization, and hard-copy publications issued by the OIE.

The OIE produces a variety of hard-copy publications on the worldwide status of animal health. A list of these publications and a description of OIE activities are provided on its web site. The guidance provided in these publications is intended to minimize dissemination of significant animal diseases from affected to unaffected regions of the world through international trade in animals and animal products. The publications describe science-based standards, guidelines, and recommendations for international trade in animals and animal products. The OIE also advises governments of ways to survey and control significant animal diseases, coordinates studies devoted to the surveillance and control of animal diseases, and works to harmonize regulations for trade in animals and animal products among Member Countries.

The OIE standards provide the scientific basis for implementation of provisions of the World Trade Organization's (WTO) Sanitary and Phytosanitary (SPS) Agreement (1) as it relates to animal health. Although recognizing that trade in animals and animal products can result in transmission of animal diseases to unaffected regions of the globe, the signatories of the WTO's SPS Agreement also recognized that global trade in animals and animal products could be affected by trade barriers disguised as health issues. To address this, they adopted the scientific provisions in the SPS Agreement.

The SPS Agreement requires that import restrictions of countries be scientifically based, transparent, adequately documented, and in harmony with international standards. Provisions of the SPS Agreement carry the force of international law. The WTO has designated the OIE as the single international organization for setting animal health standards, including zoonoses, and reporting global animal health situations and status. In addition, the SPS Agreement requires that nations strive for international harmonization of diagnostic tests, surveillance systems, import requirements, quarantine procedures, animal identification policies, vaccine standards, and risk assessment and management systems. To facilitate attainment of this goal, the OIE provides international standards, guidelines and recommendations for sanitary regulations, testing, quarantine, and health certification.

### **III. IMPORTANCE OF THE ORGANIZATION**

Although the OIE has no direct authority over human foodborne diseases at this time, its international guidelines contain standards for movement of animals and animal products that carry zoonotic diseases of significance as human foodborne pathogens. Diseases of interest that may be transmitted to humans in food include bovine spongiform encephalopathy (BSE) implicated as the likely cause of the new variant of Creutzfeldt-Jakob Disease (CJD), salmonellosis, trichinellosis, cysticercosis, leptospirosis, etc.

The OIE's publications include international standards for veterinary infrastructures to conduct surveillance, identification, and control of these diseases. Its on-line disease-reporting service provides timely information on the global spread of animal diseases that cause foodborne diseases in humans. The OIE also facilitates international harmonization of approaches to emerging issues and develops international standards for scientific risk assessments to support decision making.

The OIE's role is evolving. The Organization continually updates its goals and missions to accommodate changing world priorities and needs. For example, the 2000 5-year draft strategic plan addresses increasing interest in trade, exotic diseases, zoonoses and food safety (including resistance of bacteria to antimicrobials), and emphasis on scientific rather than political approaches. The fact that zoonoses and food safety are included on the list suggests that the OIE may play an increasingly active role in human food safety in the future.

### **IV. RISK ANALYSIS RELATED TO MICROBIOLOGY**

The OIE develops guidelines and recommendations in the following manner using the technical expertise of internationally renowned scientists.

#### **A. Annual General Session (Assembly)**

The OIE has a small permanent staff. It generates its recommendations and guidelines through the activities of the specialized Commissions and Working Groups. These bodies are composed primarily of technical specialists who identify and draft recommendations to address emerging or developing animal health and disease issues. They are expected to represent the interests of the international community by applying the best possible science and technology to questions at hand in an unbiased fashion and not to represent national interests of individual countries.

The Commissions and Working Groups identify general initiatives and submit these for prioritization.



zation at the annual meeting, designated the General Session of the International Committee. This meeting is held annually in Paris each May. In its sessions, the International Committee reviews the recommendations from the Commissions and Working Groups. Decisions are reached by majority vote of Member Countries. Although a number of observers may attend the sessions, a single country vote is placed by the Delegate, usually the Member Country's Chief Veterinary Officer (CVO).

OIE standards are adopted by consensus. If a consensus cannot be reached, standards are adopted by a simple majority. In contrast to the provisions of the SPS agreement, the OIE standards are only guidelines. It is the sovereign prerogative of individual countries to apply import measures more stringent than OIE standards if considered necessary to protect the health of livestock, poultry, wildlife or human populations, as long as the justification is transparent, science-based, nondiscriminatory, and based on scientific risk analysis.

After adoption by the General Session, the standards are published. Included in the OIE's publications of international standards are the OIE *International Animal Health Code* (2), the OIE *Manual of Standards for Diagnostic Tests and Vaccines* (3) and the *International Aquatic Animal Health Code and Diagnostic Manual for Aquatic Animal Diseases* (4). Several of the OIE's publications, including these three, are available on the OIE web site.

Even though OIE standards do not have the status of international law, import measures based on the standards defined in the Code are exempt from requirements to report changing regulations through the WTO notification process and to conduct risk analyses to support the decision. Measures consistent with OIE standards cannot be challenged within the WTO-SPS dispute resolution process.

## **B. Commissions, Regional Commissions, and Working Groups**

Commissions, Regional Commissions, and Working Groups are composed of subject specialists who address technical issues and perform a variety of specific functions. The Commissions (a) propose draft chapters for adoption at the annual General Session, (b) develop scientific reports, (c) develop a disease-specific international manual for diagnostic tests and vaccines, (d) recommend general guidelines for animal disease control, such as monitoring and surveillance procedures, quarantine methods, or regionalization and risk assessment techniques, (e) propose disease definitions, disease categorizations, and disease reporting requirements, and (f) provide scientific and technical advice upon request from the WTO trade dispute panels.

Several commissions perform functions relevant to foodborne illness in humans. One such commission is the International Animal Health Code Commission. This group develops the disease-specific guidelines for international movement of animals and animal products. This group also develops general guidelines for regionalization and risk assessment and animal disease surveillance and monitoring activities. Once adopted, these standards generated by this Commission are published in *The OIE International Animal Health Code* (2), a new edition of which is published each year in September.

Other Commissions or Working Groups with activities relevant to human food safety include the Standards Commission, which publishes *The OIE Manual for Standards for Diagnostic Tests and Vaccines* (3); the Fish Diseases Commission, which publishes the *OIE International Aquatic Animal Health Code and Diagnostic Manual for Aquatic Animal Diseases* (4) and deals with standards regarding aquatic animal health; and the Working Group on veterinary drugs.

Commissions and Working Groups meet regularly throughout the year. Substantive discussions are held with experts before documents are prepared for distribution. International standards developed by these Groups are circulated to Member Countries for comment before adoption at the annual General Session.

Standards for diagnostic tests and vaccines are published in *The OIE Manual of Standards for Diagnostic Tests and Vaccines* (3). The OIE also designates Collaborating Centers and certain laboratories as Reference Laboratories. The laboratories are intended to provide OIE Member Countries with harmonized and technical assistance on diagnostic procedures. For example, the OIE

endorses Regional Reference Laboratories that maintain and standardize diagnostic reagents and tests used in animal disease control programs. Collaborating centers have technical groups with expertise to perform risk assessments on animal health issues.

### **C. Risk Assessment and Regionalization**

The WTO SPS Agreement states that countries may impose import measures that are more stringent than OIE standards only if these measures prove necessary to protect livestock, poultry, wildlife, and human populations from disease. The SPS Agreement requires that the justification be science-based, transparent, and have a methodology that is adequately documented in a risk assessment that estimates the severity and consequences of disease introduction. The OIE Code contains a proposed model for performing import risk assessments. Included in the risk assessment model is an evaluation of the veterinary services of the exporting country. To facilitate this evaluation, the OIE provides guidelines for evaluating those services.

In addition, the Code provides guidelines for the assessment of risk according to regions or zones rather than limiting the assessment to entire countries. This practice, referred to as regionalization, permits effective localization, containment, and exclusion of existing or newly emerging (or newly introduced) disease, without unjustifiably restricting trade from adjacent zones.

## **V. ACTIVITIES RELATED TO MICROBIOLOGY**

By functioning to prevent the spread of contagious animal diseases and improve animal production through the promulgation of health standards, the OIE plays a role in the maintenance of animal health at an international level.

The OIE activities identified in this discussion, although not focused directly on microbiology as it relates to human health issues, are relevant to risk of bacterial and parasitic foodborne human diseases from various perspectives. First, they provide information on certain diseases of interest as foodborne human pathogens, as well as animal diseases that have been identified as having potential applications as bioterrorist agents or human infections spread by routes other than foodborne (e.g., anthrax, tuberculosis, Rift Valley fever). In addition, the OIE guidelines provide the basic framework for identification, evaluation of risk, and control of such diseases at both the national and international levels. Furthermore, the OIE supports Reference Laboratories, which provide Member Countries of the OIE with scientific and technical assistance. These Laboratories also provide expert advice to Member Countries on topics such as microbiology as it relates to animal disease surveillance and control.

Of particular recent relevance to foodborne diseases in humans is the OIE's role in identification of outbreaks, surveillance, and control of diseases such as BSE. The OIE issues standards for surveillance, control, and animal movement from affected countries. Revisions are under active discussion. The standards, guidelines, and recommendations are periodically reviewed. New chapters, as well as revisions of existing chapters, are presented for adoption at the annual General Session. These continuing efforts are intended to provide the most current information possible.

## **ACKNOWLEDGMENTS**

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## The Office International des Epizooties Part II

**Lester Crawford**

*U.S. Food and Drug Administration, Rockville, Maryland, U.S.A.*

### I. INTRODUCTION

Seventy-five years of rinderpest finally created the impetus for the formation of an international organization dedicated to the control and elimination of animal disease and suffering. That organization is Office International des Epizooties, or OIE. Formed in 1924, it is headquartered in Paris and comprises 158 member nations.

In a real sense, the origin of OIE can be traced to the devastating rinderpest outbreak that occurred in Great Britain in 1865. Four hundred thousand cattle were lost because of a diseased shipment of cattle from Russia. Immediately there were calls for international consultation, and 10 international congresses were convened between 1865 and 1914 for the purpose of preventing rinderpest and other devastating animal diseases. The World Veterinary Congresses, as these later became known, were primarily diplomatic and scientific enterprises that proved ineffectual in preventing recurrences of exotic animal diseases.

The need for a permanently staffed international veterinary organization was made clear when rinderpest recurred in Belgium in 1920. Most disturbing was the source of the epizootic. Zebu cattle transshipped through Antwerp from India to Brazil seeded rinderpest in Belgium, and an outbreak began that threatened to involve the whole of Europe. In this as in other outbreaks, the morbidity was nearly 100%, and the mortality of cloven-hoofed animals in the hot zone was approximately 90%.

A hastily called international conference was convened in Paris in March of 1921. Forty-two nations attended. The delegates agreed on the need to share animal health information and export health measures. It was furthermore agreed that an "international office of epizootics for the control of infectious animal diseases" be set up in Paris.

A draft international agreement was prepared after the meeting and was circulated to the 42 nations that attended the meeting as well as to all other countries. Three years later 28 countries agreed on the language of an international agreement. Finally, in 1927, 24 countries had ratified the agreement. The OIE was officially founded during its first General Session on March 8, 1927.

Interestingly, the United States of America had retreated into an isolationist mode following its failure to ratify the League of Nations treaty and was not among the countries that founded OIE in 1927. In fact, the United States did not join until 1950.

### II. EARLY INITIATIVES

The first order of business in 1927 was to organize the OIE into a functional body. The delegate from Belgium, Dr. Henri De Roo, was elected president. Dr. Emmanuel Leclainche of France was

elected Director-General, and he established the OIE office in Paris. Almost immediately there began a close relationship between OIE and the newly formed League of Nations. The Secretary-General of the League, in 1924, requested various governments to designate veterinary experts “to examine the health guarantees that could be provided by cattle-exporting countries, the facilities that importing countries could accord on the basis of these guarantees and, in general, to determine the most effective means of enabling statutory veterinary measures to be applied, taking into account the economic interests of exporting countries and without prejudicing the interests of countries wishing to protect themselves against animal diseases.” This rather grandiose statement represented an ambitious goal of the League. In the words of Leclainche, “the Economic Committee of the League of Nations thus proposed to facilitate international trade in animals and animal products to try to reverse the often highly overt tendency of numerous countries to use sanitary arguments purely for the purpose of economic protection.” The League likewise was seizing the international animal health initiative inaugurated by OIE in such a way as to imperil the prerogative of the fledgling organization.

Fortunately, OIE responded in a manner that satisfied the needs of the League and also preserved the independence of OIE itself. In effect, OIE oversaw the commissioning of appropriate experts to advise the League of Nations. Veterinary experts convened in Geneva, the home of the League, in January 1928, June 1928, May 1929, February and June of 1931, and, finally, in 1934. The purpose of these conferences was to establish the bases for international sanitary regulations to categorize infectious diseases of animals according to their importance and the necessity for issuing sanitary certificates. In the end, the very basis for controlling epizootics during transit, exportation, and importation of animal products was to be described in an omnibus document that would later be termed the “International Zoosanitary Code.” Regrettably the experts could not come to an agreement on the essential elements of this document. When all attempts at consensus failed, the draft texts were remanded to OIE. The year was 1934 and the League of Nations was shortly to have the prospect of war to consider. The failure of the OIE experts to be of service to the League was illustrative of the intransigence of science when faced with the intellectual uncertainties of international diplomacy. This embarrassing episode gave rise to an atmosphere of conservatism bordering on insularity within OIE that was not to be broken until the 1990s.

The darkest period in OIE history was during World War II. OIE was commandeered by the Nazis, and only those countries controlled by Germany were allowed to be members. No meetings were held between 1939 and 1946. Day-to-day operations were conducted by an official of the German Army and a qualified veterinarian.

Following World War II, the League of Nations was dissolved and the United Nations (UN) took its place. OIE was slated to be absorbed into the Food and Agriculture Organization (FAO) of the UN. This intention was announced in 1946, but in 1951 the UN compromised by leaving OIE intact and separate. Connectivity was assured by a series of formal agreements between OIE and the two relevant UN organizations, FAO and the World Health Organization (WHO). This 5-year fight to preserve the independence of OIE was both serious and bitter.

A number of OIE delegates from various nations lobbied hard for the preservation of OIE. The French government also took great interest in the issue because OIE had become a source of pride for France. The depth of this national feeling towards OIE stemmed in part from France’s unchallenged position as the birthplace of veterinary medicine, a position that began with the establishment in 1720 at Lyon of the first veterinary school in the world. The idea of transferring OIE from Paris to Rome to be subsumed by FAO was not pleasing to France.

The salvaging of OIE furthermore endeared the organization to the French people as a unique institution of France worthy of support. The French government supports and defends OIE. Although the OIE presidency may rotate from country to country, the unspoken quid pro quo is that the director-general and the national home of the organization must be French. This position has spawned some jingoism, but, all in all, France has generally been an accommodating host with an egalitarian attitude towards all nations and blocs of nations.

When the Treaty of Rome established the European Community in 1957, OIE lent its support

to the creation of harmonized animal health systems within the Community by providing expertise and administrative consultation. This activity essentially established the hegemony of OIE in international veterinary medicine.

By 1982, the work of OIE had become highly respected and greatly depended on. On a weekly basis, the organization reported the status of a number of diseases throughout the world. This reporting system has been of immense help to member nations in eradicating certain diseases and in preventing the spread from one country to the other. Although sometimes inaccurate, this particular function is the most important historical contribution of OIE to society in general. Inaccuracies are primarily due to the failure of a member country to report disease conditions in a timely fashion due to either inadequate national systems or unwillingness to share information. OIE has a good record of demanding proper information and taking steps to correct member countries that do not provide accurate data.

Beginning in 1982, a series of proposals were presented to OIE challenging the organization to become more involved in the wider scope of veterinary medicine including animal drugs. OIE moved cautiously into this new area because animal drugs were not specifically mentioned in the mandate or terms of reference for the organization. But two significant conferences were convened in this general area. The first, entitled "Anabolics in Animal Production," was held in Paris in February 1983. This meeting, held at OIE headquarters, comprehensively addressed the smoldering international issue of the use of hormones in livestock production. The second was held in Brasilia on "The Scientific Dimensions of an Animal Drug Residue Control Program." This meeting established the basis for the protection of the public against inadvertent food additives.

In 1983, a vote was taken in the General Assembly to include animal drugs within the purview and programs of OIE. When this failed by a large margin, several OIE member countries, recognizing that no international organization addressed the increasingly important issue of veterinary drugs, decided to attempt to establish a standing committee on veterinary drugs within Codex Alimentarius, the international food safety organization jointly managed by FAO and WHO. The Federal Republic of Germany and the United States led this initiative. Success came in 1985 when the Codex Committee on Veterinary Drug Residues in Food was officially commissioned. Somewhat later, OIE did establish an International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), which has been quite successful in standardizing requirements for the approval of these products.

In 1988 during the Uruguay Round of the General Agreement on Tariffs and Trade (GATT) negotiations, a paper was tabled by the United States that proposed rules for adjudicating alleged nontariff trade barriers based on food safety concerns. Within that paper a procedure was described wherein OIE, Codex Alimentarius, and the International Plant Protection Convention would serve as expert bodies in these adjudications. Perhaps with the memory of the unproductive OIE collaboration with the League of Nations in mind, the General Assembly balked at another international entanglement. The turning point on this issue came during the 1990 OIE annual meeting when two ministers of agriculture (France and the United States) addressed the General Assembly on this subject. The strong commitment in the U.S. paper to risk assessment of such thorny impediments to trade as foot and mouth disease was also instrumental in the final outcome of these deliberations.

OIE, Codex, and IPPC reached a consensus on becoming part of the aforementioned initiative in time to be listed as expert bodies within the treaty that created the World Trade Organization (WTO) in 1994. Today these three organizations are referred to as the "three sisters," and their world status and reputation has been tremendously enhanced as a result.

### **III. THE LEADERSHIP**

There now have been five directors-general of the OIE. The first was Dr. Emmanuel Leclainche, who served 22 years (1927–1949). He came to OIE from the National Veterinary School in Toulouse, where he was Professor of Contagious Diseases. He was widely published and truly expert

in the field of infectious diseases. A visionary leader, Leclainche founded the French Academy of Veterinary Medicine and served as President of the French Academy of Sciences.

The second director-general was the celebrated Gaston Ramon, discoverer of anatoxins and the toxoids for tetanus and typhoid. He, with Freund, discovered vaccine adjuvants. Immediately prior to his appointment, Dr. Ramon was deputy-director of the Pasteur Institute in Paris. His scientific exposition was legendary—more than 800 papers were published in his lifetime. By the time of his death in 1963, Gaston Ramon was recognized as the leading medical scientist in the Republic. He served as director-general from 1949 to 1959.

The venerable Rene Vittoz served as the third director-general from 1959 to 1980. Vittoz's career included a number of significant posts in the government of what is now Viet Nam. From 1951 to 1959, he worked at OIE, primarily on Asian matters.

Dr. Louis Blajan served as director-general from 1980 to 1990. His tenure was marked by a significant increase in membership and expansion in scope. He had worked in Morocco and Mali and had held a number of important positions in the French government and in OIE before becoming director-general.

Jean Blancou served as director-general from 1991 to 1990. This was the most important decade in OIE history. Membership increased from 108 to 158 countries, and the affiliation with the WTO gave OIE the kind of world credentials that it had been striving for since its inception. The current director-general, elected in 1990, is Bernard Vallat from France.

#### IV. THE ORGANIZATION OF OIE

The principal diseases of concern to OIE at this time are foot and mouth disease; rinderpest; peste des petits ruminants; contagious bovine pleuropneumonia; Rift Valley fever; African horse sickness; African swine fever; classical swine fever (hog cholera); highly pathogenic avian influenza; rabies; New World screwworm; bovine spongiform encephalopathy; equine morbillivirus infection; Nipah disease; and varoosis in bees.

In aquatic animals diseases of concern include viral hemorrhagic septicemia; infectious hematopoietic necrosis; epizootic hematopoietic necrosis; spring viremia of carp; infectious salmon anemia; infectious pancreatic necrosis; viral encephalopathy and retinopathy; bacterial kidney disease; furunculosis; piscirickettsia salmonis; streptococcosis; *Gyrodactylus salaris*; Perkinsosis; *Haplosporidium nelsoni*; Marteiliiosis; oyster velar viral disease; white spot disease; yellowhead disease; Taura syndrome; and vibriosis.

Wildlife diseases of greatest concern include rabies; rinderpest; canine distemper; bovine tuberculosis; foot and mouth disease; Ebola virus; Nipah virus; and Hendra virus.

Dissemination of information on animal disease developments and other items of importance is accomplished via three periodicals. Most important is the weekly *Disease Information* publication. The *Revue Scientifique and Technique* is published bi-monthly. And of course there is the annual *World Animal Health*.

Various expert committees have been commissioned. These include the International Health Code Commission, the Foot and Mouth Disease and Other Epizootics Commission, the Standards Commission, and the Fish Diseases Commission.

The next level of expert groups are termed the Working Groups. These include The Working Group on Informatics and Epidemiology, The Working Group on Veterinary Drug Registration, The Working Group on Biotechnology, and the Working Group on Wildlife Diseases.

Ad hoc groups are convened from time to time to address special issues. OIE also has established reference laboratories and a list of approved experts and consultants on various issues.

Beginning in 1991, OIE began commissioning official Collaborating Centers. Their purpose is to research specific subject areas.

Four OIE regions have been designated because of animal health needs of these geographical areas. These are the Americas, Asia and the Pacific, Eastern Europe, and the Middle East.

## V. CONCLUSION

One of the great thinkers among the OIE delegates in the formative years of the 1980s was Pedro Acha from Peru. Dr. Acha enjoyed a brilliant career with the Pan American Health Organization and the International Institute for Cooperation in Agriculture before his untimely death in 1987. It was Acha's vision that OIE become the "world veterinary parliament" and that it address *all* international veterinary issues. At a dinner in the historic Parisian restaurant Le Grand Vefour in 1983, he spoke with passion about his dream for OIE and about the societal importance of the organization. Speaking first in French, he redelivered the essence of the oration in English, Spanish, Portuguese, and Italian.

Today Pedro Acha's dream has largely been fulfilled. Just 19 years later the world has changed again, and there are new challenges, but the challenges stem from the same root causes.

The near pandemic of foot-and-mouth disease that occurred in 2001 is a grotesquerie that OIE must address. Had OIE been fully functional, these outbreaks would not have occurred or would at least have been controlled more quickly and with less damage. A variety of factors have rendered the old animal disease paradigms suspect. These include modern transport, changing climatic conditions, animal and human overpopulation in some areas, the emergence and reemergence of certain diseases, and a better informed public.

OIE needs to reexamine its models for disease control and eradication as well as its disease-reporting systems. Two categories of disease-reporting require examination: (1) what to do about the timeliness and reliability of national morbidity and mortality reporting to OIE; (2) how can OIE do a better job of reporting to member countries given the recent refinements in data transmission, storage, and reinforcement? Obviously, countries like England, Uruguay, and Taiwan were ill-served by OIE in 2001. What went wrong last year must be thoroughly analyzed with a view towards improvement.

Finally, OIE must interact more productively with kindred international organizations. It must not find itself in a position of waiting for further instruction from organizations such as FAO, WHO, and WTO. OIE should empanel standing committees that regularly explore more and better ways of addressing shared needs with these and other related organizations. And OIE needs to take a more active role in scientific analysis and generation. Outlining research needs replete with suggested protocols is a role the organization should not avoid. Achievement of Pedro Acha's vision requires an action orientation and a willingness to confront difficult, new issues.

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## Microbial Risk Assessment as a Tool for Guiding Food Safety

**Kaye Wachsmuth, Janell Kause and Carol Maczka**

*U.S. Department of Agriculture, Washington, D.C., U.S.A.*

**Eric Ebel**

*U.S. Department of Agriculture, Ft. Collins, Colorado, U.S.A.*

**Wayne Schlosser**

*U.S. Department of Agriculture, College Station, Texas, U.S.A.*

**Steve Anderson**

*U.S. Food and Drug Administration, Bethesda, Maryland, U.S.A.*

### I. INTRODUCTION

Increased awareness of the effects of food hazards on human health and the increasing importance and rapid growth of world trade have prompted regulatory officials and international organizations to consider new and improved strategies to reduce the health risks associated with pathogenic microorganisms in foods. One strategy is the use of microbial risk assessment (MRA) to guide food safety decision making. MRA is a systematic and scientifically based approach for assessing the likelihood of exposure and subsequent impact of a pathogen on human health. MRA has the potential, both nationally and internationally, to become a powerful public health tool for improving global food safety because it allows risk managers to identify the most effective strategies to control hazards to prevent foodborne illness. It is a relatively new scientific discipline that presents many challenges. These challenges are due, in part, to the dynamic nature (e.g., growth, decline, antimicrobial resistance) of microbiological pathogens as they move through the food supply from the farm to the table. As this emerging discipline continues to develop, it will further bridge traditional food safety techniques (e.g., microbiological testing) with other public health activities such as foodborne surveillance and outbreak investigations.

MRA consists of four interrelated steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (1). Hazard identification involves identifying, through collection and critical review of data and information, a pathogen that may be present in a particular food or groups of food and is capable of causing adverse health effects. Hazard characterization is an assessment of the relationship between the level of pathogen intake (dose) and the nature, severity, and frequency of illness or other adverse health effect (response). Exposure assessment involves estimating, using food consumption and prevalence data, total pathogen intake. Using information gathered in these three previous steps, risk characterization estimates the likelihood of foodborne illness or related number of deaths in a given population from exposure to pathogenic microorganisms in food, as well as the uncertainty associated with these estimates. Developing MRAs requires both public and scientific input and the balance of science and values judgments (2).

The purpose of this chapter is to describe the nature and development of various MRAs designed to inform regulatory decision making and guide international efforts to prevent foodborne illness.

## II. MICROBIAL RISK ASSESSMENT APPLICATIONS

As shown in Figure 1, MRAs have several applications nationally and internationally. At the national level, MRAs allow risk managers to evaluate the effectiveness of interventions to prevent or reduce foodborne illness, weigh policy alternatives, and select and implement appropriate public health actions. MRAs can also be used to identify data that are important for understanding the public health risks from foodborne pathogens and thereby help determine priorities for food safety research. MRAs may also be used to support industry efforts to develop more effective Hazard Analysis and Critical Control Point (HACCP) plans, by scientifically identifying hazards and critical control points (CCPs) and establishing critical control limits (3–4). MRAs can also be used to strengthen the development of risk-based performance standards, which in turn can be used to support proposed and existing regulations.

Internationally, MRAs can be used to guide the development of standards that prevent foodborne illness and promote fair trade (5). They can also ensure that countries establish food safety requirements that are scientifically sound and assist in determining equivalent levels of public health protection between countries. They can also provide a scientific basis for developing sanitary measures that achieve specific food safety goals within a country.

## III. TYPES OF MICROBIAL RISK ASSESSMENTS

In general, MRAs have been developed in response to foodborne outbreaks or surveillance data on foodborne illness associated with microbiological hazards in specific foods. While they can vary

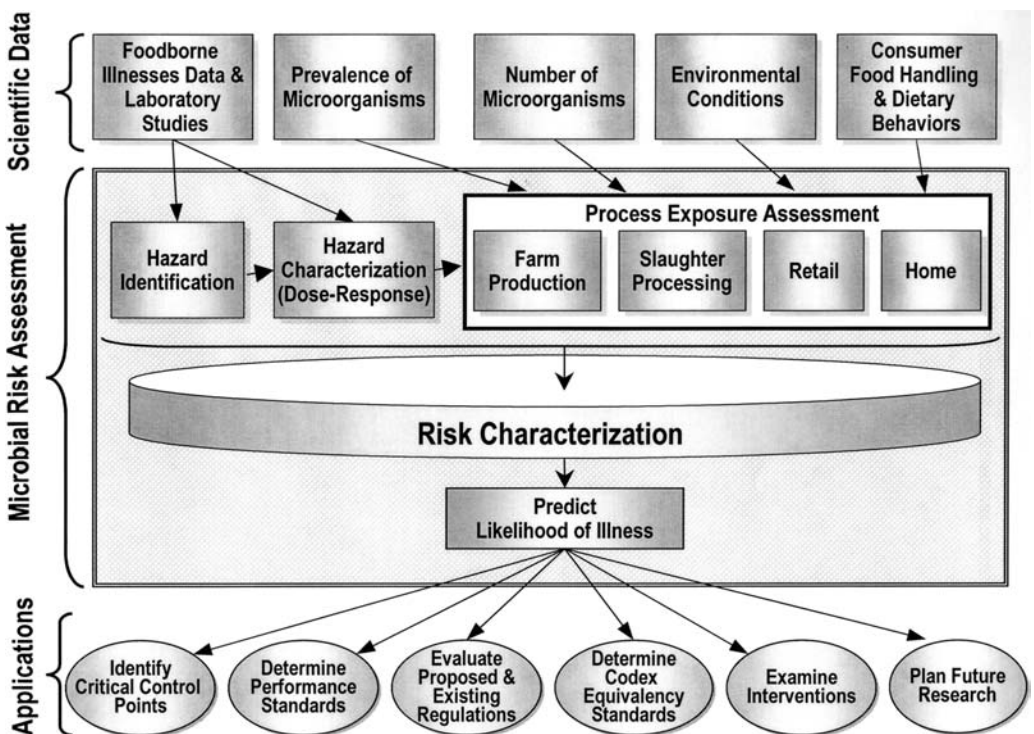


FIGURE 1 The role of microbial risk assessment in food safety.

in scope and complexity, those that have been developed can be generally classified into three types. These include “pathogen-commodity farm-to-table process risk assessments,” “pathogen-specific relative risk rankings” of food commodities, and “exposure pathway assessments.”

The pathogen-commodity farm-to-table process risk assessment is used to identify primary factors from production to consumption that influence public health risk associated with a pathogen in specific food (e.g., *Escherichia coli* O157:H7 in ground beef). These types of MRAs describe the complex nature (e.g., growth and decline) of pathogen populations in the food supply. Once the farm-to-table framework is constructed, it allows risk managers to evaluate assumptions, predict the impact of single or multiple interventions on the reduction in foodborne illness, and prioritize intervention strategies. MRAs of this type are being used in the United States as a basis for establishing food safety policy by the Department of Agriculture (USDA) and the Food and Drug Administration (FDA). The first such extensive, national MRA to be undertaken was the *Salmonella* Enteritidis (SE) risk assessment for shell eggs and egg products completed in 1998 (6). USDA has also recently completed a draft *Escherichia coli* (*E. coli*) O157:H7 risk assessment for ground beef (8). Additionally, FDA has recently completed a draft farm-to-table process risk assessment on the public health impact of *Vibrio parahaemolyticus* in raw molluscan shellfish, which identifies the primary factors from harvest through postharvest handling and processing to consumption that influence public health risk (7). International efforts include the initial development of a MRA for *Salmonella* in broilers and eggs (9) and another for *Campylobacter* in broilers (10).

When foodborne disease surveillance and outbreak data implicate a particular pathogen associated with several foods, a pathogen-specific relative risk ranking of food commodities may be used to identify those foods or food groups that pose the greatest public health risk. This comparison assists decision makers in prioritizing foods or food groups for further consideration (e.g., regulatory, voluntary, educational initiatives). The draft *Listeria monocytogenes* (Lm) risk assessment for categories of ready-to-eat foods, developed jointly by the USDA and FDA, is an example of this type of MRA (11).

Another type of MRA, the exposure pathway assessment, is used to identify those pathways likely to lead to an exposure to a foodborne pathogen or agent. This type of MRA focuses on the identification and likelihood of exposure to a foodborne hazard through various exposure routes rather than on the likelihood or severity of illness. Like pathogen-commodity farm-to-table process risk assessments, it too can be used to examine interventions to reduce foodborne exposure to a hazard. An example of this type of MRA is the bovine spongiform encephalopathy (BSE) risk assessment, commissioned by USDA and conducted by Harvard University’s School of Public Health (12).

#### **IV. PROCESS FOR DEVELOPING MICROBIAL RISK ASSESSMENTS**

The process for developing national and international MRAs involves public input from initiation to development and evaluation. MRAs continually improve as more relevant data are incorporated and with critical review. Once a national or international MRA is developed, it can be used along with other policy considerations to target food safety efforts (e.g., policies, guidelines, standards, or educational efforts) to effectively reduce foodborne illness.

##### **A. National Process**

In a regulatory setting, the scope and complexity of a MRA is determined by risk assessors and risk managers working together, along with public input, to identify food safety public health concerns and policy objectives (13). This process is known as “problem formulation” and is a critical step prior to developing a MRA (14). Once food safety concerns and policy objectives have been established, risk assessors begin developing a baseline MRA that can be used to inform regulatory decision-making.

As shown in [Figure 1](#), several types of scientific and public data are collected, evaluated, and incorporated into the baseline MRA. Sources of data used in the hazard identification include clinical

trials, foodborne illness surveillance and outbreak data, laboratory animal studies, studies of microbial characteristics, ecological studies characterizing the interaction between microorganisms and their environment, and studies on the historical presence and level of microorganisms in foods. In the exposure assessment, consumption data are linked with food contamination data to determine the prevalence and level of microbiological contamination in food consumed by an individual. Consumption data include the amount and frequency of the food being consumed. Demographic data are used to characterize the exposed population. Data used to estimate the prevalence and level of microbiological contamination in food include initial extent of contamination, effect of competitive microbiological flora within food, impact of cross-contamination and of sanitation and controls during production and processing, effect of storage and handling during distribution and retail, and the influence of consumer storage, handling and preparation behaviors. To describe the severity and duration of an adverse public health effect associated with exposure to a pathogen in food (i.e., hazard characterization step), pathogenic virulence and infectivity as well as host susceptibility data are needed. If quantitative data (e.g., human clinical challenge and animal feeding studies) are available, then a dose-response relationship is estimated and the public health endpoints, such as infection or illness, are explicitly considered.

After the data are incorporated, the baseline MRA is “validated” by comparing model estimates with surveillance data. For example, baseline MRA estimates for the prevalence of a pathogen in a food are compared to similar estimates from national microbial sampling surveys. Likewise, the MRA estimates of the number of foodborne illnesses are compared to similar estimates from national public health surveillance systems. National foodborne illness surveillance estimates are considered the “best” estimate of the magnitude of the public health problem in the case of acute illnesses associated with microbial pathogens (15). When baseline MRA estimates for foodborne illness overlap with estimates produced using surveillance data, the baseline MRA is considered to accurately reflect our current state of knowledge.

Following the development of the baseline MRA, it is again presented to the public for further critical evaluation and specific data are requested from stakeholders. The public input process enables stakeholders to participate in the decision-making process and ensures that the science supporting decision making is transparent (5). Public input and review is an iterative process and may result in subsequent reformulation of the problem, development of another type of MRA, or incorporation of additional data into the model.

After the baseline MRA has been received public review and input, it can be further refined and then used to identify control points and as a predictive tool to evaluate the public health benefits of potential mitigation strategies. The evaluation of the relative influence of MRA inputs on MRA outputs is referred to as “sensitivity analysis.” Few sensitivity analyses for MRAs have been conducted to date because they are complex, require significant computer resources, and it is difficult to track the effects of changes in MRA inputs on MRA outputs. Once sensitive MRA inputs have been identified, however, the MRA can be used as a predictive tool to evaluate proposed and existing regulations, examine interventions, and help set performance standards. By modeling inputs, the effects of alternate assumptions on health risk can be examined. To ease the computational burden and provide rapid responses to these “what-if” questions, MRAs are typically computerized. Spreadsheet applications and available “add-ins” (e.g., computer macros) allow generation of complicated probabilistic models that can be particularly useful in predicting the impact of changes in food processing.

## **B. International Process**

MRAs are also being developed to guide international food safety (e.g., to assist in the development of sanitary and phytosanitary measures) and to promote fair trade. This is a relatively new endeavor. Recently, the Codex Alimentarius Commission (CAC), a joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) international body with the responsibility for developing food safety standards, established principles and guidelines for the development of interna-

tional MRAs (16). These guidelines require the development of international MRAs through ad hoc expert consultations convened by FAO and WHO. This process begins with a call for data and the selection of experts to summarize and interpret data on hazard characterization or exposure assessment for specific product-pathogen combinations. The resulting documents are then subject to peer review and public review prior to their presentation to the Codex committee responsible for drafting provisions on hygiene applicable to specific food items or food groups, the Codex Committee on Food Hygiene (CCFH), at its annual meeting. The CCFH reviews the MRA documents and further formalizes its risk management question. As a final step in this process, the FAO/WHO ad hoc expert consultation is again assembled to develop a risk characterization document. This document integrates the findings of the exposure assessment and the hazard characterization and takes into consideration the risk management questions raised by CCFH. The primary goal of these ad hoc expert consultations is to develop risk assessments that will enable broad standardization and at the same time leave room for regional differences.

The communication process during the development of international MRAs mirrors that of national MRAs in many countries. That is, communication is frequent and ongoing between risk assessors and risk managers, but the roles of these groups are functionally separate in order to achieve unbiased risk assessments. Communication is also ongoing with stakeholders, including member countries and consumers, to ensure that the type of MRA to be developed, data and analyses selected, and interpretation of results is transparent.

Following completion of the risk assessment, a risk management option assessment will be performed. It is envisioned that this will ultimately lead to selection of a preferred management option(s) and implementation of management decisions. Such management decisions will be monitored and reviewed in order to ensure that food safety goals are being achieved on an ongoing basis. If the MRA yields unsatisfactory findings, risk managers may request a re-evaluation of specific inputs to the MRA or may commission a new MRA. Both of these requests will involve interaction among risk managers, risk assessors, and stakeholders to adequately formulate the statement of the problem.

## **V. SPECIFIC EXAMPLES OF MICROBIAL RISK ASSESSMENT**

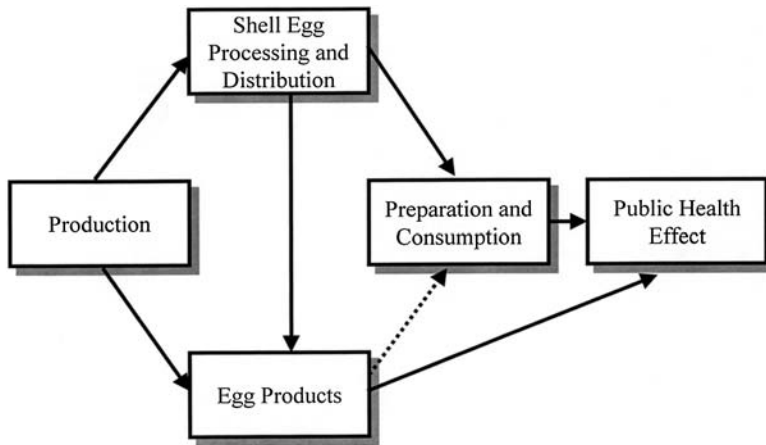
### **A. Regulatory Microbial Risk Assessments**

#### *1. Salmonella Enteritidis in Egg and Egg Products*

The *Salmonella* Enteritidis (SE) risk assessment for shell eggs and egg products, developed jointly by the USDA and FDA, is a pathogen-commodity farm-to-table process risk assessment (6). It models the likelihood and level of SE in eggs from the farm to the table and the subsequent nature and extent of human illness associated with consuming these eggs. This MRA was initiated in response to an increasing number of human illnesses attributed to the consumption of SE-contaminated eggs, despite prior regulatory interventions and safe egg handling education campaigns to mitigate these illnesses. Its purpose was to provide a baseline for identifying those mitigation strategies most likely to reduce the occurrence of SE infection in humans from eggs.

The SE risk assessment is comprised of five modules (Fig. 2). Each module represents both a stage in the farm-to-table continuum and a step in the MRA process. Four of the modules—shell egg production, shell egg processing and distribution, egg products processing and distribution, and preparation and consumption—address exposure assessment. Each module provides an estimate of the increase or decrease in the number of SE organisms in eggs as they pass through storage, transportation, processing, and preparation, respectively. The public health outcomes module integrates hazard characterization with the exposure assessment results to characterize the risk—incidence of illness and four clinical outcomes, as well as the cases of reactive arthritis—associated with SE infection following the consumption of meals containing eggs.

Data used to characterize variables in the baseline MRA include: flock size, molting status,



**FIGURE 2** Farm-to-table MRA for SE in eggs and egg products.

and ecology of SE in layer hens for the shell egg production module; storage, washing, packaging, and transportation conditions for shell eggs in the shell egg processing and distribution module; processing, storage, and handling conditions affecting internal and cross-contamination of egg products (whole eggs, yolk, albumen) in the egg products processing and distribution module; consumer storage, handling and cooking practices and consumption patterns (i.e., serving size and frequency) in the preparation and consumption module; and dose-response information from *Salmonella* feeding trials and foodborne outbreaks in the public health module. Variables in the MRA model were expressed as distributions rather than as point estimates to better express the variability in estimated values. The SE risk assessment estimates were calculated in a spreadsheet using a technique referred to as Monte Carlo simulation (17).

Egg products were not modeled in the MRA beyond pasteurization because there was no epidemiological evidence associating egg products with human illnesses from SE (18,19). Also, there is evidence that egg products are primarily contaminated by cross-contamination during processing whereas the focus of this MRA is on eggs internally contaminated with SE (20,21).

The baseline MRA estimates an average production of 2.3 million (i.e., 1 in 20,000) SE-contaminated shell eggs per year and predicts an average of 661,633 human illnesses per year from the consumption of these eggs. The MRA estimates that approximately 94% of these cases will recover without medical care, 5% will visit a physician, an additional 0.5% will be hospitalized, and 0.05% will result in death. Additionally, it was estimated that approximately 3% of consumers that became ill due to SE in eggs would be expected to develop chronic reactive arthritis subsequent to the original illness.

To validate the baseline MRA, estimates for the annual production of SE-contaminated shell eggs and related human illnesses were compared to estimates from a microbial sampling survey and a national public health surveillance system, respectively (22). The baseline MRA estimate of 2.2 million SE-contaminated eggs produced annually in the United States was similar to estimates from a survey of SE-contaminated eggs provided by the California Department of Food and Agriculture (I. Gardner, personal communication, 1998). The California survey found an average of one out of 1416 eggs to be contaminated with SE, which is equivalent to an annual production of about 2.7 million SE-contaminated eggs per year. The average number of human illnesses estimated by the MRA baseline was also similar to the median number of illnesses estimated per year by national foodborne illness surveillance systems (i.e., a median number of 504,082 cases per year, ranging between 254,000 and 1,167,000 cases per year) (22). Such agreement between the baseline MRA

and surveillance data estimates suggests that the SE risk assessment is reasonably accurate in its depiction of the number of cases of human illness per year due to SE-contaminated eggs.

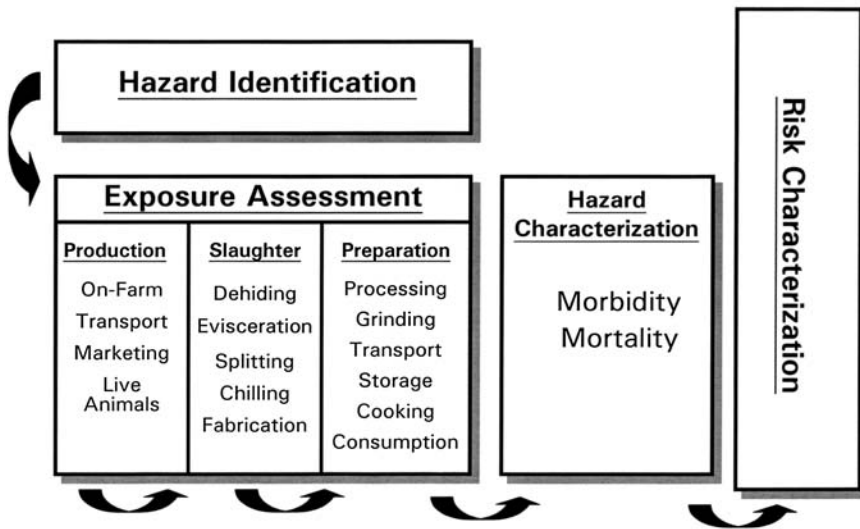
A sensitivity analysis was conducted for each module of the SE risk assessment to identify those factors that had the greatest influence on the prevalence and level of SE in eggs. Sensitive variables included: the within-flock frequency of producing SE-contaminated eggs and the prevalence of SE-contaminated flocks in the shell egg production module; internal contamination of shell eggs rather than cross-contamination in the egg products processing and distribution module; variables associated with time and temperature (e.g., storage and handling practices) for both the shell egg processing and distribution module and the preparation and consumption module; and the proportion of eggs that are pooled (broken and combined into a single container for later use) in preparation and consumption module. Each of these “sensitive” variables, along the farm-to-table continuum, is a potential control point for mitigating SE-related illnesses.

The SE risk assessment has been used to guide national policy to reduce, with the intent to eliminate, SE in eggs as a source of human illness. The Egg Safety Action Plan developed by the President’s Council on Food Safety is based on the SE risk assessment, which indicated that multiple interventions would be required to reduce human illnesses associated with SE-contaminated eggs by more than 25% (23). Specifically, the SE risk assessment evaluated four mitigation scenarios for comparison of their individual and combined effects on the number of human illnesses. These scenarios included: reducing the amount of time eggs are stored in homes, institutions, and at retail; reducing the time and/or temperature at which eggs are stored; reducing the number of SE-contaminated flocks as well as those with a high prevalence of SE; and reducing the number of contaminated eggs available to consumers by diverting SE-contaminated eggs from the shell egg market to the egg products market. None of these mitigation scenarios alone reduced the predicted number of human illness by greater than 25%. However, when the SE prevalence in the largest flocks and storage times in homes, institutions, and at retail were reduced, the predicted occurrence of human illness declined by more than 25%.

## 2. *Escherichia coli* O157:H7 in Ground Beef

The draft *E. coli* O157:H7 risk assessment for ground beef, developed by the USDA, is a pathogen-commodity farm-to-table process risk assessment (7). It was initiated in response to heightened public awareness of the association of *E. coli* O157:H7 with foodborne outbreaks associated with severe illness and death. The development of more sensitive (selective) diagnostic tests revealed that *E. coli* O157:H7 was present in cattle, on carcasses, and in ground beef more frequently than previously thought (24). Despite increased efforts to prevent *E. coli* O157:H7 in ground beef through monitoring programs and consumer education initiatives focused on safe cooking practices for hamburgers, *E. coli* O157:H7 continues to be associated with large foodborne outbreaks (25,26). The purpose of this MRA is ultimately to help identify those variables, under current practices of production, slaughter, processing, transportation, storage, and preparation, that have the greatest influence on the risk of illness associated with *E. coli* O157:H7 in ground beef. This will allow risk managers to identify potential areas where mitigation strategies could be applied.

The MRA models the prevalence and level of *E. coli* O157:H7 in ground beef from the farm to the table and the subsequent likelihood and severity of human illnesses associated with consuming undercooked ground beef (Fig. 3). In the hazard identification step, epidemiological data, unpublished FoodNet data, and a case-control study of a large *E. coli* O157:H7 outbreak implicated ground beef (e.g., hamburgers, meatballs, and meat loaf) as a primary vehicle associated with human illnesses—bloody diarrhea, hospitalization, hemolytic-uremic syndrome—and death from *E. coli* O157:H7 (P. Sparling, USDA, personal communication, 1999; 27,28). In the hazard characterization step, attempts were made to quantify the nature and severity of the adverse health effects (i.e., illness or death) associated with the level of *E. coli* O157:H7 present in ground beef (29). However, available data needed to describe the relationship between the number of *E. coli* O157:H7 consumed (dose) and the likelihood and severity of illness (response) are lacking. To characterize this relation-



**FIGURE 3** Farm-to-table MRA for *E. coli* O157:H7 in ground beef.

ship, the *E. coli* O157:H7 risk assessment uses a novel approach—the “envelope method” (30). In the envelope method, epidemiological data (i.e., FoodNet surveillance data and foodborne outbreak data) and data from human clinical studies are used to construct minimum and maximum dose-response curves for *E. coli* O157:H7. These minimum and maximum dose-response curves were derived by fitting human clinical data for enteropathogenic *E. coli* and *Shigella dysenteriae* stains to two separate beta-Poisson distributions, respectively. The most likely dose-response curve (i.e., a dose-response curve within these two separate beta-Poisson distributions) was derived by using national public health surveillance data and data from a large *E. coli* O157:H7 outbreak associated with consumption of hamburgers from a fast food chain (27,28). The exposure assessment step is comprised of three modules—production, slaughter, and preparation—and uses Monte Carlo probabilistic techniques to model the prevalence and concentration of *E. coli* O157:H7 in live cattle, carcasses, meat trim, and ultimately, in a single serving of cooked ground beef (17). Data for this step include: herd and within-herd prevalence of *E. coli* O157:H7; slaughter processing conditions, including decontamination steps; consumer and retail storage times and temperatures contributing to the growth of *E. coli* O157:H7 in ground beef; and consumer demographics and consumption patterns. In the risk characterization step, results from the dose-response model, derived from the envelope method, along with results from the exposure assessment were used to estimate the number and severity of human illnesses resulting from the consumption of contaminated servings of ground beef.

There are several outputs from this draft baseline MRA. The production segment estimates that 3–6% of breeding herds and 11–16% of feedlot cattle are infected with *E. coli* O157:H7. The slaughter segment estimates that an average of 33% of 2000 lb bins (combo bins) of beef trim contain at least one *E. coli* O157:H7 organism. Most contaminated combo bins, however, will contain only a few *E. coli* O157:H7 organisms. The preparation segment estimates that about 90% of grinder loads contain at least one *E. coli* O157:H7 organism. Overall, the exposure assessment estimates that an average of 15 out of every 1 million prepared ground beef servings are contaminated with *E. coli* O157:H7. Of the 15,904 human illnesses predicted annually by the CDC, the following severe outcomes are estimated: 1636 cases of bloody diarrhea, 353 hospitalizations, 84 cases of hemolytic uremic syndrome, and 11 deaths (28).



The baseline MRA was constrained (anchored) at several points to ensure that its estimates were consistent with observed data. The slaughter module estimates of the prevalence of *E. coli* O157:H7 on carcasses—0.3–0.8% in breeding cattle carcasses and 0.9–2.4% in feedlot cattle carcasses—were close to the prevalence of 0.2% estimated from Food Safety and Inspection Service (FSIS) carcass sampling data (31). In the preparation module, FSIS monitoring data were used in a Poisson distribution to estimate the prevalence of *E. coli* O157:H7 in grinder loads of ground beef (32). In this draft baseline MRA, the estimate of 15,904 illness resulting from *E. coli* O157:H7 in ground beef was derived from analysis of public health surveillance data and incorporated into the hazard characterization step (28). Additionally, the production module was validated by comparing the draft baseline MRA estimates of the prevalence of *E. coli* O157:H7 in live cattle to similar estimates derived from a Canadian study (33). Overall, the Canadian study found 12% of feedlot cattle and 2% of breeding cattle infected with *E. coli* O157:H7. These estimates were comparable to the draft baseline MRA estimates of 11–16% *E. coli* O157:H7 prevalence in feedlot cattle, but had only marginal overlap with the draft baseline MRA estimates for breeding cattle. By anchoring the estimated number of illnesses to public health surveillance data and validating the estimates of *E. coli* O157:H7 in live cattle, this draft baseline MRA can be used to reasonably depict the prevalence and level of *E. coli* O157:H7 in beef trim, grinder loads, and cooked ground beef servings.

Currently, the *E. coli* O157:H7 risk assessment is being refined to incorporate the effects of seasonality on the prevalence and level of *E. coli* O157:H7 on the farm, at slaughter, and through retail. At present there are limited consumer behavior data to capture the effects of seasonality on storage, handling, and cooking practices for ground beef. Information on the impact of seasonality on consumption patterns for ground beef is also being requested from the USDA Agricultural Research Service. Once the MRA has been refined, it will be publicly available for further input and comment. It is anticipated that once the baseline MRA is complete, it can be used to identify, using sensitivity analysis, those variables in production, slaughter, and preparation that have the greatest influence on the likelihood of human illnesses from *E. coli* O157:H7-contaminated ground beef. Given the identification of possible control points along the farm-to-table continuum, mitigation strategies can be evaluated on the basis of reducing this risk.

### 3. *Listeria monocytogenes* in Ready-to-Eat Foods

The draft *Listeria monocytogenes* (Lm) risk assessment, developed jointly by USFDA and USDA, is a pathogen-specific relative risk ranking of food commodities (11). This risk assessment provides analyses and models that estimate the potential level of exposure of three age-based population groups (i.e., elderly, newborns and fetuses, and an “intermediate age” group) to Lm-contaminated ready-to-eat foods in 20 food categories and relate exposure to these contaminated foods to the likelihood of listeriosis among these populations. The purpose of this draft MRA is to systematically examine available scientific data and information in order to estimate the relative risks of serious illness and death that may be associated with the consumption of different types of ready-to-eat foods contaminated with Lm. The results of this MRA will assist regulatory agencies in prioritizing which ready-to-eat foods require further consideration to further reduce foodborne listeriosis.

The hazard identification step of this draft MRA identified those at risk for listeriosis, the primary foods involved in transmitting Lm, the conditions under which Lm survives, and the primary environmental sources of Lm. In this step, foodborne outbreak data and active foodborne disease surveillance data (i.e., from the Foodborne Disease Active Surveillance Network) were used to characterize the population at risk for listeriosis. These data identified people with suppressed immune systems (e.g., those with AIDS or diabetes), fetuses and newborns, and the elderly as those most at risk. Other data used in this step include (a) ecological studies concerning environmental sources of Lm, (b) laboratory studies about the conditions under which Lm survives, grows, and declines, and (c) data from scientific and medical literature to identify foods that are historically associated with contamination by Lm. Foods were categorized into 20 groups under five categories—seafood, produce, dairy, meats, and combination foods—contaminated by Lm.

The exposure assessment step estimated how often consumers ate ready-to-eat foods contaminated with Lm and the number of organisms likely to be present in those foods. Consumption and food contamination data were used to estimate the foodborne exposure to Lm. Consumption data came from two nationwide surveys: the Continuing Survey of Food Intakes by Individuals and the Third National Health and Nutrition Examination Survey (34,35). These surveys provide nationally representative data on the amount of food consumed by an individual, the number of servings of a food consumed per year, and demographic characteristics of the consumer. Food contamination data on the prevalence and level of Lm in ready-to-eat foods at retail were gathered from published scientific literature and published and unpublished government and industry documents. There is no systematic, quantitative survey related to Lm contamination in foods. The draft exposure assessment also collected information on consumer behavior practices associated with ready-to-eat foods and quantitative data on storage times and temperature for these foods to model postretail growth of Lm in ready-to-eat foods.

The hazard characterization step describes the relationship between the number of Lm organisms consumed (dose) and the likelihood of illness occurring in combination with the severity of the illness resulting from that dose (response). Data from animal studies were modified in an attempt to account for variation in virulence among Lm strains and differences in susceptibility between laboratory mice and humans and to estimate the shape of the dose-response relationship. These data were adjusted to fit the number of listeriosis fatalities observed in national epidemiological data to derive a dose-response relationship for each age group considered.

The risk characterization step combined the results of the exposure assessment and the hazard characterization to produce estimates of the likelihood of listeriosis from a serving of each of these foods and an estimate of the likelihood of contracting listeriosis from consuming these foods over the course of a year. The foods were then ranked relative to each other on the basis of the predicted relative risk per serving of food and based on the relative risk per annum basis. This risk characterization was developed using a two-step computer modeling process. In the first step, two-dimensional Monte Carlo techniques were used to calculate the “most likely” model estimates and their attendant uncertainty (17). The second step of the modeling process involved ranking the foods in relation to the relative risk they pose to each age group (i.e., fetuses and newborns, elderly, and the “intermediate age” group) based on a single serving of food. This was again accomplished using computer simulation techniques where the “most likely” relative risk ranking was generated for each food category. When the number of servings for each food was considered, the relative risk of each food category on a per annum basis was determined.

A key determinant in characterizing risk to the consumer is the predicted relative risk per serving of food, i.e., the probability that consuming one serving of a food will result in listeriosis. In this draft MRA, preliminary results indicate that the following ready-to-eat foods presented the highest per-serving relative risk ranking of listeriosis among all age groups: pâté and meat spreads, fresh soft cheeses, and smoked seafood. Ready-to-eat foods with a moderate per-serving relative risk ranking were cooked ready-to-eat crustaceans, deli meats, and deli salads. Those with the lowest per-serving relative risk rankings were ice cream and frozen dairy products, aged cheese, fruits, and vegetables. It is important to note that these preliminary relative risk rankings do not represent an absolute risk of illness from these ready-to-eat foods, but provide a comparison of the risk from ready-to-eat foods relative to each other. In addition to the estimated relative risk per serving for each food category, the Lm risk assessment also considered the predicted relative risk of the food categories contributing to the incidences of listeriosis on a per annum basis. This preliminary relative risk ranking is heavily weighted by the frequency with which foods are consumed over the course of a year. Because some ready-to-eat foods are consumed more often throughout the year (e.g., frankfurter versus pâté), they present a greater overall public health risk. Those foods with the highest per annum relative risk ranking were deli meats, deli salads, and pasteurized fluid milk. Those foods with a moderate per annum relative risk ranking were hot dogs (reheated), dairy products, and smoked seafood. Those foods with the lowest per annum relative risk ranking were the same as those ranked on a per-serving basis.

Several gaps in data and information were identified during the development of the Lm risk assessment, including outbreak data on the amount of Lm in foods, data on the number of stillbirths and miscarriages due to foodborne listeriosis, information on the health status of individuals who consume ready-to-eat foods, information on consumer storage and preparation practices for hot dogs, and studies on growth of Lm in ready-to-eat foods and under various conditions. Filling these data gaps to refine this MRA would provide a more accurate relative risk ranking of ready-to-eat foods for listeriosis. For example, USDA is currently collecting consumer behavior data for the storage and preparation of hot dogs in order to better characterize the impact of storage time on the growth of listeria on refrigerated hot dogs and the proportion of hot dogs consumed without further reheating. It is possible that hot dogs that are stored longer or consumed without reheating will pose a greater relative risk than is currently predicted by this MRA. Additional data and scientific input and comment will improve the value of this MRA as a tool to prioritize regulatory efforts in reducing or eliminating foodborne listeriosis.

#### 4. *Bovine Spongiform Encephalopathy in Cattle*

The bovine spongiform encephalopathy (BSE) risk assessment for cattle was conducted by the Harvard Center for Risk Analysis (12). This MRA evaluates possible routes by which cattle and humans may be exposed to the BSE agent in the United States. Although BSE has not been identified in the United States, this MRA will assist the USDA in evaluating the effectiveness of current control measures in preventing the disease from entering or occurring in the country. It will also facilitate the identification of additional measures that might further reduce exposure of the cattle population and American public to the BSE agent.

BSE is a neurodegenerative disease that most likely spread among cattle in the United Kingdom and Europe through consumption of BSE-contaminated feed (36–38). In 1996 the emergence of a new disease in humans, called variant Creutzfeldt-Jakob disease (vCJD) (39), was thought to be linked to the consumption of BSE-contaminated beef. To date more than 90 cases of vCJD have been reported (40).

In 1997, the FDA Center for Veterinary Medicine instituted a feed ban prohibiting the feeding of ruminant meat and bone meal (MBM)-based feeds to cattle. Since feed is the major vehicle of transmission for BSE, the BSE MRA examines possible routes of exposure to potentially contaminated BSE tissue through noncompliance with the ruminant-to-ruminant feed ban and the possibility of mislabeling and cross-contamination of feed with ruminant MBM and the misfeeding of cattle with ruminant MBM-based feed. The routes by which the BSE agent could contaminate beef food products during production and processing and result in human exposure are also examined in the study. Of concern are brain, spinal cord, and central nervous system tissue, which are considered “high-risk” tissues because together these tissues contain at least 90% of the BSE agent in an infected animal. The BSE MRA evaluates each stage of beef production to determine the extent to which these “high-risk” tissues may be incorporated into beef products destined for human consumption.

The foundation of the BSE risk assessment is a mathematical model developed in C++ programming language. It can be used to understand a variety of scenarios by which cattle and humans can be potentially exposed to the BSE agent. The MRA is dynamic and follows the changing demographics of the U.S. cattle population, while simultaneously tracking changes in the transmission and levels of the BSE agent (if present) throughout cattle and beef production, on an annual basis or over a period of several years.

Variables used in the MRA model capture the frequency and levels of contamination that pose the potential for exposure if BSE were present in the United States. The input variables are represented by distributions to capture the variability in the data. Sources for the data include scientific literature, government reports, survey data, and expert elicitation. Data used for the potential cattle exposure portion of the model include U.S. cattle population demographics, birth and death rates, transmission, rendering and feed production, and feeding practices. There is the potential for human exposure to the BSE agent if the disease were identified in U.S. cattle. Data for the potential human exposure portion of the model include antemortem and postmortem inspection, pneumatic stunning,

carcass missplitting, advanced meat recovery systems, and the distribution of the BSE agent in various tissues.

The dose-response relationship for cattle is based on the ingestion of cattle oral ID<sub>50</sub> units (defined as the estimated number of infectious units in infected tissue that would be expected to result in half the cattle population becoming infected). Again, the BSE risk assessment only estimates the potential for human exposure should BSE be detected in the U.S. cattle population. Because a dose-response relationship is not known for humans, any estimate for risk to the U.S. population would be extremely uncertain.

Preliminary results from the baseline scenario suggest that the risk of BSE for the U.S. cattle population is very low and that human exposure is even lower. Sensitivity analysis indicates that if BSE were present at some level in the United States, then the pathways that are predicted to have the greatest implications for cattle exposure are: rendering inactivation, mislabeling of ruminant-based feed, comingling in a mixed ruminant/nonruminant feed facility, and misfeeding with ruminant-based feed. An important pathway of potential exposure of the American public would be the consumption of “high-risk” tissues such as brain and spinal cord. The relatively large contribution of each of these pathways to exposure suggests that further mitigations at these points in the farm-to-table continuum might yield additional reductions in the levels of potential exposure to the BSE agent.

The BSE risk assessment also helped to identify data gaps and research needs. Addressing these data needs could enhance the accuracy of the predicted exposure in the pathways examined. For instance, better survey information on the level of compliance with the current ruminant-to-ruminant feed ban, the occurrence and levels of nervous system tissues in certain beef products, and the efficiency of cattle-to-human transmission of the BSE would reduce the uncertainty of both the cattle and human exposure estimates.

One approach used to validate the accuracy of the predictions made by the mathematical model will be to compare the results to risk estimates obtained for the cattle population from a country, such as Switzerland, known to have BSE. If the model can reliably predict the progression and number of BSE cases using country-specific cattle demographic and production data, then the results from the model should be a reasonable estimation of the risk for the U.S. cattle population.

The BSE risk assessment will be an invaluable tool that can be used by the USDA to determine the effectiveness of current control measures in preventing the disease from occurring or entering the country. The risk assessment can guide policy makers in the development of national policies that might further reduce potential exposure of the cattle population and American public to the BSE agent.

## **B. International Microbial Risk Assessments**

In support of the CAC, FAO and WHO began a joint expert consultation on risk assessment of microbiological hazards in foods in early 2000. The purpose of this consultation was to provide advice on risk assessment of microbiological hazards in foods from an international perspective (as suggested by the 32nd session of the CCFH). Specific hazards considered were *Salmonella* species in broilers, *Salmonella* Enteritidis in eggs, and *Listeria monocytogenes* in ready-to-eat foods. These product-pathogen combinations were selected because they are microbial food safety problems of worldwide notoriety. Other product-pathogen combinations identified for future consultation include *Campylobacter* in broilers and *Vibrio* species in seafoods.

The overarching goals of these consultations are to review the state of the art in microbial risk assessment, identify data gaps, and illustrate differences in modeling approaches. For each product-pathogen pair, the consultation conducts a hazard characterization, which includes a short synopsis of hazard identification, an exposure assessment, and a risk characterization. Since it began its work in 2000, the expert consultation has developed hazard characterizations and exposure assessments for *Salmonella* Enteritidis in eggs, *Salmonella* species in broilers, and *Listeria monocytogenes* in

ready-to-eat foods. The expert consultation is also planning to complete generic risk characterizations for these MRAs in 2001.

The hazard characterizations of these MRAs consist of describing, summarizing, and evaluating relevant data and constructing appropriate dose-response equations. Exposure assessments for *Salmonella* Enteritidis in eggs and *Salmonella* species in broilers are structured as farm-to-table analyses, whereas exposure assessments of *Listeria monocytogenes* in ready-to-eat foods are structured as retail-to-table. Both the hazard characterization and exposure assessment are derived using published research information typically indicative of industrialized countries. Surveillance data are adjusted to account for sensitivity and specificity of methods. No attempt is made to compare different data sets to detect differences between countries. Risk characterizations have been drafted using the best available dose-response model(s) and generally specified exposure assessments.

Review of the hazard characterizations and exposure assessments conducted to date demonstrate that there are many data gaps related to these product-pathogen pairs. For example, better data are needed regarding dose-response. Data are also needed to distinguish host, pathogen, or product variability. An important conclusion of these consultations is that standardization of modeling methods should be a goal for both exposure assessment and hazard characterization. This process has begun for hazard characterization. In addition, sensitivity analysis will be addressed as a part of the risk characterization analysis currently underway. Unlike previously completed MRAs, which have attempted to validate or anchor risk estimates with varying degrees of success, no formal method for validation of models was proposed as part of these efforts.

Feedback from CCFH regarding the exposure assessment and hazard characterization work already completed has been favorable. The risk characterizations currently underway are in direct response to CCFH questions, and they are to be reviewed at the next (34th) session of CCFH. It is envisioned that these MRAs will be used to set limits for health risks in foods, determine equivalent levels of public health protection between countries, and provide a framework for considering mitigations and risk reduction strategies.

## VI. CONCLUSIONS

Microbial risk assessment is an emerging public health tool that promises to provide a sound scientific basis for developing international standards and guidelines for food safety and for supporting risk-based national policies and practices to prevent foodborne illness. As described, the types of MRAs developed to date can be classified into three categories: pathogen-commodity-specific farm-to-table risk assessments, pathogen-specific relative risk ranking of food commodities, and exposure pathway assessments. Each of these MRAs is a tool that can be used to guide various types of food safety decision making—whether prioritizing foods for further regulatory consideration (e.g., Lm risk assessment) or targeting prevention and control strategies to reduce specific foodborne illness. The process of developing MRA requires ongoing public and scientific input to ensure transparency and take into consideration stakeholder viewpoints in the development of food safety standards, guidelines, and regulations. Only a handful of MRAs addressing foodborne pathogens have been developed to date. It is envisioned that as this tool matures, it will be used to address many more pathogens, including those not yet considered (e.g., viruses).

MRA has highlighted the need to prevent and control foodborne pathogens from the farm to the table and linked traditional food safety techniques (e.g., microbiological testing) with public health approaches (e.g., foodborne disease surveillance and outbreak investigations). MRAs developed to date may help serve as a powerful predictive tool to efficiently allocate resources to effectively reduce foodborne illness. There is, however, much work to be done to develop MRAs that address foodborne illness, including gathering relevant data from developed and developing countries as well as validating MRAs and conducting sensitivity analyses.

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# Hazard Analysis and Critical Control Point Systems

**George P. Hoskin, William R. Jones, Brett Podoski, and Leslie Bluhm**

*U.S. Food and Drug Administration, College Park, Maryland, U.S.A.*

## I. INTRODUCTION

The concept of the Hazard Analysis and Critical Control Point (HACCP) system was introduced to the food industry during the 1960s to meet requirements for manned space flight. The U.S. Army Natick Laboratories, in conjunction with the National Aeronautics and Space Administration (NASA), contracted the Pillsbury Company to design and produce the first foods to be used in space. Zero gravity poses numerous challenges, including that of preventing food particles from dispersing such that they could be aspirated or could foul sensitive instrumentation. While struggling with problems such as this, the Pillsbury Company also undertook the development of a system to assure that the foods they produced could not cause illness.

The food industry's traditional quality control methods soon proved to be inadequate for the task Pillsbury had undertaken. The "zero defect" goal that NASA desired in order to guarantee the safety of the foods astronauts would consume in space could not be achieved with existing methods. The product sampling and failure rate necessary to provide this desired level of food safety would have been prohibitively expensive. Pillsbury discarded its standard quality control methods and began an extensive program, in conjunction with NASA and the U.S. Army Natick Laboratories, to evaluate food safety. They soon realized that they would have to develop and maintain exacting control over all raw materials, processes, work environments, and personnel. In 1971, at the U.S. National Conference of Food Protection, Pillsbury introduced HACCP as a preventive system that enables manufacturers to produce foods with a high degree of assurance that they were produced safely while reducing dependence on finished product sampling and testing. To address serious botulism problems in the canning industry, the FDA incorporated HACCP principles into its low-acid canned foods regulations in 1973.

As a management system, HACCP provides a more structured and scientific approach to the control of identified hazards than that achievable by traditional inspection and quality control procedures. By identifying hazards inherent in processing a specific product and then implementing preventative measures that can be monitored, HACCP provides a logical basis for controlling product safety and offers a number of advantages. Most importantly, HACCP:

- Places responsibility for ensuring food safety on the food manufacturer or distributor

- Identifies hazards and prevents them from leading to the contamination of food

- Is based on sound science

- Permits more efficient and effective government oversight, primarily because the record keeping allows investigators to see how well a firm is complying with food safety laws over a period of time rather than on a given day



Helps food companies compete more effectively in the world market  
Reduces barriers to international trade

The HACCP approach quickly attained international recognition as the most effective means of controlling foodborne disease. It is endorsed by the joint FAO/WHO Codex Alimentarius Commission, the U.S. National Advisory Committee on Microbiological Criteria for Foods (NACMCF), the European Union, several other country governments, and (independent of regulatory influence) a number of food processing companies seeking improvements in their production systems.

One of the key advantages of the HACCP concept is that it enables the processors, importers, and regulators to adopt an approach whereby potential hazards are identified and controlled in the manufacturing environment, thus preventing product failure. HACCP provides a proactive, preventive approach to food safety, rather than a reactive, random one. As such, it was adopted by the FDA for the cumbersome task of improving seafood safety. This accomplishment exemplifies the broad applicability and versatility of the overall approach.

The following summarizes this adoption of HACCP as a mandatory requirement for seafood imported to or produced in the United States. It is intended to describe the intent and operation of seafood HACCP requirements from both user and regulator points of view. However, it cannot cover every aspect of HACCP guidance and is not intended nor should it be used as a substitute for HACCP training.

## **II. BACKGROUND**

On December 18, 1995, the U.S. Food and Drug Administration published a final rule, in Title 21 of *The Code of Federal Regulations*, Part 123, requiring that seafood produced in or shipped into the United States be produced under a HACCP system. Enforcement of the rule was implemented 2 years later in order to provide time for U.S. seafood processors, and processors supplying U.S. importers, to design and implement individual plans. U.S. importers were afforded time to develop the verification systems they needed to demonstrate that seafood imported after the implementation date was produced in compliance with the new regulation. The time was also used to train federal, state, and local regulators in the performance of HACCP inspections.

The rule was created due to concern over the safety of the U.S. seafood supply and to address the inherent unreliability of a massive federal inspection and testing program where any item not actually tested could still be suspect. The solution lay in establishing a formalized system of preventive controls that would allow seafood suppliers, as well as state and federal regulators, to have a high degree of confidence in the safety of the seafood. By adopting the HACCP system, a seafood supplier would identify what hazards could make a product unsafe, how to prevent them, and how to document that control had taken place. Seafood safety, under the new regulations, would be attributable to good practices, rather than good luck, through routine adoption of and adherence to the following basic HACCP principles:

- Principle 1: Conduct a hazard analysis
- Principle 2: Determine the critical control points (CCPs)
- Principle 3: Establish critical limits
- Principle 4: Establish monitoring procedures
- Principle 5: Establish corrective actions
- Principle 6: Establish verification procedures
- Principle 7: Establish record-keeping and documentation procedures

## **III. CHARACTERISTICS OF THE RULE**

The rule is published in the Monday, December 18, 1995, issue of the *Federal Register* (FR, Vol. 60, pp. 65197–65202). The brevity of the regulation, at just over five pages, reflects an intention

**TABLE 1** Summary Outline of 21 CFR Part 123—Fish and Fishery Products<sup>a</sup>

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Subpart A—General Provisions
Definitions (includes descriptions of “critical control point,” “processor,” “seafood,” and other significant terms)
Current Good Manufacturing Practice (includes reference to 21 CFR Part 110)
Hazard Analysis and Hazard Analysis Critical Control Point (HACCP) plan
Corrective actions
Verification (describes actions to be taken by industry and regulators; includes the concept of HACCP plan validation)
Records (describes records must be kept and how long they must be retained)
Training (qualifies the need for training and flexibility of what it may entail)
Sanitation control procedures (stipulates implementation of standard procedures and their monitoring)
Special requirements for imported products
Subpart B—Smoked and Smoke-Flavored Fishery Products
General
Process controls
Subpart C—Raw Molluscan Shellfish
General
Source controls (specifies that sources must be approved by competent authorities)

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<sup>a</sup>When embarking upon HACCP Plan development or other action or analysis, the actual rule must be consulted.

to describe an overall outcome to be achieved rather than to stipulate detailed requirements. To implement the regulations, seafood processors and importers must undertake a thoughtful analysis of the products they handle and must develop and monitor controls designed to prevent the occurrence of safety hazards they have found reasonably likely to occur in their products and processes. Key aspects of the rule are that it is not prescriptive, it covers safety only, basic sanitation is a prerequisite, all sources of U.S. seafood are to operate under HACCP, records are essential, and, finally, it augments rather than replaces existing regulations. The organizational structure of the rule is summarized and partially annotated in Table 1.

#### **IV. SANITATION REQUIREMENTS**

One realization in developing the rule was that good manufacturing practices regarding sanitation and cleanliness are necessary to produce wholesome food, but are not appropriately identified as critical control points in a HACCP system. Good sanitation practices have to be in place before product- and process-specific HACCP plans can be developed, in that a HACCP plan and its implementation would be ineffective in an unsanitary setting. Breaches in sanitary control may occur at any stage, time, and location during production. Some breaches are clearly openings for the introduction of hazardous agents should they be present, but neither the concept of locating a critical control point, nor establishing critical limits, describes these situations adequately. For example, hand washing is a significant sanitation precaution, but where would a critical control point be placed in the process flow and what critical limit would be set? Given the overarching and repetitive practices comprising sanitation precautions such as this, sanitation integrates best with HACCP operation as part of a prerequisite condition.

Analysis of the sanitation provisions of the Good Manufacturing Practices requirements in 21 CFR Part 110 of the *Code of Federal Regulations* identified eight broad areas requiring emphasis

**TABLE 2** Sanitation Affecting Safety

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As stated in 21 CFR Part 123, processors must monitor conditions and practices during processing frequently enough to ensure conformance with those conditions and practices specified in 21 CFR Part 110 that are appropriate to the food being processed and relate to the following:

- (1) Safety of the water that comes into contact with food or food contact surfaces, or is used in the manufacture of ice;
  - (2) Condition and cleanliness of food contact surfaces, including utensils, gloves, and outer garments;
  - (3) Prevention of cross contamination from unsanitary objects to food, food packaging material, and other food contact surfaces, including utensils, gloves, and outer garments, and from raw to cooked product;
  - (4) Maintenance of hand washing, hand sanitizing, and toilet facilities;
  - (5) Protection of food, food packaging and food contact surfaces from adulteration with lubricants, fuel, pesticides, cleaning compounds, sanitizing agents condensate, and other chemical, physical, and biological contaminants;
  - (6) Proper labeling, storage, and use of toxic compounds;
  - (7) Control of employee health conditions that could result in the microbiological contamination of food, food packaging materials, and food contact surfaces, and
  - (8) Exclusion of pests from the food plant.
- 

in order to prevent the inadvertent introduction of hazards in seafood (Table 2). Seafood processors are required to monitor the identified sanitation conditions and practices during processing and to maintain these records along with corrective action records. Each of the following activities is considered an aspect of processing for the purposes of this regulation: handling, storing, preparing, heading, eviscerating, shucking, freezing, changing into different market forms, manufacturing, preserving, packing, labeling, dockside unloading, and holding. Factory ships as well as shore-based plants are considered processors. Operations which might appear to be processing procedures, but which are comprised of onboard activity normally associated with fishermen handling the catch for delivery to shore, are excluded. Specifically, heading, gutting, bleeding, icing of fish, and shucking of scallops onboard a harvest vessel are considered normal fishing practice and are not regarded as processing for the purposes of this regulation.

## **V. HAZARD IDENTIFICATION**

The HACCP process seeks to identify safety hazards that are reasonably likely to occur. A record of illness associated with a particular product or process, such as scombrototoxin illness, demonstrates that a hazard is reasonably likely to occur. In some cases, however, such a record may not exist for a hazard that causes illness, even though the hazard may be reasonably likely to occur. For example, scombrototoxin is likely to form in herring under conditions of temperature abuse, although industry practices already in place for most herring products have precluded such toxin formation. Under the HACCP regulation, even those critical control points that may already exist as industrial standard practice must be specified, the critical limits must be met, and records of monitoring these limits must be kept. The rule classifies hazards into nine categories: natural toxins, microbial contamination (bacteria, viruses, protozoa), chemical contamination, pesticides, drug residues, decomposition toxins (notably scombrototoxin), parasites, unapproved food and color additives, and physical hazards. The inclusion of unapproved food and color additives reflects the intent of the Federal Food Drug and Cosmetic Act to regulate such additives by legally defining them as unsafe until they have been approved for use. Similarly, only drugs approved by FDA may be used in the aquaculture of seafood animals raised in or imported into the United States, although certain exceptions may be made for some drugs used in aquaculture in other countries.

Processors should recognize that consideration of specific hazards also includes, as the principles of HACCP stipulate, consideration of the end use of the product. For example, parasites pose a safety hazard in fish intended for raw consumption, but not necessarily in fish intended for cooking. Heavy parasite burdens, however, might still render fish intended for cooking unwholesome and unfit as food and therefore adulterated, although this would not constitute a safety concern and so is not handled by a HACCP regulation.

In order to aid processors and importers, FDA has developed a *Fish & Fishery Products Hazards & Controls Guidance*, now in its third edition (1). The intent of the guide is to help HACCP plan developers recognize the hazards that need consideration, and the controls that should be implemented, for their products and processes. This guide is available through the Internet on the FDA home page. It is intended to be a “living document,” which is periodically revised as new information becomes available. In addition to providing information on hazards that may be characteristic of particular species and harvest environments, it also provides information on potential hazards associated with types of processing. It contains instructions and forms which, although not required, can be used to help develop product- and process-specific HACCP plans. Background information as well as information regarding HACCP training and the use of the guide is also available on the Internet from a variety of sources (see, e.g., Refs. 2–4).

## VI. APPLICATION OF HACCP TO THE CONTROL OF BACTERIA

While the HACCP system provides a structure in which to analyze products and processes in order to control hazards, its implementation does not necessarily create new processing procedures that are more effective for the control of hazards. Even with the implementation of a HACCP plan, it remains necessary to control hazards such as bacteria through the best available process mechanisms. For some products and bacterial hazards, there may be no sure controls available, or the potential controls may be too impractical to apply, as is the case for the production of molluscan bivalve shellfish intended for raw consumption. In general, the bacterial hazards likely to occur are well recognized. These may originate from contamination of the water where the shellfish are grown, or they may be naturally present in such water. The options available in this example include harvest water quality control, depuration, and other forms of postharvest processing, as summarized below.

1. *Harvest water control.* The need to avoid contamination by bacteria from human and other animal waste is well established, but absolute avoidance of such bacteria cannot be assured through harvest water control because it is impractical for all sources of contamination to be totally eliminated. Similarly, human health risk can be reduced but not eliminated through programs intended to reduce the hazard to an acceptable level. Thus, in lieu of quantitative statements describing absolute limits for bacteria, these programs depend on the ability to determine whether the human health risk from bacterial contamination has been reduced to an acceptable level. Control for bacteria which occur naturally in the environment, rather than being introduced by human activity (e.g., *Vibrio vulnificus*), remains more difficult for any products which do not have a cooking step as part of the processing or before the point of consumption.
2. *Depuration.* This postharvest approach to hazard reduction is a process by which harvested shellfish are placed in clean water to permit the purging of their gastrointestinal contents under controlled conditions. A related approach is the practice of relaying shellfish to clean estuarine waters to promote self-cleansing through normal physiological processes. Neither approach has been shown to be effective for the removal of viruses or for eliminating naturally occurring *Vibrio* species.
3. *Other forms of postharvest processing.* Freezing and holding under prescribed conditions that can significantly reduce levels of some naturally occurring pathogens, thermal processing that can reduce bacteria to virtually undetectable levels, and irradiation that elimi-

nates viable bacteria completely are among other postharvest processes which may reduce bacterial pathogens in molluscan shellfish. The freezing process is highly effective against naturally occurring *Vibrio* species but does not achieve complete elimination of them. The heating process causes textural changes that some consumers find unacceptable for a raw product while also leaving some level of risk from any bacteria that may remain. Irradiation is more effective at killing bacteria, but is not approved for this use. Finally, each of these processes kills the shellfish, so the treated product is somewhat different from the traditional shucked-at-consumption product and must be handled differently from the time of treatment to the point of consumption; e.g., wet storage is not a possibility.

In considering the human health risks associated with bacteria in shellfish, the relative contributions of exposure level, various strains of potential pathogens, and wide-ranging host susceptibility factors remain to be fully elucidated. A full understanding of the synergistic relationships involved is even more elusive. Therefore, HACCP controls designed to achieve a reduction of bacteria to acceptable levels, rather than to prevent or eliminate bacterial contamination, are appropriate to molluscan shellfish. However, due to the lack of consumer acceptance, the inability to stipulate an acceptable level of shellfish-borne bacterial hazards, the unreliability of current control options, and other practical reasons, there is no postharvest process that may be regarded as a sufficient critical control point. Nevertheless, application of one or more of the possible controls will reduce risk and should be undertaken. Other standards, such as those involving harvest water quality and postharvest handling, are stipulated by shellfish control authorities. Ultimately, if thorough cooking is unacceptable to the consumer, microbiological risks will remain.

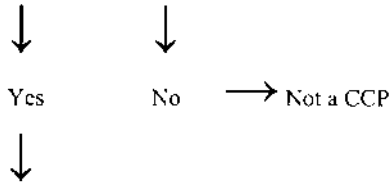
In most cases, the question is what to do upon discovering that an appropriate critical control point, control process at that point, or appropriate critical limit to evaluate that control process has not been established. The *Fish & Fisheries Products Hazards & Controls Guidance* contains a Critical Control Point Decision Tree, derived from that developed by the U.S. NACMCF (Fig. 1). This flow diagram can be followed to determine if a HACCP plan can be, or has properly been, designed for a given product and processing operation. The diagram reflects that the process should be redesigned as necessary to include the needed HACCP controls. In practice, some production processes may not be able to assure bacteriological safety of their products through the certainty of a documented HACCP operation. Rather, an uncertain level of risk will remain. That risk can be reduced through prudent actions, and these should be followed and documented. Again, risk associated with bacteria on raw products will remain, and it is important that this risk be recognized by both regulators and consumers.

A simpler situation is exemplified by a process or product with a bactericidal component as a normal processing step. In such a case, the development of a HACCP plan might be relatively straightforward, although the hazard analysis may prove difficult and the proof of effective control measures may require specific studies tailored to a firm's operations. For example, in the canned food industry, bacterial control is provided in the retort cooking operation. By adopting an empirically determined and carefully validated kill step requirement as a scheduled process for low-acid canned foods, canned food controls ensure that *Clostridium botulinum*, a hazard deemed through HACCP analysis to be reasonably likely to occur, is eliminated.

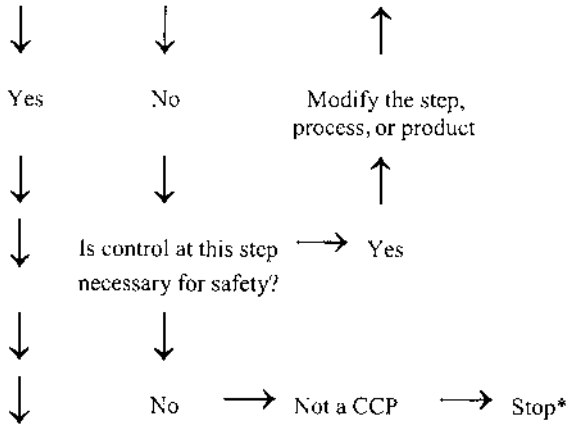
## VII. VERIFICATION

Verification is essential to assuring effective implementation of the HACCP regulation. The necessary procedures have two purposes: to ensure the HACCP plan is up to date (at least annually) and to ensure ongoing implementation is effective. Both FDA verification and processor or importer verification are critical components of the process. These verifications must be designed to determine the accuracy of the HACCP plan and to show whether it is being followed. The establishment of a Memorandum of Understanding (MOU) between the United States and the government of a country

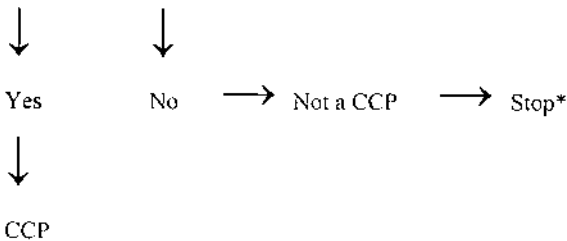
**Q1.** Does this step involve a hazard of sufficient risk and severity to warrant its control?



**Q2.** Does a control measure for the hazard exist at this step?



**Q3.** Is control at this step necessary to prevent, eliminate, or reduce the risk of the hazard to consumers?



\* Proceed to the next step in the process.

**FIGURE 1** CCP decision tree.

exporting a product to the United States would serve to remove the obligation for an importer to undertake importer verification. However, affirmative steps for the import of any product must be covered by that MOU. In the absence of an MOU, importer verification is of particular importance to foreign firms exporting products to the United States. These firms will be dealing with the U.S. importer directly, and the importer is required to obtain proof that seafood exported to the United States has been produced in accordance with the HACCP regulation. Both domestic processors and importers must implement and maintain written verification of procedures to ensure that the product

is processed in accordance with 21 CFR Part 123. Verification procedures, at a minimum, shall list the following:

1. *Product specifications addressing food safety for each product.* Examples of such specifications include no residues of drugs in fish from aquaculture (or, where a residue tolerance exists, no residues above that tolerance) and histamine levels below 50 ppm. The example of histamine specifications requires elaboration as it can illustrate several practices that domestic processors or U.S. importers may choose to adopt in setting their specifications and in conducting their own sampling and analysis. A histamine level of 50 ppm is regarded by FDA as indicative of decomposition, but not necessarily of a health hazard. The FDA regards a level of 500 ppm or above as sufficient to characterize a product as a health hazard (although some individuals may become ill after consuming fish with histamine levels below this level). However, if histamine is present in a particular lot of fish and sample testing reveals levels at or above 50 ppm, then not only is the fish decomposed and unacceptable for reasons outside of the HACCP requirements, but there may be fish in that lot with levels higher than 500 ppm. Further testing by the domestic processor, importer, or FDA would have some statistical probability of revealing such levels. In considering the establishment of specifications, it is important to recognize that the process of decomposition, and of histamine formation in particular, may continue even at refrigeration temperatures (even though bacterial growth is inhibited, the bacterial enzymes necessary for histamine production are already present). In such a case, a marginally acceptable product at receipt might quickly become hazardous after receipt. For this reason, it would be wise to establish histamine requirements that not only ensure a product is within compliance for reasons of wholesomeness, but also would protect against safety hazards due to variability within lots.
2. *Written procedures stating the affirmative steps.* The rule provides six suggestions to the importer for undertaking affirmative steps. Table 3 presents these as they are stated in the rule. An importer may hire a competent third party to carry out the affirmative steps in their written plan, but the importer must maintain records, in English, that document these affirmative steps. These records are to be maintained for one year for perishable products and for 2 years for shelf-stable products.

The rule stipulates special requirements for imported products but does not prescribe the affirmative steps that an importer must take. Instead, the rule clearly states that the importer is free to

**TABLE 3** Importer Affirmative Steps

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- (A) Obtaining from the foreign processor the HACCP and sanitation monitoring records required by this part that relate to the specific lot of fish or fishery products being offered for import;
  - (B) Obtaining either a continuing or lot-by-lot certificate from an appropriate foreign government inspection authority or competent third party certifying that the imported product is or was processed in accordance with the requirements of this part;
  - (C) Regularly inspecting the foreign processor's facilities to ensure that the imported fish or fishery product is being processed in accordance with the requirements of this part;
  - (D) Maintaining on file a copy, in English, of the foreign processor's HACCP plan, and a written guarantee from the foreign processor that the imported fish or fishery product is processed in accordance with the requirements of this part;
  - (E) Periodically testing the imported fish or fishery product, and maintaining on file a copy, in English, of a written guarantee from the foreign processor that the imported fish or fishery product is processed in accordance with the requirements of this part; or,
  - (F) Other such verification measures as appropriate that provide an equivalent level of assurance of compliance with the requirements of this part.
-

take any steps that would serve to assure that the product arriving for import is in compliance (Table 3, item F). The exporter and U.S. importer have a mutual need to present adequate proof to inspectors that the requirements have been met. A U.S. importer may request that a foreign exporter provide an English version of a HACCP plan in order to satisfy the importer's requirements for verification. However, the exporter may find that producing an English translation is difficult, particularly on short notice. For reasons such as this, flexibility is provided in the regulation for importer verification such that an agreement between the two parties might satisfy the intent of the regulation.

FDA will verify an importer's compliance with the rule by conducting HACCP verification of imports. This may be accomplished through field examination, sample collection, document review after notification of import, or document review at the importer's place of business. If no HACCP verification is presented from the importer, or such verification is inadequate, the shipment and/or future shipments may be detained without physical examination (DWPE). FDA also conducts foreign inspections, but the primary basis for establishing compliance with 21 CFR Part, 123 is through inspection of importers and the determination of compliance as evidenced by the importer's verification procedures and records. When physical examination reveals no violations (including those pertaining to wholesomeness, labeling, and other relevant requirements for full compliance with the Federal Food, Drug, and Cosmetic Act) and the HACCP documentation is adequate, the shipment is released. If HACCP documentation is absent or inadequate, the lot is detained and future shipments of that product or similar product(s) from the exporter to the importer will be detained. The FDA District Office responsible for finding the violation will recommend DWPE for the affected product(s).

It is important to recognize that the DWPE decision does not necessarily affect all products from the specified foreign processor. An Import Alert, describing the establishment of a DWPE, is specific for the combination of a particular importer and a specific product from a particular foreign processor. There may be other products from that processor for which the importer does have adequate HACCP verification, and these products would not be covered in the Import Alert. If, however, the foreign processor is found to have inadequacies in a HACCP plan, in the monitoring of sanitation procedures, or in corrective action records, the foreign processor could be placed on an Import Alert through which all products from that processor would be subject to DWPE. Once an importer provides documentation of verification, the FDA District Office responsible for finding the violation recommends removal from the DWPE order. If the DWPE order resulted from the importer's failure to have and provide adequate HACCP documentation, then there is no requirement that five consecutive shipments must be in compliance upon entry, as is the case for products that have been placed under DWPE orders because of laboratory determined adulteration.

## VIII. SUMMARY

The diversity of sources of seafood, varieties of seafood, and the innumerable processes involved in its production pose substantial food safety challenges and demonstrate the tremendous utility of the HACCP process. The implementation of the far-reaching seafood HACCP program involved a 2-year preparation period and one year of implementation. Now all suppliers of seafood, U.S. and foreign, are expected to have established and be operating under HACCP safety controls and sanitation provisions. Much to the program's credit, there have been no serious interruptions in seafood trade since the enforcement date of December 18, 1997.

As the program continues to evolve, with an ever-increasing number of people becoming trained and guidance being continually refined, seafood safety continues to serve as a leading example of HACCP system utility. As such it plays a pivotal role in international trade, allowing foreign suppliers to improve the flow of trade in seafood by improving seafood safety. To do so, exporters should ensure that their products have systematically met the U.S. importer's product specifications, particularly for safety. Second, exporters should provide the evidence for HACCP safety controls and for sanitation controls to the U.S. importers so they can demonstrate to the U.S. inspectors that the



products have met the requirements of 21 CFR Part 123. Third, exporters should work cooperatively with their seafood inspection authorities to establish a clear record of compliance with safety requirements through HACCP application. Fourth, exporters should work cooperatively with their government seafood regulators to provide the information necessary to establish a Memorandum of Understanding, or similar agreement on seafood inspection and safety assurance, between the U.S. government and the exporting country's government.

New challenges to the U.S. food supply have prompted the FDA and others to consider adopting a HACCP-based food safety system on a wider basis. In 1998 the U.S. Department of Agriculture established a pathogen-reduction HACCP program for raw meat and poultry producers, and HACCP continues to find additional applications to food safety, most recently for fruit and vegetable juices in a final rule released January 19, 2001(5). The FDA will continue to conduct pilot HACCP programs with volunteer food companies in order to determine the degree to which HACCP can become a food safety standard in other areas of the food industry as well.

## ACKNOWLEDGMENTS

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