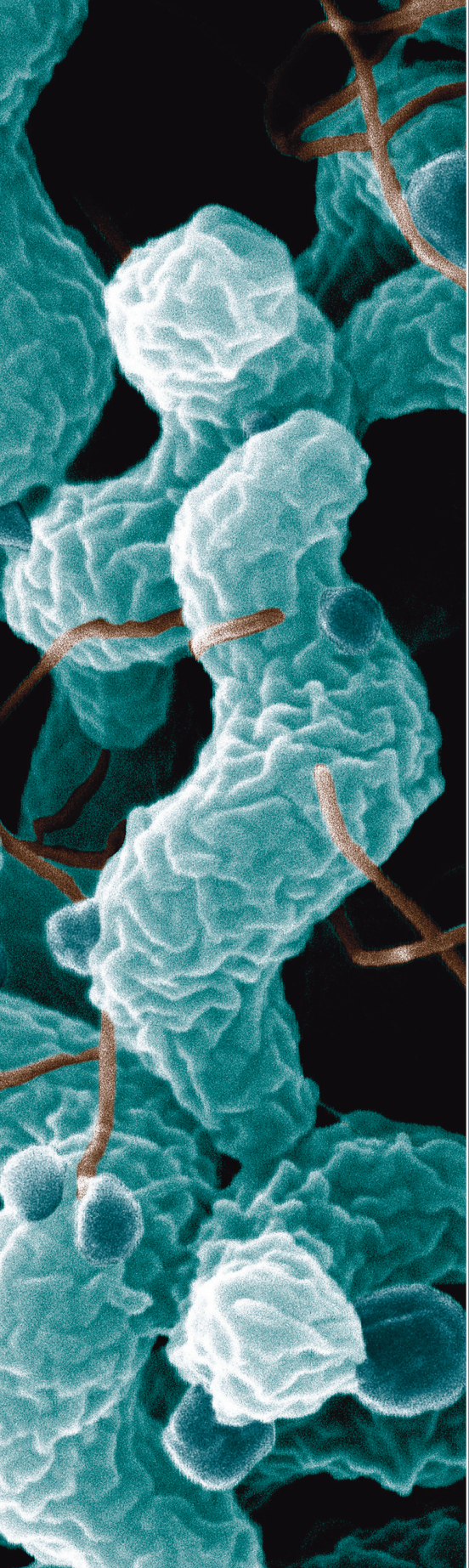

Guide to Foodborne Pathogens

Second Edition

Edited by
Ronald G. Labbé
Santos García

WILEY Blackwell

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1 Globalization and epidemiology of foodborne disease

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1.1 Introduction

Infectious and toxigenic pathogens transmitted through food have been recognized for over 100 years. By the 1950s, the main pathogens of concern in the UK and the US were *Salmonella*, *Staphylococcus aureus* and *Clostridium perfringens*. Botulism had also been understood as a dangerous disease related only rarely to commercially canned food, home canning of vegetables, or associated with traditional marine mammal products in the Arctic. Therefore, for most public health officials, foodborne disease, or food poisoning as it was called then, was generally considered to be an inconvenience for a day or two, and more of a nuisance than a threat to life. Not much was known in other countries because of a lack of any systematic reporting program. In fact, there was little interest or research being carried out on acute foodborne disease agents. We knew from outbreaks that most of the situations could have been avoided if there was proper time and temperature handling and storage of food, especially meat and poultry. It seemed that once staff in foodservice establishments became better educated, these problems with *Clostridium, Salmonella*, and *Staphylococcus* would resolve themselves. However, by the time that the 1980s came in, we were beginning to be a little more concerned with agents like *Campylobacter*, *E. coli* O157:H7, *Listeria monocytogenes* and *Yersinia enterocolitica*. However, it took several years for health authorities to recognize that these had the potential to cause serious complications or death, could be transmitted by a variety of products, and that there were limited control mechanisms in place to reduce such foodborne disease. Large outbreaks in the US arising from *Listeria monocytogenes* in 1985 and *E. coli* O157 in 1993 resulted in changes to food safety policy in the US and other countries, specifically aimed at these organisms. Now, it is recognized that a variety of pathogens in many different types of foods can cause illnesses that may be life-threatening. These include *Cyclospora*, *Cryptosporidium, E. coli* O157 and other shigatoxin/verotoxin-producing *E. coli*, multidrug resistant *Salmonella*, *Shigella* and small round structured viruses (SRSV), mainly norovirus, in produce, dairy products, eggs, ice

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cream, and shellfish. Current surveillance systems are only capable of detecting a few of these pathogens. However, with DNA typing systems, like PulseNet, more interstate and international outbreaks are being detected.

1.2 Globalization of foodborne disease

There appears to be a general increase – or at least a plateauing – for foodborne disease cases throughout the world, even though new regulations and educational strategies are being adopted nationally and internationally. One of the reasons for this is that surveillance of foodborne and waterborne disease has been very limited in its ability to detect cases other than small clusters of ill persons in the same general geographic area. The traditional passive system of letting outbreak reports be sent to a central source in a very few countries has been the source of our knowledge for decades, but it is far from adequate. Outbreaks tend to be only investigated and written up if they are large enough. Many household illnesses are never documented. Therefore, we are more familiar with mass catering or restaurant outbreaks or those involving a well-publicized processed product. Even with these limitations, we have learned much about the types of implicated foods for which we can anticipate problems even if we are not ready to initiate targeted control programs.

The globalization of the food supply is another issue that can increase the risk of foodborne disease. Changes in farming practices, with larger operations and faster throughput, the drive to increase profit by recycling all animal materials, and the difficulty in disposing of manure, all lead to the increased likelihood of contamination of raw animal products. There has been much more intensive rearing of animals, which allows transmission of pathogens even if the animals themselves are not affected. Treatment of flocks and herds with antibiotics is primarily for growth enhancement but extensive applications have led to increased antimicrobial resistance in the gut flora of animals, and combined with inappropriate use for self-treatment of human infections is increasingly a problem for the population worldwide.

Environmental sources of contamination, such as rodents in barns and gulls in fields, are being recognized as important links in the transmission chain in zoonotic diseases. New varieties of strains appear in human cases, but may originate in environmental or animal-raising conditions where genes can be transferred from one organism to another. Large-scale aquaculture is another rapidly-expanding industry in many parts of the world where fish and shellfish are raised in close proximity to contamination sources and are prone to carry enteric viruses or bacterial pathogens, as well as seafood toxins if they are present in the aquaculture areas. We have also moved rapidly from local manufacturers producing our food to national industries and now international trade with wholesalers packaging products in ways appealing to local populations. The larger market size and wide geographic distribution of products means that, if problems occur, many people are at risk and extremely large outbreaks with thousands of cases have occurred. One example is the 2011 *Escherichia coli* O104:H4 outbreak with more than 4000 cases and 50 deaths; these were mainly in Germany but many people, including visitors to Germany, were affected in another 15 nations. The source was eventually tracked to fenugreek seeds from Egypt sprouted in Germany but not before the Spanish cucumber industry was largely destroyed over the outbreak period as it was initially fingered as the most likely vehicle for the pathogen's transmission. This illustrates how trace-back of a product to its source becomes difficult, especially if the originating company is in a foreign country.

Immigrants and travelers introduce people to new types of foods, and the public today wants a wide choice of products to eat. This leads to many companies trying out new

products or modifying traditional ones, with each step a potential for the introduction or growth of a pathogen. The consumer wants more varieties of food and in packages that require the minimum of preparation. This means that many types of ready-to-eat (RTE) packaged food may be stored in a refrigerator for long periods of time. This allows various organisms, such as *Listeria*, *Yersinia*, and *C. botulinum* type E, if present, to grow slowly to reach numbers that can cause illness. Therefore, it is perhaps not surprising that we see an increase in foodborne disease throughout the world, especially as we are living in an age with a rapidly growing and migrating society. The demographics of the population are also changing due to our ability to live longer and improved public health services and medical treatment. However, as a result of this there is an increase in the immunocompromised group, especially those who are aging. These people are at risk from infections by opportunistic pathogens, for which we have no or limited surveillance mechanisms and laboratory capabilities to detect.

1.3 Measuring the impact of the burden of foodborne disease

Ways to improve the surveillance system include linking newer epidemiological approaches with molecular fingerprinting of clinical and food isolates. In the US and many other countries around the world, the PulseNet laboratory typing of strains by molecular techniques is possible by comparing isolates from across the country to look for identical strains and possibly detect common sources. The FoodNet program in several sites allows detailed investigative studies in consumer food hygiene and physician diagnostic practices, food preferences, and case-control studies when illnesses have occurred. For each outbreak, there is much information that can be gathered in if the incriminated food is available. Counts of pathogens in food, for example, are useful for determining doses and quantitative risk assessments. Since we know that surveillance data are very much underestimated, more realistic figures can be made, even if they are not precise, such as the recently estimated 48 million foodborne cases in the US (range, 28.7–71.1 million). From these, the economic burden of disease can be estimated. Although these are crude figures, they put the costs of foodborne disease and potential control measures into perspective. For instance, in the US the cost of six foodborne bacterial diseases alone totalled US $2.9–$6.7 billion in the 1990s, excluding industry losses and legal suits; these values would much higher today. Surveillance must not only be national but also continental and eventually global. EnterNet (formerly Salm-Net) allows tracking of *Salmonella* and other enteric pathogen isolates throughout Europe and a few other countries. There is logic for blocks of free-trade countries to compare systems and eventually adopt a common one, not to rapidly track international outbreaks and stop them, but to learn from the experience and understand the factors that led to them occurring.

The World Health Organization (WHO) has established a Foodborne Disease Burden Epidemiology Reference Group (FERG) which is charged to:

- assemble, appraise and report on the current, the projected as well as the averted burden of foodborne disease estimates, with the burden expressed in Disability-Adjusted Life Years (DALYs);
- conduct epidemiological reviews for mortality, morbidity and disability in each of the major foodborne diseases;

- provide models for the estimation of the burden of foodborne disease where data are lacking;
- develop cause attribution models to estimate the proportion of diseases that are foodborne; and, most importantly
- use the FERG models to develop user-friendly tools for burden of foodborne disease studies at country level.

Data generated from these will allow prioritization of food-pathogen safety issues for future research, prevention and control measures.

1.4 Investigation of foodborne disease outbreaks

To determine the agent and vehicle for a outbreak, an epidemiological investigation is carried out through epidemiological associations and laboratory analysis. Awareness of a foodborne disease outbreak by public health authorities is triggered by: consumer complaints; government laboratory-based surveillance for reportable diseases, food monitoring programs; and industry alerts and recalls. However, by the time an investigation is under way, many people may be exposed to the contaminated product and, in some cases, the outbreak may be over. Since most alerts start at the local level with one or two complaints, the appropriate agency has to make a decision when to assemble a team of experts to follow up with these. More often, one food or environmental health inspector will conduct telephone interviews with the complainant(s) and may visit any establishment(s) suspected to be the causative location by those who have fallen ill. In many cases, the last foodservice facility visited will be the prime suspect in the complainant's mind but, through judicious questioning, the inspector may identify other scenarios or consider there is no link between the illness and food. If further investigation is warranted, the inspector may request assistance to conduct more interviews and analyze food and environmental samples. For multiple reports of illness seemingly associated with one food, a case definition is desirable. Case definitions are defined based on: the characteristics of the illness, the pathogen, toxin or other agent, if known; symptoms typical for that agent; time range for when the illnesses occurred; the regional distribution of the cases; and any other pertinent laboratory or epidemiological data. It is important to locate the extent of the outbreak as soon as possible. This is done by: monitoring for more consumer complaints and checking emergency room records; reviewing surveillance and laboratory reports; and asking lab personnel and health officials in other jurisdictions to report any potential cases that fit the case definition even if they are not yet confirmed. At this stage, an epidemic curve will demonstrate the occurrence of a point-source outbreak (the food was all eaten in one time period) or one with a continuing source (exposure to a population over a longer period of time); plotting such a curve also indicates that cases have a short or long incubation periods.

By this time, it should be possible to generate a reasonable hypothesis about the causative agent and its source. Initially, any enteric infection can arise from exposure to food, water or a colonized/infected person or animal, or even touching surfaces that have been contaminated by the above. Before any food is suspected, drinking and recreational water uses and other activities such as visiting a petting zoo or playing with pets need to be ruled out. Initially, it may be difficult to formulate an hypothesis because of limited data. People do not remember all the details of their illnesses or what they ate and did days or weeks ago. It may be necessary to ask about food preferences, including what is typically or rarely eaten as

well as what they ate over seven days prior to their illnesses. Food ingredients, including spices, herbs and garnishes, are easily forgotten or never noticed at the time. It may be more time consuming to probe further, but checking foods in refrigerators, freezers and pantries, and reviewing shopper card purchases can yield useful supporting data.

Hypotheses can be tested by epidemiological means and laboratory analyses. All the obtained data must be reviewed to determine the probability that the illness came from one contaminated food source. The food consumed most frequently by the victims is a good place to start, although a contaminated ingredient may be in more than one food. Statistical approaches are used to link those ill with specific foods, e.g., food-specific attack rate tables, chi-square, odds ratio. A dose–response relationship may be worth following up if the quantities of food and beverages consumed are known (e.g., did those ill eat more of the suspect food?). Consider whether the distribution of potential food items matched the geographic spread of cases. Based on these data, ideas may be possible on the likely contamination points in the food chain, i.e., production, transportation, storage, preparation of food. Laboratory analyses of recovered food samples may be fruitful in isolating an agent. Ideally, the laboratories should be competent and certified, and use the appropriate culture methods to isolate the agents. Clinical and food isolates can be compared for similarity, with the likelihood enhanced if serotyping, phage typing or molecular typing methods are used. PulseNet comparisons are normally done at the state/provincial and national level. However, confirmation by laboratory methods is not always possible, because the sample may be wrongly selected or in inadequate amounts, contaminated by the producer or the consumer, or simply not available. Also, the laboratory staff may not have the required culture media or be sufficiently expert to isolate a pathogen or its toxin, even if it is present. Thus, it is not always possible to categorically state the cause of an outbreak even though all the epidemiological findings clearly point to foodborne transmission. In fact, investigators identify a specific food as the source of illness in only about half of the foodborne disease outbreaks in the US and other countries. If the outbreak has apparently ended (no more cases are reported), the source of the outbreak is declared as unknown. However, if indications of illnesses are still occurring, investigators must keep gathering information and studying results to find the food that is causing the illnesses and remove it from the market. This may require an environmental assessment to discover how the food was contaminated.

An environmental assessment could point to one food facility or multiple food facilities. In the former case, contamination was likely in that facility or during transportation to it, where employees, kitchens, and environmental sources would be investigated in more detail. The investigation of ill and well persons and the facility, e.g., restaurant or retail store, will narrow likely sources of contaminated food. In a commercial establishment, a history of regulatory compliance and violations may give clues to one or more contamination scenarios. Where more than one facility has been implicated, the contamination probably occurred earlier in the food production chain and this would require a trace-back approach, a more complex form of investigation. This requires checking on the food suppliers and the delivery systems. The further back in the food chain, the more likely other facilities will have received the contaminated food and a trace-forward is then required. Interstate/provincial sources and imported food may have to be investigated. If the contamination source is close to the beginning of the food chain, many agencies and even countries may be involved (multi-state/multi-province/international outbreak). The environmental assessment report will recommend control procedures to stop the outbreak, e.g., recalls and embargoes, and recommend prevention strategies to prevent future contamination. The source trace-back should suggest ways to control the outbreak and prevent similar outbreaks from happening in the

future. In some larger outbreaks, the authorities all the way up to the national level are familiar with details of the outbreak because they have been involved in the overall investigation. However, many outbreaks are small and local but, since every good investigation yields important information on causes and trends, they should be documented. Thus, even small outbreaks should be written up in a standardized format and copies sent to state/provincial or federal/national authorities for record keeping. Analyses of these over time will indicate how well existing prevention and control programs are working, and point to priorities for reducing food contamination and thus foodborne disease.

1.5 Vehicles frequently implicated in foodborne illness

In this section on foods associated with outbreaks, some recent but also some older ones are briefly described to show that many of these types of problems have occurred over decades. They also reflect a wide range of scenarios that may be encountered in any country. Zoonotic diseases are the most difficult to control because there are many entry points for the pathogens into the food chain. In contrast, there is a better chance of reducing foodborne diseases spread by human carriers such as *Staphylococcus aureus* where an organism must come from a food worker and grow to large numbers before the enterotoxin is produced. In fact, there has been a reduction in staphyloccocal food poisonings in the UK, the US and Canada. It is more difficult to prevent enteric virus transmission because the infectious dose is very low and no growth time is necessary, but better hygiene by food workers and restricting infected workers to non-food areas will help keep the contamination rate down. Food workers, knowingly or asymptomatically, have caused large outbreaks, particularly from norovirus and *Salmonella*. There are some more recent trends where the detection of multistate outbreaks involving a widespread distribution of a contaminated product is now feasible with molecular typing techniques even though the number of cases may be low in any one state or region.

1.5.1 Meat and poultry

It has been recognized for many decades that *Salmonella* has been transmitted by meat and poultry. Typically, limited food safety knowledge has led to undercooking, improper cooling, or cross-contamination. The following are some examples. In 1998, over 50% of guests at a church supper in Maryland were ill after eating ham (750 cases and one death). The hams had been packed too tightly into cooking pots, creating uneven cooking conditions. Then they were packed too tightly into the freezer, which prevented rapid cooling and gave the salmonellae present an opportunity to grow. One slicer was used to cut all the hams without any sanitation between the slicing process, thus allowing the transmission of salmonellae onto many of the ham pieces served. In Alaska in 1992, leftover improperly cooked roast pig was taken home by some picnic goers and only those who microwaved the meat to reheat it (10) versus those who used an oven or skillet for the same purpose (20) were infected. Microwaving was also an issue in 2007 when consumption of commercially sold pot pies caused 425 cases of salmonellosis in a multi-state outbreak because the labeling was insufficiently clear to indicate that some of the ingredients were uncooked, and the microwave reheating process used was insufficient to kill the *Salmonella*. In Texas, from May to October 1995, 59 persons were ill from *S*. Agona infection. All those who lived in

San Antonio and Houston had eaten food in a San Antonio Mexican restaurant. The same pulse field gel electrophoresis (PFGE) strain was isolated from machacado (air-dried raw beef), which had been shredded in a blender and cross-contaminated other foods such as salsa. Austin residents had eaten machacado at a similar Mexican restaurant. Both restaurants had purchased the beef from the same supplier in Corpus Christi. Without PFGE typing of isolates, these outbreaks would not have been detected. Poultry still causes many outbreaks including ones with widely distributed products. For instance, in 2011, there were two US multi-state outbreaks involving turkey (124 cases in 34 states of *Salmonella* Heidelberg from ground turkey, and 12 cases in six states of *S*. Hadar from turkey burgers).

Acute illnesses are not the only problems arising from salmonellosis and other enteric diseases. For example, in 1984, 423 provincial police officers were infected with *S*. Typhimurium after eating contaminated meat sandwiches provided to them as they lined a route in Ontario, Canada, for the Pope's visit; 6.4% had acute reactive arthritis (RA) with one third resolving within four months, and the majority continued to have mild symptoms in their joints for the following five years. Four had sufficient damage to force them to change jobs. The risk factors identified were severe diarrhoea at the time of the outbreak and presence of specific genes that coded for HLA-B27 and HLA-CREG antigens. In an Australian outbreak in March 1997, of those ill after eating pork rolls produced by a bakery, two died and 3% developed RA. One half of those with arthritis (1.5%) continued to have it 12 months later. In another outbreak in August from the same product, 5% developed RA. The difference in the percentage affected may be due to the ethnic background of those ill who may have a differing proportion of susceptible genes (Vietnamese in March and Cambodian in August). Sequellae such as RA are more frequent and are more long lasting than previously recognized.

Multidrug-resistant *Salmonella enterica* serotype Typhimurium definitive phage type 104 (DT 104) has emerged during the last decade as a global health problem because of its association with animal and human disease. Multidrug-resistant strains of this phage type were first identified from exotic birds in the UK in the early 1980s and in cattle and humans in the late 1980s but have since become common in other animal species such as poultry, pigs and sheep. Since 1991, this phage type has been second only to *S*. Enteritidis phage type 4 as the principal agent of human salmonellosis in the UK. The DT 104 epidemic has now spread globally, with several outbreaks since 1996 in the US and Canada. No effective treatment of infected animals has yet been found. In humans, there is a relatively high mortality rate of 3%, especially in the elderly. It is spread from farm to farm by water and is difficult to eradicate since it survives well in both dry and wet environments. It has been found in beef, pork, salami, chicken and cereals, and foodborne disease outbreaks have been associated with hamburgers, sausages and sausage rolls. This pathogen can be present in poultry flocks and other meat animals and extend down the food chain from farm to fork. Because of its ability to colonize many farm animals and be in the environment, its impact will likely be worse than most other serovars including *Salmonella* Enteritidis worldwide.

Campylobacter has been associated in outbreaks and epidemiological studies with undercooked chicken and meat, as well as from other sources. A case-control study in four cities in New Zealand bears this out. Risk factors were: raw or undercooked chicken; any chicken prepared in a sit-down restaurant; unpasteurized milk or cream; overseas travel; rain water; contact with puppies and calves. Barbecued or fried chicken was associated with illness whereas baked or roasted chicken was protective, although the reason for this was not determined, unless the former was more typically undercooked. Although cases of sporadic campylobacteriosis are frequent, large *Campylobacter* outbreaks from poultry or meat are

relatively rare. In the following example, chicken was the source of the pathogen, but it was other foods that caused the illness through cross-contamination. In 1996, at least 14 patrons of a restaurant in Oklahoma were ill with campylobacteriosis after eating lasagna and lettuce. Raw chicken had been cut up on a cutting board and the lettuce and lasagna contaminated by the cook's unwashed hands, utensils or counter top. Because the infectious dose of *Campylobacter* is low (500 cells), one drop of chicken exudate can be enough to infect a consumer. The workers had frequently used the same towel to dry their hands. The investigation emphasized the importance of clean up and sanitation of preparation areas and of handwashing facilities. In 2010 in Scotland, a chicken liver paté was improperly cooked and caused 24 cases of *Campylobacter* gastroenteritis at a wedding reception. The raw chicken livers were mixed with a red wine reduction and raw eggs, heated using a bain marie (water bath) to a core temperature of 65 °C and then immediately removed from the oven and cooled for 15 minutes. According to the UK Food Standards Agency advice, if liver is cooked at 65 °C, it should be held at this temperature for at least ten minutes to ensure adequate cooking.

E. coli O157:H7 was first identified as a foodborne pathogen in 1982 from two outbreaks involving hamburgers served in fast food restaurants of the same chain in Michigan and Oregon. Reports of other outbreaks followed with undercooked ground meat as the most frequently-associated vehicle. By 1985, the most serious outbreak then reported occurred in a London, Ontario, home for the aged where 70 were ill and 17 died after eating ham sandwiches probably contaminated by an infected food worker. The most significant US outbreak occurred in 1992–1993 in four western US states with a total of over 700 cases and four deaths. Hamburgers from a fast food chain had been insufficiently cooked. Many children were affected which caused sufficient concern for the government to make the pathogen an adulterant in ground beef so that no level could be tolerated. This rule did not, unfortunately, stop the outbreaks which could involve very large amounts of ground beef. For instance in 2002, contaminated ground beef was linked to 28 *E coli* O157:H7 infections in Colorado and six other states. The manufacturer recalled 18.6 million pounds of ground beef products that were produced at the processing plant identified as the source.

The most deadly outbreak of *E. coli* O157:H7 to date occurred in Scotland in November and December 1996, with nearly 500 cases and 20 deaths, mainly elderly people. Those ill had eaten cold cooked meats, meat sandwiches and cooked steak in gravy prepared by the same butcher, and distributed to many locations including nursing homes. As a result of this episode, numerous changes in food hygiene were initiated in the UK. These recommended changes were not sufficient, however, to prevent the death of a child in Wales in 2005, when 157 persons, mainly schoolchildren, suffered from *E. coli* O157:H7 infection because school meals supplied by a caterer butcher were cross-contaminated through dirty equipment and carelessness by employees and management. These two outbreaks remain those with the largest number of cases in UK history. In 2011, 14 persons in five US states were infected with *E. coli* O157:H7 in Lebanon bologna; this product is a fermented, semi-dry sausage, similar to salami. Salamis and other sausages have caused *E. coli* (O157 and non-O157) outbreaks in Australia, Canada, the US and Europe. There are also risks to hunters; 11 persons were ill after eating home-made jerky prepared from a deer that had been shot. The hunters were not hygienic in storing or dressing the carcass and preserving the meat. *E. coli* O157:H7 was found in the patients, jerky, uncooked deer meat, a saw used on the carcass, deer hide and deer fecal pellets. *Salmonella* has also been implicated in sausage outbreaks. Since this pathogen in food is primarily derived from the feces of domestic animals, it is not surprising that

petting zoos have been the source of infections. One example is from North Carolina, when in 2005 82 visitors to a petting zoo at the North Carolina state fair were infected.

In late 1998, at least 100 people contracted listeriosis after eating hot dogs and other processed meat cold cuts in several US states. There were at least 20 deaths and miscarriages. *L. monocytogenes* in the processing facility had contaminated the already cooked hot dogs before packaging. In 2007 in the EU, *L. monocytogenes* was found rarely in various meat products – pork, 2.2%; red, mixed or unspecified meat, 2.5%; 3.0% in RTE broiler meat – with less than 1% of samples exceeding the 100 cfu/g limit. However, some countries did have samples much more frequently contaminated: Germany, Greece, Italy, Poland and Slovenia reported presence of *L. monocytogenes* in samples of 25 g in 11.0%, 20.7%, 13.6%, 62.9% and 16.7%, respectively. A European Food Safety Authority (EFSA) study showed that *L. monocytogenes* was still a problem because of poor practices in the use of slicing machines for meat products, inadequate storage temperatures, the lack of an effective HACCP system, and lack of education and training of food workers. Storage temperatures in retail and domestic refrigerators were found to vary significantly, especially for the domestic refrigerators. Despite more awareness, the largest recent outbreak involving listeriosis in a meat product was the Canadian Maple Leaf outbreak in 2008 when over 50 persons were infected and at least 22 died. The use of large commercial slicing machines that were difficult to clean was a major contributing factor, leading to biofilms and contamination of the sliced deli products. Many deli meat products were contaminated, including salami brands. In 2009–2010, a smaller listeriosis outbreak occurred in Ontario from a fermented salami product. These two outbreaks have led manufacturers to consider ways of limiting or preventing growth of *L. monocytogenes* in these types of products. One approach is the development of specific starter cultures with multiple organisms to prevent the growth of *Listeria* and spoilage organisms under a wide range of temperature conditions while maintaining a reducing atmosphere to preserve the traditional colour and flavour of the salami-type products.

Clostridium perfringens toxico-infections are relatively frequent, and it is the pathogen third-most frequent cause of foodborne illnesses in the US. Most of the outbreaks involve slow cooling of large quantities of stews and soups where the spores have a chance to germinate and outgrow to numbers sufficient to cause abdominal cramps and diarrhea. In an institution in England, 12 joints of lamb were thawed and roasted for five hours and stacked on a single tray to cool before refrigeration. The next day, some of the meat was removed from the refrigerator and sliced, and left at room temperature for several hours before being served. This was repeated the next day with the rest of the lamb; 12 staff and residents were ill with cramps and diarrhea after an incubation period of 9–16 hours. *Clostridium perfringens* was found in the stools of the cases and in the leftover lamb ($>10^5$/g). Improper cooling of the joints and storage periods at ambient temperatures allowed growth of the pathogen. In London in 2009, 93 guests at two weddings suffered *C. perfringens* gastroenteritis after eating either a curried chicken or lamb dish that had not been cooled sufficiently quickly by the caterer, or reheated properly later. Also in this case the food was not refrigerated during transportation between the caterer and the wedding reception sites, and the food worker preparing the items had fecally-contaminated hands.

Foodborne outbreaks caused by norovirus (previously called small round structured virus – SRSV or Norwalk-like virus – NLV) typically involve an infected food worker preparing RTE foods which may include meat and poultry but more often multi-ingredient foods like hors d'oeuvres. One example is a caterer with a norovirus infection who transmitted it to 67 people attending an international AIDS conference in Wales. The food handler, who had boned the cooked chicken with bare hands, had been ill two days earlier.

1.5.2 Fish and shellfish

Cholera remains a threat in much of the world with limited sanitation and potable water. The seventh pandemic began when cholera was first documented in Peru in 1991 and rapidly spread to other Latin American countries with millions of cases. *V. cholerae* O1 was found in water, plankton, sewage and seafood. Street-vended foods, crabs, shrimp, ceviche and beverages containing ice were all vehicles of transmission. The economic loss from lost markets and tourism, absence from work, medical care and the value of lives lost was high. In Hong Kong in May 1998, 34 cholera cases occurred after they had been to restaurants where hotpot (raw meat and seafood dipped into a communal boiling broth and eaten with a raw egg and sauces) was served. Live seafood from polluted water was probably the source of cholera, as found in a 1994 outbreak when tank water for fish and lobsters contained *Vibrio cholerae*. Cholera can also affect trade, as in Mozambique in 1997. The country had a yearly catch of 20,000 tonnes, with more than 50% exported to the EU. When cholera caused over 30,000 cases and 780 deaths starting in August of that year, the EU banned import of Mozambican fish, costing the country US$240 million.

Vibrio parahaemolyticus is a pathogen that occurs naturally in warm waters, including North America. It is resident year round in the Gulf of Mexico and increases in number during the summer months. One example of an outbreak in that region occurred in July 1998, when over 400 persons in five states were ill from *V. parahaemolyticus* from contaminated oysters harvested in Galveston Bay, Texas. The pathogen was identical to ones found in SE Asia, and probably came from dumped ballast water. Texas had a heat wave with no water running into the Bay and any discharge would stay in same area allowing oysters to be contaminated. Sales were stopped if counts were >10,000/g, but this did not contain the outbreak which continued until December. The same strain caused illnesses in September in Oyster Bay, Long Island, New York, affecting an oyster festival. The warmer water and less oxygen from fertilizer run-off may have allowed the vibrios to multiply. *V. parahaemolyticus* has spread to more northern waters in North America because of increasing seawater temperatures, causing illnesses, as illustrated by two examples. In July–August 1997, 209 persons were infected with *V. parahaemolyticus* on the US west coast from California to British Columbia after eating oysters and other shellfish. The water temperature then was 1–5 °C above normal. This was the first *V. parahaemolyticus* outbreak in Canada linked to food. Another outbreak occurred in 2005 in Juneau, Alaska, when cruise ship passengers ate locally-harvested oysters growing in waters of >15 °C. This outbreak was 1000 km further north than any other previous US outbreak. Although outbreaks from *V. parahaemolyticus* are rarer in Europe, an emergent virulent serotype (O3:K6) is being more frequently found there. In July 2004, a *V. parahaemolyticus* outbreak with this serotype occurred among guests at several weddings in Spain after they ate boiled crabs at a restaurant. The crabs had been imported live from the UK, processed under unhygienic conditions, and stored at room temperature for several hours before being eaten.

Vibrio vulnificus is also a warm-water pathogen, more frequently found in tropical regions, and occurs more with wound infections and sporadic food-associated cases rather than large outbreaks. In 1994 in Sweden, the summer was unusually hot and water temperatures reached 17 °C. That year *V. vulnificus* was first observed after a bather was infected. Since then, some summers have been just as warm and other cases have been reported, and one individual died. *Vibrio vulnificus* septicemias occur each year as a result of consuming oysters harvested in the Gulf of Mexico during the summer months when the water is warm enough to allow rapid growth of the pathogen. Illnesses and deaths have also been documented

in Singapore, and probably all populations in tropical areas that eat undercooked or raw seafood are at risk of infections from this species and other vibrios.

E. coli infections are rarely associated with fish or shellfish but one unusual outbreak in Japan illustrates that this is possible. In 1998, 62 cases *E. coli* O157:H7 infection were reported in four separate locations after they ate salted salmon roe distributed to many sushi shops. The salmon roe had been soaked in a liquid seasoning consisting of soy sauce (79.0%), water (14.0%), chemical seasoning (6.5%), synthetic sake (0.3%), and a fermented seasoning (0.2%); its salt content was equal to a 13% NaCl concentration. This salted salmon roe is a popular component of Japanese sushi. Research subsequently showed that the strains isolated from the roe and patients entered the viable but nonculturable (VNC) state in the high salt concentration. Yet they were capable of causing illness at a concentration of 0.73–1.5 MPN/10 g of the processed salmon roe. Previously, this traditional way of preparation was thought to be safe from pathogen growth or survival. The normal isolation methodology would not have been successful in recovering these strains. Unfortunately, the company was liquidated, and the owner was arrested on criminal charges.

Listeria monocytogenes can be present in marine waters especially if there is agricultural runoff or sewage effluent, and seafood products found to contain the organism may be recalled. However, listeriosis is only occasionally linked to seafood, as in New Zealand in 1992 when two women contracted the disease after eating smoked mussels, which resulted in the death of newborn twins. The contamination was traced to the processor; the product had been widely distributed and other cases were probably ill in England. In Sweden, nine persons suffered from listeriosis from June 1994 to June 1995 after they had eaten fermented or smoked rainbow trout produced by one manufacturer. In Canada in 1996, six cases were linked to imitation crab meat.

Improperly canned food is always at risk for botulism, whether from underprocessing or from post-process contamination. These are rare in recent years but holes in some cans of Alaska canned salmon resulted in two small outbreaks in England and Belgium respectively, killing a total of three persons and affecting trade not only in Alaska but also Canada. Stored uneviscerated fish in various countries has also led to botulism outbreaks, with one of the biggest being in Egypt in 1991 which resulted in at least 20 deaths from ingestion of locally-made *faseikh*. Although outbreaks from commercial food are relatively few today, foodborne botulism remains a serious threat, as illustrated by recent botulism episodes in Finland from inappropriately processed or stored fish products including vacuum-packed smoked whitefish. Botulism is also well-documented arising from consumption of fermented marine mammal parts in Inuit communities in Alaska and northern Canada, and fermented salmon eggs in northwest coast First Nations/Native American communities. Parasites have been found in shellfish, such as *Cryptosporidium* in oysters in Chesapeake Bay near river tributaries where there may be sewage discharge or farm runoff. No cryptosporidiosis cases have been linked to the ingestion of raw shellfish, but the potential exists.

Because, for most countries, much of the seafood eaten is imported (for the US it is over 50%), there are risks of contracting illnesses from pathogens in the originating country, like *L. monocytogenes* in the New Zealand mussels episode, *Clostridium botulinum* in canned salmon or tuna, or from *Salmonella* derived from aquaculture practices in developing countries. Therefore, a vigorous inspection program by importing countries should be in place. Preferably, this should be based on HACCP rather than random samples and microbiological testing.

Enteric viruses have long been associated with shellfish. In early 1988, the residents in the Shanghai area were offered clams from a newly-discovered bed. Unfortunately, many of

the fishing boats did not have adequate toilet facilities on board and partially treated sewage from shore communities was also entering the sea, allowing the hepatitis A virus to enter the harvesting area and be taken up by the filter-feeding clams. Most consumers prepared the clams by only scalding them with boiling water, which was not sufficient to kill the virus. As a result, nearly 300,000 estimated cases of hepatitis A occurred. It was reported that industrial production in the city fell by about 17% in February, largely because of the large number of ill workers.

Scombroid or histamine poisoning arises in certain types of fish (such as tuna, mackerel, bonito, marlin, mahi-mahi, anchovies and sardines) that were inadequately refrigerated or preserved after being caught and prone to spoilage where histidine is converted to histamine by bacterial action. Scombroid poisoning can be confused with allergic reactions to food because some of the symptoms are similar (rapid onset, flushing, headache). A scombroid outbreak with 15 cases occurred in Spain from a fresh tuna sold at a hypermarket in June 1994. Typical symptoms of facial flushing, headache, diarrhea, nausea and abdominal pain occurred 45 minutes after the tuna was eaten. Histamine was found in leftover tuna samples and in urine specimens from the cases. Such illnesses are not uncommon every year, caused not only by fresh or frozen fish but by canned fish like tuna. In August 2003, an outbreak of scombroid fish poisoning occurred at a retreat centre in California with 42 persons ill after eating escolar fish. Individuals who ate at least two ounces of fish were 1.5 times more likely to develop symptoms and more of them than those who ate less. Samples of fish contained markedly elevated histamine levels (from 2000 to 3800 ppm). This was one of the largest reported outbreaks of scombroid fish poisoning in the US and was associated with a rare vehicle for scombroid fish poisoning, escolar.

Seafood toxins are found locally in molluscs and fish throughout the world, and illnesses frequently occur but are not always documented. Most of these originate from naturally toxic plankton that are ingested by shellfish, and include paralytic shellfish poison (saxitoxins and related toxins), diarrhoetic shellfish poison (okadaic acid and dinophysistoxins), and amnesic shellfish poison (domoic acid). The first two affect fisheries in the Americas, Europe, Asia and many tropical countries. Once illnesses occur or levels of toxins reach a certain limit, harvesting stops until the toxin level in the shellfish is below the limit or the toxic algae are no longer abundant – but not all countries have monitoring programs. PSP shellfish contamination regularly occurs every year on the northern west and east coasts of Canada and the US because of the presence of *Alexandrium* blooms, and illnesses are sometimes reported from recreational harvesters. In contrast, DSP poisoning has rarely occurred in North America, despite the presence of low levels of the causative *Dinophysis* dinoflagellate. Two small outbreaks affected those eating mussels in Nova Scotia and Newfoundland in the early 1990s, and there have been several reports of sporadic cases from the east coast. In 1997, the first incident of diarrhoetic shellfish poisoning (DSP) was recorded in the UK with 49 cases that may have been caused by water temperature changes. More significantly, in the summer of 2011 over 60 persons were ill after eating mussels harvested in British Columbia and shipped to four other provinces. It is not known what caused that *Dinophysis* bloom though water temperatures were high. The outbreak was quickly contained through a recall by the Canadian Food Inspection Agency, but this outbreak raises the question of the safety of local aquaculture industry, since this may be a recurring problem. Usually in DSP outbreaks, the amounts of toxin present are not known, but this was determined in 2009, following 11 outbreaks of DSP poisoning involving 45 individuals who had consumed mussels harvested in northwestern France. The incriminated batch was still available and

consumption data were known. Both okadaic acid (OA) and DTX-3 (closely related to OA) were present. The batch of source mussels contained 1261 µg OA equivalents/kg shellfish, eight times higher than the European regulatory limit of 160 µg OA eq/kg shellfish flesh. This analysis also supported the lowest observable adverse effects level (LOAEL) to be 50 µg OA eq per person.

Domoic acid is found in shellfish worldwide, but has only caused one outbreak. This was in Canada in 1987, when contaminated mussels affected at least 107 persons, several of whom were left with permanent memory damage, and caused three deaths. As for PSP and DSP, monitoring programs are in place for domoic acid in many countries, either in shellfish or for the phytoplankton themselves. A more recent biotoxin is azaspiracid, which was first identified after an outbreak in the Netherlands caused by Irish mussels in 1995, and over 20 different analogs of the polyether compound have since been identified. The dinoflagellate responsible, *Azadinium spinosum*, was discovered only recently in 2009. Azaspiracids not only cause gastroenteritis but may have carcinogenic properties, which is giving concern to regulators. Ciguatoxin is a seafood toxin present in many varieties of tropical fish but currently there are no routine diagnostic tests to detect it. Although its effects are mainly in regions where these fish are caught, consumption of exported fish in temperate regions has resulted in several outbreaks. For instance, in Texas, 17 crew members of a Norwegian ship suffered from ciguatera poisoning after eating a barracuda caught off the Bahamas, and medical help had to be sought. There is some evidence that toxic plankton are spreading throughout the world, though climate change affecting water temperatures, ballast water being dumped in harbors or increased aquaculture. This may have happened in the Hong Kong area recently, when the causative organism of ciguatera poisoning, *Gambierdiscus toxicus*, was found for the first time in 1998. That same year, at least 71 suspected ciguatera cases occurred after they ate tiger grouper; most were hospitalized briefly. Authorities warned people not to eat fish larger than 1.5 kg. Illnesses typically occur where tropical fish are frequently eaten, as in the Pacific and Indian Oceans and the north Caribbean Sea. However, such fish served in restaurants have also caused cases in temperate regions where the disease is less well-known.

Toxins are increasingly being found in new areas and new toxins are discovered. In New Zealand in 1993, illnesses from neurotoxic shellfish poisoning (NSP) and paralytic shellfish poisoning (PSP), previously unrecorded, were documented because of water temperature changes caused by the El Niño Southern Oscillation (ENSO) effect. One incident of apparent ciguatera poisoning affected over 500 people who ate a shark in 1993 in Madagascar, causing 98 deaths. In fact, it was two heat-stable liposoluble toxins, carchatoxin A and B, isolated from the shark that seemed to be the cause of the illnesses. In Australia, 20 cases of mild foodborne disease were associated with the consumption of small cockles from New South Wales beaches hundreds of miles apart. An unknown algal toxin was suspected, and a ban was placed on harvesting. Haff Disease was first reported by people living by the Baltic Sea and associated with ingestion of burbot in the 1930s. The same disease affected people in the US from 1984 to1986 after they ate buffalo fish. In 1997, six cases developed muscle aches, rigidness or stiffness, and weakness. One patient had muscle weakness for six months. A heat-stable toxin was suspected.

1.5.3 Egg products

In recent years, *Salmonella* in eggs has been a major problem for public health agencies. *Salmonella* Enteritidis (SE), particularly phage type (PT) 4, infects egg-laying poultry

flocks and some of the eggs contain the organism. The risk of illness is more when the egg is used as an ingredient in a food eaten by many people rather than when it is a single egg. Many outbreaks have been reported around the world, and have been associated with foods such as omelets, quiche, meringues, desserts and cakes with egg ingredients, egg nog, and ice cream. For instance, five persons suffering from SE PT 4 infection in England had attended the same gym and consumed a protein-based beverage composed of milk powder and a raw egg as a body-building drink. The value of the drink to these persons outweighed any risk of enteric illness. In one of the largest SE outbreaks, in 1994 there were an estimated 224,000 cases in several US states, following consumption of ice cream. The premix had been contaminated during transport in tankers which had previously been used for carrying raw liquid egg. It was determined that, for some cases, the infectious dose was no more than 28 cells. A risk assessment in 2002 for *S*. Enteritidis in eggs estimated that only 1 in 20,000 (0.005%) eggs were contaminated with S. Enteritidis. However, with over 90 billion eggs produced each year in the US there is a potential for 4.5 million contaminated eggs entering the market each year. Based on storage, consumption and dose response data from a 2005 risk assessment, there are an estimated 175,356 SE illnesses each year from eggs, of which 1440 are hospitalized, and 75 die.

Between 1986 and April 1990, in Argentina, 35 outbreaks of SE affected 3500 persons, largely through consumption of insufficiently-cooked poultry and eggs used in mayonnaise. A similar type problem occurred in Brazil where, in 1993, 280 of 400 patrons of a restaurant suffered from SE infections after eating potato salad with a raw egg dressing; 10^6 *Staphylococcus aureus* cfu/g was also found in the salad which probably contributed to the discomfort of those ill. Even as late as 1998, the use of raw eggs was still leading to outbreaks in the UK after over a decade of public awareness; one family was ill after a member prepared a lemon soufflé at a cookery course in a college. It was only after the outbreak that the college agreed not to use raw eggs as ingredients in prepared foods. Although the risk of contamination of eggs has been reduced in recent years, one large outbreak shows that, unless the producers are vigilant, large outbreaks are still possible. Beginning in May 2010, the CDC identified a nationwide, four-fold increase in the number of SE isolates through PulseNet, with reports of approximately 200 SE cases every week during late June and early July (Fig. 1.1). Epidemiologic investigations conducted by public health officials in California, Colorado and Minnesota revealed several restaurants or events where more than one person ill with this type of SE had eaten, and that shell eggs were the most likely source of the infections. These eggs had come from a single egg company in Iowa, and subsequently 500 million eggs were recalled nationwide. From 1 May to 30 November 2010, approximately 1939 SE illnesses were reported as likely to be associated with this outbreak. The epidemic curve demonstrates the extent of the outbreak over more than six months. From the nearly 600 environmental samples taken, 11 were found positive for the same strain of SE in feed, egg wash water, farm traffic areas and manure; rodents were also found in the egg-laying area that could have spread the contamination.

Factors contributing to the contamination were: failure to prevent unwanted animals (including rodents) coming into the poultry houses; manure pits too close to the chickens, blocking doors; fly maggots in the manure pits and multitudes of flies in the houses; no practices against the introduction or transfer of SE between and among poultry houses; no changing of protective clothing when employees moved from one house to another; and failure to clean and sanitize equipment.

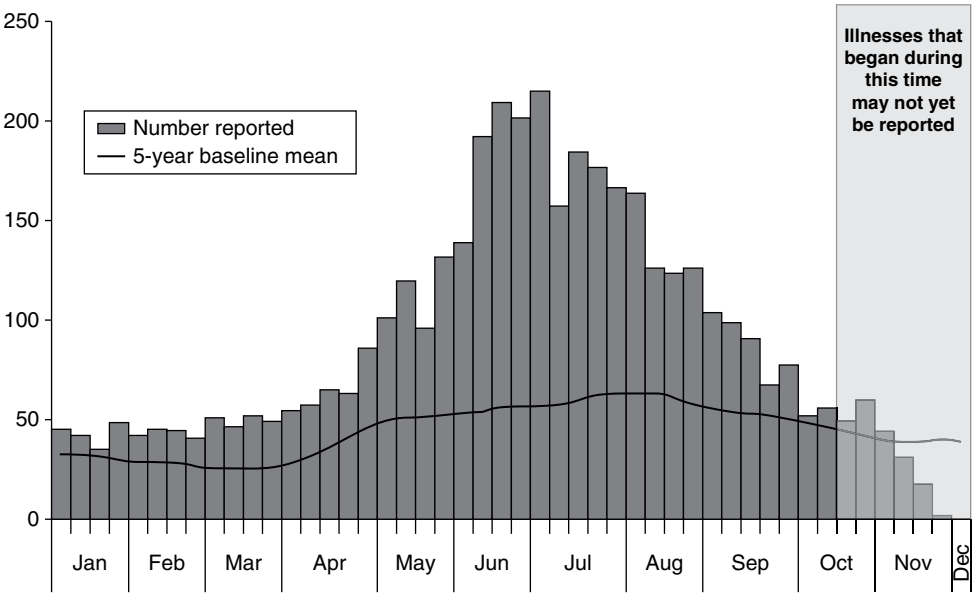

Figure 1.1 Number of *Salmonella* Enteritidis cases matching PFGE pattern JEGX01.0004 reported to PulseNet, United States, 2010. http://www.cdc.gov/salmonella/enteritidis/epi_curve.html

1.5.4 Dairy products

Outbreaks from untreated milk have been well documented, with *Salmonella*, *E. coli* O157 and *Campylobacter* being the most frequent pathogens. A large *Campylobacter* outbreak of 72 cases occurred in 1992 at an outdoor festival at an English farm at which local unpasteurized milk was sold. A similar sized outbreak occurred in 2001 in Wisconsin when 75 people drinking raw milk from one organic dairy farm with 36 cows suffered from *Campylobacter* gastroenteritis. Those ill had participated in a cow leasing program to try and avoid regulatory action since the state prohibits the sale of raw milk. The farm also gave away unpasteurized milk to tour groups, and more cases could have occurred but were undocumented. In a smaller campylobacteriosis outbreak in South Carolina in 2011 eight persons were ill after drinking raw milk. Since raw milk sales are permitted in that state, the farm continues to sell its product, especially as FDA could not find any *Campylobacter* in the samples the inspectors took. The farm produces about 80 to 100 gallons of milk per day and sells raw milk for $6 a gallon.

Occasionally, pasteurized milk has been implicated in outbreaks. In 1985, over 200,000 persons were ill from milk contaminated with *Salmonella* Typhimurium produced in one large Illinois dairy. The cause was never discovered but assumed to be a cross-connection fault that allowed contamination of the pasteurized milk. The dairy closed permanently and there were high settlement costs. In 2000, an outbreak of multidrug-resistant *Salmonella* Typhimurium infections occurred in Pennsylvania and New Jersey with 93 confirmed cases. A case-control study implicated pasteurized milk from a dairy, and an inspection indicated the potential for contamination after pasteurization. Dairy cattle were the likely reservoir. The milk had been adequately pasteurized but conditions in the plant likely allowed post-process contamination. There was excessive condensation throughout the processing and packaging areas, allowing condensation droplets to fall into open containers, several machines leaked raw milk onto the floor, and raw skim milk was held in a silo at >10 °C.

Many small outbreaks have been attributed to *E. coli* O157:H7 in raw milk and occasionally raw milk cheese though environmental contamination typically arising from the cows themselves. Risk factors for contracting the associated disease also include direct or indirect contact with farm animals or animal manure. Farming families may develop immunity to the organism. There is also concern for non-O157 serotypes. In 1994, 17 people were ill after drinking a brand of pasteurized milk in Montana. The pathogen was a non-O157 STEC (*E. coli* O104). Coliform counts exceeded 10/100 ml milk, and fecal coliforms were isolated from post-pasteurization pipes and surfaces. One new pathogen of concern is *Streptococcus zooepidemicus*, which causes acute pharyngitis, glomerulonephritis, meningitis, septicemia and death. There have been outbreaks in Romania, Austria, England and Australia from raw milk consumption. In one of these, delivered milk, meant to be pasteurized, was substituted with bottled raw milk because of supply shortages.

Reconstituted skim milk powder containing *Staphylococcus aureus* toxin caused a very large outbreak in Osaka, Japan, in June 2000. Although many staphylococci were seen under the microscope they had all been killed by the processing, but any enterotoxins produced earlier would still be effective. In fact, more than 13,000 persons were affected by the action of staphylococcal enterotoxin A (SEA). The average total intake of SEA per capita was estimated to be approximately 20–100 ng. Since this is considered a low amount to cause illness, other enterotoxins not looked for could have been present and increased the ingested dose. *S. aureus* if present in pasteurized skimmed milk under room temperature will grow rapidly and some kind of processing failure may have occurred, in this case through a power outage, to allow the organism to grow (≥500,000 cfu/ml) and produce toxin.

L. monocytogenes was declared an adulterant in RTE food by the US FDA after a major outbreak from Mexican-style soft cheeses produced in California killed 18 adults and 10 newborns in 1985. Since then, several cheese-associated listeriosis outbreaks have occurred in North America, Europe and elsewhere despite more rigorous attention to better cheese-making hygienic conditions and regulatory oversight. A commercial cheese (acid curd) made from pasteurized milk caused a large listeriosis outbreak with 189 cases in Germany from October 2006 through February 2007. The *Listeria monocytogenes* outbreak strain was identified in humans and in cheese samples from a patient's home and from the production plant. Recent European food safety alerts due to *Listeria*-contaminated cheeses more often concerned products made from pasteurized or heat-treated milk than from raw milk. Between June and December 2008, 38 confirmed cases of listeriosis in cheese in Quebec led to 38 hospitalizations, of which 14 were pregnant and gave birth prematurely. Two adults died and there were some perinatal deaths. The trace-back of food eaten found two cheese plants producing soft cheeses contaminated with the same *L. monocytogenes* PFGE type, and in early September recalls were conducted affecting more than 300 retailers which had received the cheeses. The incriminated product was a soft washed rind cheese made with pasteurized milk, but other cheeses made from both pasteurized and raw milk were found to contain nonepidemic strains of *L. monocytogenes* indicating a high degree of insanitation in some facilities. In 2010, in the US, three outbreaks were associated with raw milk cheese products and one with pasteurized cheese (with *Brucella*, *Campylobacter*, *E. coli* O157:H7, and *L. monocytogenes*). In two of these, the cheeses had been stored for the permitted >60 days before sale, indicating that this decades-old safety measure is not that effective. Overall, the types of cheese and etiologic agents involved in outbreaks in the US attributed to cheese varied depending on whether the milk used to make the cheese was pasteurized. Outbreaks associated with unpasteurized cheese were more frequently due to pathogens transmitted from animals and their environments, whereas pasteurized cheese outbreaks were associated

with contamination by food handlers. Understanding the sources and routes of contamination can help to improve the safety of cheese products.

Butter is an unusual vehicle for foodborne illness, but from December 1998 to February 1999, 18 people in Finland contracted listeriosis after eating locally-produced butter. Sixteen of these had septicemias and four died. The same PFGE strain of *L. monocytogenes* serotype 3 was isolated from the cases, the butter and the producing dairy plant. Although botulism from dairy products is rare, some serious outbreaks have been documented. In 1989, the largest outbreak in the UK took place after commercially-prepared hazelnut yogurt contaminated the nut paste containing *C. botulinum* toxin. In Italy, in 1996, eight persons, mostly children, and one death occurred after tiramisu made with mascarpone cheese was eaten. In 2011, korma (curry) sauce made with yoghurt, onions, nuts and spices and stored in glass jars paralyzed two children in Scotland from *C. botulinum* toxin.

1.5.5 Vegetables and fruits

Fresh fruit and vegetable consumption has risen 50% in the US since 1970. Premixed salad sales have risen substantially. Processors are now venturing into new areas, sometimes with little understanding of the risks of transmitting pathogens and the requirements for producing safe food. Many products are certified as organic and chemical free but there are no control measures regarding the presence of manure or cattle feces. Fresh produce, therefore, is becoming increasingly a vehicle for transmitting enteric diseases of many different types, and because there is no terminal heat step, consumers are vulnerable even to low doses of pathogens. Control strategies are limited because there are many stages in the production where pathogens can enter the food chain, and for imported products, there is little knowledge of the agricultural practices involved in their production. There have been many multistate and international outbreaks involving fresh produce. In Japan, in 1996, more than 6300 cases of *E coli* O157:H7 occurred among schoolchildren who ate radish sprouts contaminated at four large growers. Sprouts were also implicated in US multistate salmonellosis outbreaks in more recent years (119 ill from alfalfa sprouts in 1999; 648 ill from mung beans in 2005; 25 ill from alfalfa and spicy sprouts in 2011). Also, in the summer of 2011 a massive outbreak in Germany caused by sprouts was eventually traced back to fenugreek seeds imported from Egypt; the causative organism was *E. coli* O104:H4, a particularly virulent strain that affected over 4100 persons in 15 countries and caused the deaths of 50 of them. The source of the outbreak was particularly difficult to trace and originally cucumbers from Spain were suspected and Europeans refused to eat them and much other produce; eventually a sprouting operation in Germany was the most likely source but no *E. coli* O104 was found there, and the Egyptians denied that the fenugreek seeds were the contaminated source. The EU government offered €210 million ($306.2 million) to affected European farmers who lost money during the outbreak but the economic impact was far greater than the estimated at $2840 million for human losses from sick leave, etc., alone.

Cantaloupes have been contaminated with *Salmonella* for decades. A case-control study showed that 22 persons were ill from May to June 1998, after eating *Salmonella* Oranienberg-contaminated cantaloupes imported into Ontario from the US, Mexico and Central America. Advice to consumers was to thoroughly clean melons with potable water before cutting, prepare cut melons using clean and sanitized utensils and surfaces, and to hold cut melons at ≤7 °C until served or sold. In 2011 cantaloupes were implicated in two multistate outbreaks in the US (Del Monte melons with 20 ill in 10 states, and Jensen Farms product affecting 139 persons in 28 states). The second outbreak, which lasted from July to October

with a recall in September, was considered the worst outbreak in US history since 1924 (when typhoid in raw oysters from New York City killed 150 people) with 29 deaths from *L. monocyogenes*. Other recent large multistate outbreaks were 202 ill (four dead) from *E. coli* O157:H7 in bagged spinach in 2006; 1438 ill from *Salmonella* Saintpaul in tomatoes/peppers in 2008; 106 ill from *Salmonella* Agona in papayas imported from Mexico in 2011). The spinach outbreak, coupled with previous outbreaks from lettuce, triggered the US FDA and the leafy green industry to reduce the risk of contamination. However, as with any crops grown outdoors, there are multiple potential sources of contamination (e.g., irrigation water, flooding, runoff from nearby fields with domestic animals, wild bird and animal droppings, fecal contamination by workers and other humans or pets). Chlorination in the wash water has a limited impact on pathogen decontamination during processing. The human element is always a possibility for contamination as demonstrated in a 1986 *Shigella* outbreak in Texas, where an infected food worker was responsible for contaminating lettuce during its shredding. Another issue is the vigilance of distributors along the supply chain. In 2009, outbreaks in Sweden, Norway and Denmark were caused by *Shigella dysenteriae* type 2 in sugar snaps imported from Kenya. The supply chain involved several companies and distributors with international certification and quality standards that failed to detect potential contamination scenarios at the source or to isolate the pathogen from the product until the outbreak was over. *Shigella* must come from a human source since this pathogen does not colonize animals.

In 1996, 1400 *Cyclospora* infections in 15 US states and Ontario resulted from the consumption of fresh Guatemalan raspberries. Similar outbreaks occurred in 1997 with over 500 persons ill from fresh raspberries, also from Guatemala. In 81% of the 1996 outbreaks and most of the 1997 outbreaks, the raspberries had been rinsed before they were eaten. In 1998, the US banned imports of Guatemalan raspberries but Canada did not follow suit, and in May 1998, 13 clusters of 192 *Cyclospora* cases occurred in southern Ontario. Control measures on farms either were not effective or they were not directed against the true source. The Guatemalan industry had to spend funds to use chlorinated water irrigation systems, develop HACCP plans, and improve worker hygiene, but the market for North America was essentially lost. *Cyclospora* outbreaks are not limited to berries. In 2005, a *Cyclospora* outbreak affected 122 persons who ate an uncooked tomato and cheese appetizer containing fresh basil originating from a Mexican farm in a Quebec restaurant. The attack rate was estimated at nearly 90% with diarrhea, nausea, fatigue and abdominal cramps being the main symptoms. Similar outbreaks with contaminated basil were reported from the Washington, DC, area in 1997, Missouri in 1999, and British Columbia in 2001. *Cyclospora* outbreaks have also been associated with snow peas and mesclun lettuce.

A product not normally associated with illness is nuts. Yet there are many examples where different types of nuts have been contaminated with *Salmonella* and were responsible for illnesses in the US and Canada over the last 10 years. California almonds caused *Salmonella* Enteritidis outbreaks in 2001 and 2003/2004. In the first outbreak with five cases in West Virginia, *Salmonella* was traced back to three California farms. In the second outbreak, 29 cases occurred in 12 states and Canada, and the *Salmonella* was found to be present on two-huller-shellers used to shell almonds during the period the contaminated almonds were produced. There is now a mandatory program requiring all raw almonds to be pasteurized. In 2009, two million pounds of California pistachios were recalled because of contamination by four different serotypes; no known illnesses were associated with consumption of the pistachios which were present in a wide range of foods, including cakes, cookies, puddings, trail mix, snack bars and ice cream. In 2010, an outbreak of *E. coli* O157:H7 was associated

with eating in-shell California hazelnuts (also known as filberts) mostly purchased from bulk bins in grocery stores; the outbreak strain was also found in mixed nut samples. Another outbreak of *E. coli* O157:H7 with 13 cases occurred this time in three Canadian provinces from walnuts imported from the US from January to April 2011. Raw shelled walnuts were sold in bulk and packages. In November 2011 there was a confirmed outbreak of *Salmonella* Enteritidis with 42 cases associated with eating Turkish pine nuts purchased from bulk bins at a grocery chain. As expected, some of the pine nuts were eaten as an ingredient in prepared foods, such as Caprese salad, asparagus with pine nuts and pesto. Shopper card records were helpful in identifying pine nut purchases.

In February 2010, 272 people in 44 states and DC were infected with *Salmonella* Montevideo in an Italian-style sausage. On investigation it was found that the black pepper in the salami carried the *Salmonella*. As a result, not only was the meat recalled but also all the batches of black and red pepper from the contaminated source. Because the main *Salmonella* Montevideo outbreak PFGE pattern commonly occurs in the US, there was difficulty separating outbreak strains from unrelated sporadic cases. Interestingly, the USDA regulates salami, and the FDA oversees black pepper and other additives.

Processed vegetables and fruits have also been implicated in foodborne disease, but to a lesser extent than fresh or minimally processed products. Botulism has been documented from several types of vegetable products. In Japan, in July 1998, five people were ill after eating bottled green olives containing type B toxin, with another six ill from the same product in August. In Italy, in August 1998, a woman drank a soup bottled locally and suffered from type A botulism. In the UK, in April 1998, two people were ill and one died after eating home-bottled mushrooms from Italy containing type B toxin. Four persons were ill in two US states in 2007 from consuming canned hot dog sauce which was underprocessed, allowing survival and subsequent growth of *C. botulinum*. Scottish health officials recalled 47,000 jars of a curry sauce made in England after two persons suffered from botulism in November 2011. Botulism from home-bottled low-acid vegetable product and occasionally from commercially-canned products has been documented for decades, but it still occurs as illustrated above. Less typical is botulism from baked potatoes. In El Paso, Texas, in 1994, 30 people suffered from botulism after eating a potato dip or egg plant dip at a Greek restaurant. The potatoes had been baked in the oven and left at room temperature, still in the foil, for 18 hours before being used in the dip. Foil allowed anaerobic conditions for surviving *C. botulinum* spores on the skin to germinate and grow into the potato. Shared utensils spread the organism to the eggplant dip. There have been five other botulism outbreaks from baked or boiled potatoes in the US since 1978. These items are now considered a potentially hazardous food.

There have been many peanut butter-related outbreaks and recalls in the last 20 years. The first outbreak was in Australia in 1996 with 15 cases. In the Australian outbreak, 57 cases of *Salmonella* Mbandaka in three states were associated with consumption of the implicated peanut butter. Settlements in 1998 ranged from $500 to $50,000, totalling $7 million for the most seriously affected. The first US instance of *Salmonella* traced to peanuts was a ConAgra brand in 2006–2007 with 648 people in 47 states culture-confirmed with *Salmonella* Tennessee. In 2008/2009 the Peanut Corporation of America was the source of the 2008–2009 outbreak of *Salmonella* Typhimurium, involving thousands of products with peanut butter as an ingredient. There were 691 illnesses, nine deaths, and a recall of over 3800 products costing billions of dollars. In both cases, water in the processing area allowed growth of the *Salmonella*. An outbreak of salmonellosis caused by *Salmonella* Agona affected 211 persons with 47 hospitalized in 11 states from April to May 1998. Most cases were aged under

10 or over 70. Toasted oats cereal was the implicated food. A listeriosis outbreak in 1997 with 1594 cases arose from consumption of salad with canned corn served in Italian schools by a mass catering establishment. The same strain of *L. monocytogenes* (group 4) and DNA profiles were found in 123 hospitalized patients, left over corn, and floor and sink drains of the catering establishment. The pathogen grew in corn at 25 °C after six hours.

Fruit products are also being increasingly implicated in outbreaks involving different pathogens. In 1995, 62 cases of salmonellosis were associated with unpasteurized orange juice. Four *Salmonella* serovars were found in juice samples, unwashed fruit surfaces and amphibians beside the processing facility.

In 1993, consumers of apple juice in Massachusetts contracted cryptosporidiosis seemingly from cysts in cattle manure contaminating the apples. Apple juice outbreaks were also documented in Canada and the US in 1980, 1996 and 1998 where *E. coli* O157 was the confirmed or suspected agent. In the largest of these with over 61 cases and one death, a widely-marketed commercial product was involved. A batch of rotten apples had been included in the lot pressed on the day the juice was contaminated, despite the inspector's advice to reject it. The impact on the company was high with a fine of $1.5 million, payments in millions of dollars made to the families of the ill people, and over $1 million in business losses. Several of the parents of the ill children become lobbyists for stricter food safety.

Hepatitis A virus (HAV) outbreaks have been linked to strawberries and green onions. In the 2001 Pennsylvania outbreak associated with one restaurant, 555 persons with hepatitis A were identified, including at least 13 foodservice workers. Several characteristics of the way food was prepared and served in the restaurant could have contributed to the size of the outbreak, including, (1) multiple opportunities for intermingling of uncontaminated and contaminated green onions kept in a common bucket for five days with the ice in which they had been shipped, and (2) serving contaminated items with a relatively long shelf life (e.g., mild salsa) to a large number of patrons over several days. Since HAV is only transmitted through the human fecal-oral route, workers as sources are typically suspected. Green onions require extensive handling during harvesting and preparation for packing. Contamination of green onions could occur (1) by contact with HAV-infected workers, especially children, working in the field during harvesting and preparation, and (2) by contact with HAV-contaminated water during irrigation, rinsing, processing, cooling, and icing of the product. Green onions and strawberries might also be more vulnerable to contamination because plant surfaces are particularly complex or adherent to viral or faecal particles.

1.6 High-risk populations

Susceptible persons include children under the age of five, pregnant women, the elderly, persons who have an impaired immune system (e.g., people taking immuno-suppressive drugs, undergoing cancer therapy or infected with the human immunodeficiency virus (HIV), which causes AIDS). These persons should avoid raw foods of animal origin such as undercooked poultry, eggs and meats, unpasteurized milk or cheese or yogurt made with raw milk, dishes prepared with raw or undercooked eggs, raw or undercooked molluscs and crustacea, any cooked food which has been cross-contaminated by raw food after cooking or as a result of poor personal hygiene, and any food stored above the recommended safe temperature. Other populations at risk are those in refugee camps and camps for alien workers, and those with specific customs that compromise food safety, such as eating feasts at funerals even during cholera epidemics, where people share plates, eat with their hands and have poor standards

of hygiene. Some high-risk populations are unpredictable, such as those exposed to bioterroism where enteric pathogens may be put into food in random locations. Travelers to countries where food hygiene is not understood or practised well are exposed to a higher risk of infection than at home, even if the location they are staying in seems to be high class.

1.7 Policies to reduce foodborne disease

Policies around the world on how to reduce foodborne disease vary. Most traditional regulations may have specific bacterial counts that cannot be exceeded, and a few may ban products, for example if pathogens like *Listeria monocytogenes* are in RTE foods or *E. coli* O157:H7 is found in ground meat (zero tolerance). The emphasis today is more on preventative measures such as HACCP, either voluntary or mandatory in meat, poultry and/or seafood operations. Warning labels are now being put on packages of raw meat and poultry to advise consumers to cook well, avoid cross-contamination, etc. For juices, these may be pasteurized or a warning label may indicate the risks of drinking unpasteurized products. Because of the concern over high numbers of foodborne illness relating to products prepared in the home from ingredients purchased in retail stores, a few supermarket chains are promoting food safety by educating consumers to keep food safe at home, and working with government to prevent contaminated food from entering the distribution system. In Sweden, great emphasis is put upon eliminating the source of the pathogen, e.g., depopulating flocks with *Salmonella*, testing animals and feed, sanitary slaughter, hygienic practices, and import control. These types of controls come with a substantial cost factor.

Governments are recognizing there has to be improved surveillance and new approaches to this are being undertaken in different countries. For example, such approaches include measuring the burden of infectious intestinal disease or establishing sentinel sites for case-control and other epidemiological and laboratory studies, including DNA fingerprinting of strains. In the UK, the government is considering making farmers directly liable for compensation for any illness caused by the food they produce. This means that a victim of foodborne disease could sue a farmer if the source of the infection can be traced to a particular foodstuff and farm; the plaintiff would be required merely to establish a causal link but not prove any negligence on the part of the farmer. The principle of strict liability has applied to manufacturers but up until now not to primary producers. This raises a number of questions, such who would be responsible for illnesses arising from bulked food or where raw products of animal origin were mishandled in a home or a restaurant. Certainly, the role of the food worker to have more effective hygienic practices is important, an issue that involves management culture and practices as well as the convenience of hand hygiene sites and new disinfectant and pathogen barrier technologies.

The Council for Agricultural Science and Technology has issued two publications on foodborne pathogens. The first has been widely quoted and contributed to US policy. The second, published in 1998, gives specific recommendations in goal setting, research needs, production control and education. Some are listed below, and although some progress has been made in the last 14 years, these are still valid today.

Goal setting

1. Base food safety policy on risk assessment and include risk management and risk communication strategies.

2. Base food safety regulations on risk assessment and risk management.
3. Set federal food safety goals and priorities: criteria include
   – the numbers of acute illnesses
   – numbers of chronic complications
   – numbers of deaths and disabilities
   – types of food products implicated
   – types of production, harvesting, or processing deficiencies or handling errors identified
   – impact on high-risk populations
   – economic losses to society.

Research recommendations

1. Improve reporting of foodborne disease by pathogen, by food, and by contributory factors.
2. Expand existing database on food animals, foods and pathogens.
3. Conduct epidemiologic studies to establish the cause of illness.
4. Improve and regularly update foodborne disease estimates and their costs.
5. Find mechanisms of chronic illnesses and populations at elevated risk from chronic disease associated with foodborne pathogens.
6. Develop rapid, accurate detection methods for pathogen detection in foods.
7. Use dose-response modeling in the risk assessment process.
8. Identify food and pathogen/toxin associations in order to establish controls to minimize the risks.
9. Support pathogen research to understand more about the agents causing foodborne disease, e.g., biofilms, virulence factors, factors contributing to contamination, survival and growth

Production control

1. Require producers to adopt effective preharvest intervention strategies to enhance public health, including foodborne pathogen control practices from food source to consumption
2. Harmonize international food safety standards

Education

1. Educate the general public and food handlers for safe food preparation and handling, especially for high-risk populations.
2. Use and evaluate food labelling to communicate safe food preparation.
3. Provide risk information on food choices to susceptible persons.

1.8 Conclusion

Foodborne disease is an increasing concern in all countries. Because of lack of investment in surveillance and research in infectious diseases over decades, we have to react to problems today rather than anticipate them. For instance, we have limited knowledge on virulence

factors and their transfer between organisms, such as Shigatoxin/verotoxin production from *E. coli* to *Citrobacter* and enteroinvasive properties from *E. coli* to *Klebsiella*. Antimicrobial resistance to antibiotics is preventing adequate means of eliminating enteric pathogens from the gut. Therefore, we are seeing new varieties of pathogens, some of which become important such as *E. coli* O157:H7 and increasingly non-O157 STEC/VTEC strains, and others we are still not certain about for the normal population but can infect the immunocompromized person, such as *Aeromonas*. Governments need to collaborate with limited resources within each country and within blocks of countries, and work with interested stakeholders to develop meaningful policies. Countries need to take recommendations and research conclusions from other countries and adapt these to their own situation, through scientific experts with appropriate resources to produce the relevant policies, risk assessments, and methods to reduce foodborne disease. At present, no country can claim that the battle against the foodborne pathogen is won. Can existing long-term strategies of production control (on farm and HACCP), import inspection, trade agreements, third-party auditors and consumer education substantially reduce foodborne disease or will new problems continually arise to keep the numbers up? If governments are not committed to put resources into new ways to reduce, or at least stabilize, the impact of enteric pathogens, the world's population of 7 billion persons will be continually exposed to new and old hazards. One issue that can be expected is that as surveillance methods improve, we can expect more outbreaks to be identified and this may be an exercise in risk communication to inform the public that these advances are eventually going to improve food safety. Will the 21st century be looked upon as a time when foodborne disease is effectively contained, or will it expand to new products and from unexpected sources and economic downturns limit research and control polices, so that it will become in unpredictable ways a threat to the lives of our children and the increasingly aging population?

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2 *Staphylococcus aureus*

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2.1 Introduction

The first time *Staphylococcus* was associated 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, in the United States in Michigan in 1907, and in the Philippines in 1914. In 1930, Gail Dack and his colleagues at the University of Chicago were able to demonstrate that the cause of a food poisoning that occurred from the consumption of a contaminated Christmas sponge cake with cream filling was a toxin produced by the isolated staphylococci.

Staphylococci are ubiquitous and impossible to eradicate from the environment. Many of the 32 species and subspecies in the genus *Staphylococcus* are potentially found in foods, due to environmental, human and animal contamination. Several staphylococcal species, including both coagulase-negative and coagulase-positive, have the ability to produce highly heat-stable enterotoxins that cause gastroenteritis in humans. *S. aureus* is the etiologic agent predominantly associated with staphylococcal food poisoning.

The growth and proliferation of *Staphylococcus aureus* in foods present 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 in a specific food-poisoning episode, determine whether a food or ingredient is a source of enterotoxigenic staphylococci, and demonstrate post-processing 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 represent a potential hazard because of the absence of competitive organisms that might 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

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staphylococci 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 *Staphylococus intermedius* and *Staphylococcus hyicus* have also been shown to be enterotoxigenic. Normally considered a veterinary pathogen, *S. intermedius* was isolated from butter blend and margarine in a food-poisoning outbreak. A coagulase-negative *Staphylococcus epidermidis* was reported to have caused at least one outbreak. 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 that are exposed to temperatures that permit the growth of *S. aureus*. This is especially true for fermented meat and dairy products. Though the potential is there, it is only when improper fermentation takes place that the development of staphylococcal enterotoxin occurs.

In processed foods in which *S. aureus* is destroyed by processing, the presence of *S. aureus* 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 that cause *S. aureus* to come 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 vehicle 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 and/or demonstrating the presence of staphylococcal enterotoxin in food. Neither the absence of *S. aureus* nor the presence of small numbers is 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 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.

2.2 Nature of illness

2.2.1 Symptoms

The onset of symptoms in staphylococcal food poisoning is usually rapid (2–6 h) 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 may not always 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 two days; however, it is not unusual for complete recovery to take three days and sometimes longer in severe cases. Death from staphylococcal food poisoning is very rare, although such cases have occurred among the elderly, infants and severely debilitated persons.

2.2.2 Dose

A toxin dose of less than 1.0 µg in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when *S. aureus* populations exceed 10^5 g^{-1}. However, in highly sensitive people, a dose of 100–200 ng is sufficient to cause illness.

2.3 Characteristics of agent

2.3.1 Organism

Staphylococcus aureus is a spherical bacterium (coccus) that on microscopic examination appears in pairs, short chains, or bunched, grapelike clusters. The organisms are Gram positive. Some strains can produce a highly heat-stable protein toxin 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. The *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 required among the B vitamins. When grown anaerobically, they appear to require uracil.

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 7–47.8 °C with an optimum temperature for growth at 35 °C. The pH range for growth is between 4.5 and 9.3, with the

Table 2.1 Characteristics of Staphylococcus species

	Species[a]			
Property	***S. aureus***	***S. intermedius***	***S. hyicus***	***S. epidermidis***
Pigment	+[b]	–	–	–
Coagulase	+	+	+/–	–
DNase	+	+	+/–	–
Hemolysis	+	+	–	+/–
Mannitol (anaerobic conditions)	+	–	–	–
Acetoin	+	–	–	+
Clumping	+	+	+/–	–
Hyaluronidase	+	–	+	–
Lysostaphin	HS[3]	HS	HS	SS[4]

[a]Abbreviations: HS, high sensitivity; SS, slight sensitivity.
[b]Over 90%

optimum between pH 7.0 and 7.5. As is true with other parameters, the exact minimum growth pH is also dependent on the degree to which all other parameters are at optimal conditions. With regard to water activity (A_w), the staphylococci are unique in being able to grow lower than other nonhalophilic bacteria. Growth has been demonstrated as low as 0.83 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. *Staphylococcus aureus* grows best at an A_w of >0.99 and growth at low-A_w values depends on other growth conditions being optimal.

Staphylococcus 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. 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 2.1. 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), a heat-stable phosphodiesterase that can cleave either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) to produce 3′-phosphomonucleosides. Thermonuclease is much more heat stable than ribonuclease and is useful in speciating staphylococci.

2.3.2 Enterotoxins

There are five classical enterotoxin serotypes: SEA, SEB, SEC1,2,3, SED, and SEE and the more recently described SEG, SEH, and SEI; all exhibit emetic activity. There are also SE-like enterotoxin serotypes, SElJ-SElU; these SE-like designations have not been confirmed to exhibit emetic activity. The different SE serotypes are similar in composition and biological activity, but are different in antigenicity and are identified serologically as separate proteins.

The staphylococcal enterotoxins are single-chain proteins that are antigenic with molecular weights of 26,000–29,000. They are 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, so remain a potential health hazard when they appear in canned foods. Their other general properties such as amino acid composition and immunological characteristics have been readily described elsewhere.

2.4 Epidemiology

The epidemiology of foodborne disease is changing in intensity and concept 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. 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 defining the chain of events that allowed contamination with an organism in large enough numbers to cause illness. This approach provides strategies for preventing similar occurrences in the future.

2.4.1 Frequency of illness

Staphylococcus aureus is the cause of sporadic food poisoning episodes around the world. Domestically, *S. aureus* is one of the top five pathogens contributing to foodborne illness. In the US, it is estimated that staphylococcal food poisoning causes approximately 241,148 illnesses, 1064 hospitalizations and six deaths each year (Fig. 2.1). The true incidence of staphylococcal food poisoning is unknown for a number of reasons, including: poor responses from victims during interviews with health officials; misdiagnosis of the illness, which may be symptomatically similar to other types of food poisoning (such as vomiting caused by *Bacillus cereus* emetic toxin); inadequate collection of samples for laboratory analyses; improper laboratory examination; and, in many countries, unreported cases.

2.4.2 Diagnosis of human illness

In the diagnosis of staphylococcal foodborne illness, it is essential to have proper interviews with the victims and gather and analyze epidemiological data. 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 be useful when

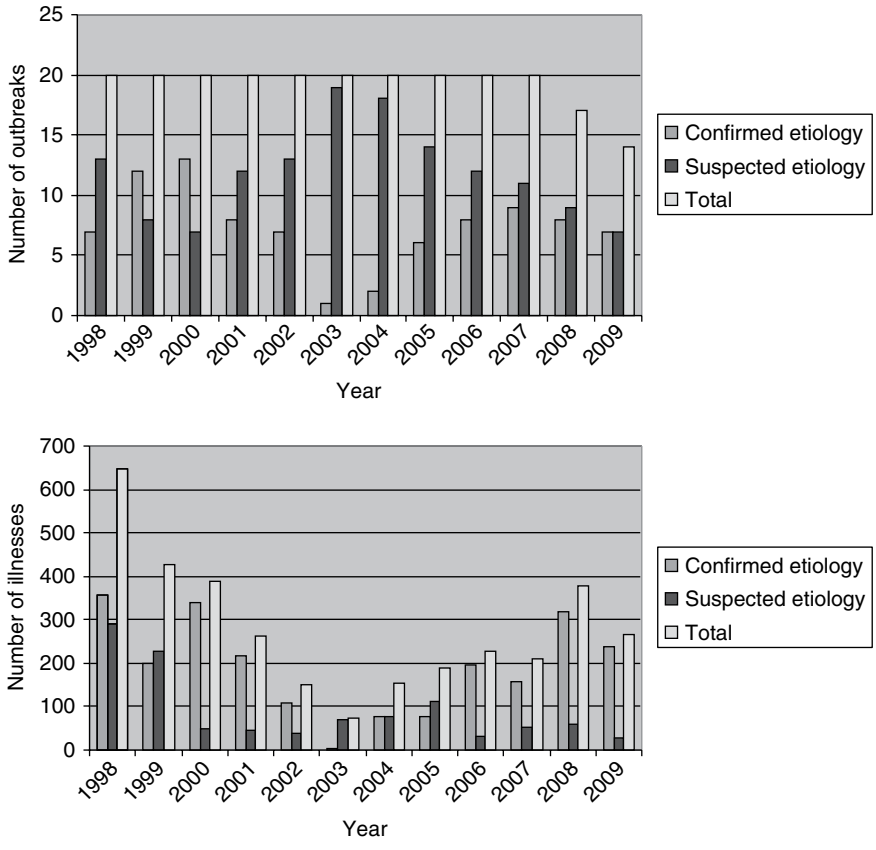


Figure 2.1 Comparative numbers of reported outbreaks and illnesses caused by staphylococcal species in the United States, 1998–2009. During an 11-year period (1998–2009) in the United States 231 outbreaks involving 3374 cases were attributed to staphylococcal intoxication. Shown are the comparative numbers of outbreaks and the number of cases attributed to staphylococcal foodborne illnesses from 1998–2009 in the United States. *Source*: Compiled from Foodborne Outbreak Online Database (FOOD). http://wwwn.cdc.gov/foodborneoutbreaks/

viable staphylococci can be isolated from the incriminated food, from victims, and 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 and DNA restriction fragment polymorphism of ribosomal RNA (rRNA) genes referred to as ribotyping.

2.4.3 Vectors of transmission

Staphylococci exist in air, dust, sewage, water, milk, and 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%

Table 2.2 Reported Vehicles of transmission causing staphylococcal foodborne outbreaks and illnesses in the United States, 1998–2009

	Number of outbreaks (Illnesses)												
Food type	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**Totals**
Baked food	0 (0)	1 (4)	2 (119)	3 (17)	6 (20)	0 (0)	1 (20)	1 (15)	1 (4)	1 (3)	2 (7)	0 (0)	15 (209)
Beef	2 (4)	1 (5)	1 (7)	1 (2)	1 (8)	2 (5)	1 (2)	0 (0)	2 (5)	1 (2)	1 (40)	0 (0)	13 (80)
Poultry	2 (25)	0 (0)	2 (6)	2 (15)	1 (2)	3 (8)	2 (5)	0 (0)	3 (6)	3 (29)	0 (0)	4 (51)	22 (147)
Pork	3 (32)	5 (131)	4 (51)	4 (188)	3 (44)	2 (10)	2 (6)	6 (135)	3 (73)	3 (97)	3 (57)	2 (77)	40 (901)
Fish	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	1 (2)	3 (32)	1 (2)	1 (23)	0 (0)	8 (63)
Deli meats	2 (54)	0 (0)	0 (0)	1 (5)	4 (4)	2 (8)	0 (0)	0 (0)	2 (6)	0 (0)	0 (0)	0 (0)	11 (77)
Mexican food	3 (24)	1 (50)	1 (17)	0 (0)	3 (52)	0 (0)	1 (4)	1 (2)	0 (0)	1 (12)	2 (5)	0 (0)	13 (166)
Chinese food	0 (0)	3 (10)	0 (0)	0 (0)	2 (7)	1 (4)	0 (0)	1 (3)	0 (0)	1 (5)	1 (2)	0 (0)	9 (31)
Dairy	1 (30)	0 (0)	0 (0)	1 (3)	1 (8)	1 (3)	0 (0)	0 (0)	2 (38)	0 (0)	0 (0)	0 (0)	6 (82)
Salad	3 (447)	2 (28)	2 (103)	0 (0)	1 (3)	0 (0)	5 (66)	0 (0)	2 (58)	0 (0)	0 (0)	2 (58)	17 (763)
Pasta	1 (19)	1 (19)	1 (2)	0 (0)	0 (0)	1 (5)	1 (5)	0 (0)	1 (2)	1 (9)	1 (17)	0 (0)	8 (78)
Other	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	1 (6)	4 (18)	0 (0)	0 (0)	1 (5)	1 (66)	8 (97)
Unknown	1 (9)	4 (166)	6 (79)	8 (34)	1 (2)	6 (29)	6 (39)	6 (15)	1 (3)	8 (51)	4 (223)	5 (15)	56 (665)

Source: Compiled from Foodborne Outbreak Online Database (FOOD). http://wwwn.cdc.gov/foodborneoutbreaks/

or more of healthy individuals. This incidence is even higher for those who associate or 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].

2.4.4 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 poisoning. The types of food incriminated in food-poisoning outbreaks in the United States from 1998–2009 are listed in Table 2.2.

2.4.5 Typical food-poisoning outbreak

Recently, 1364 children became ill out of a total of 5824 who had eaten lunch served at 16 elementary schools in Texas. 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 a chicken salad. The afternoon of the day preceding the lunch, frozen chickens were boiled for 3 h. 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 am and 10:30 am, where it was kept at room temperature until served between 11:30 am 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 also 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 the *Staphylococcus*, more rapid cooling of the chicken, and adequate refrigeration of the salad from the time of preparation to its consumption.

In another foodborne outbreak, almost 300 children experienced symptoms of vomiting, nausea, abdominal cramps and diarrhea several hours after eating food prepared by caterers contracted by one of the county school's department of education. Testing of food samples and patient samples confirmed that the illnesses were consistent with staphylococcal food poisoning. The foods served that day included milk, cantaloupe and egg salad sandwiches. Standard microbiological cultures and biological assays revealed high numbers of enterotoxigenic *S. aureus* and the presence of preformed SED from the egg salad sandwiches.

2.4.6 Atypical food-poisoning outbreaks (thermally processed food)

In 1989, multiple staphylococcal foodborne diseases were associated with the consumption of canned mushrooms at which time the product was sterile at the time of consumption.

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 the 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.

Queens, New York On February 28, 48 people became ill a median of 3 h 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.

McKeesport, Pennsylvania On April 17, 12 people became ill with gastroenteritis a median of 2 h 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 the 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.

2.5 Detection and identification

2.5.1 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 grapelike 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

Table 2.3 Partial list of commercially-available nucleic acid and serological-based assays for detection and confirmation of *S. aureus*

Trade name	Assay format	Company
AccuProbe	Probe[a]	Gen-Probe, San Diego, CA
GENE-TRAK	Probe	Gene-Trak Hopkinton, MA
Genevision	PCR	Warnex
S. aureus	PCR	Biotecton Diagnostics
Microscreen	Latex agglutination	Microgen
ANI *S. aureus*	Latex agglutination	ANI Biotech
Staphylo-slide	Latex agglutination	Becton Dickinson, Cockeysville, MD
Aureus Test	Latex agglutination	Trisum, Taipei, Taiwan
Staphlococcus aureus Visual Immunoassay	ELISA	TECRA 3M, St. Paul MN
Staphaurex	Latex agglutination	Rhone Poulenc, Glasgow, United Kingdom
Staphylase	Latex agglutination	Unipath—Oxoid Ogdensburg, NY
Slidex[b]	Latex/RBC agglutination	BioMérieux, Marcy-l'Etoile, France
Mastastaph	Latex agglutination	Mast Laboratories
Staphytest	Latex agglutination	Unipath—Oxoid Division, Ogdensburg, NY
Avistaph	Latex agglutination	Omega Diagnostics
Staph Latex Test	Latex agglutination	Difco Laboratories, Detroit, MI
Staphytect Plus	Latex agglutination	Oxoid, Hampshire, UK
Bactident Staph	Latex	Merck

[a] Used for identification of pure culture isolates.
[b] A combined latex and hemagglutination test. RBCs sensitized with fibrinogen; latex particles sensitized with monoclonal antibodies to protein A or immunogens on the surface of *S. aureus* strains.
Source: Feng, 2007. Rapid methods for the detection of foodborne pathogens: current and next generation technologies, In: Doyle, M. P. and Beuchat, L. R., *Food Microbiology Fundamentals and Frontiers*, 3rd ed. Washington D.C.: ASM Press, pp. 911–934.

commercially-available nucleic acid and serological-based assays for the detection and confirmation of *S. aureus* are listed in Table 2.3.

2.5.2 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 from 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 phosphase;
(i) the production of thermonuclease; and
(j) 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.

Table 2.4 Partial list of commercially-available substrate media for the detection of *S. aureus*

Trade name	Assay format	Company
Baird-Parker Agar	Media	Remel
Petrifilm	Media-film	3-M
CHROMagar	Media	Becton Dickinson
RIDA COUNT	Media-sheet	R-Biopharm

Source: Feng, 2007. Rapid methods for the detection of foodborne pathogens: current and next generation technologies, In: Doyle, M. P. and Beuchat, L. R., *Food Microbiology Fundamentals and Frontiers*, 3rd ed. Washington D.C.: ASM Press, pp. 911–934.

2.5.3 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 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 (Table 2.4). 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.

2.5.4 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 Food and Drug Administration's (FDA) *Bacteriological Analytical Manual* (1998).

2.5.5 Enrichment isolation method

The Most Probable Number (MPN) method 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.

2.5.6 Differential characteristics

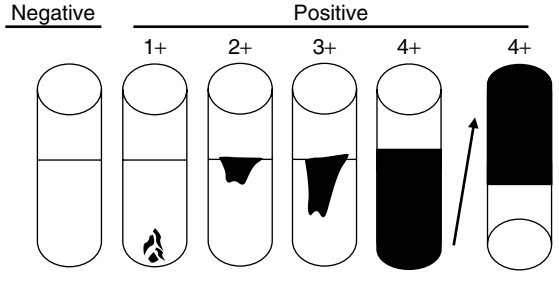
Staphylococcus aureus is differentiated from the other staphylococcal species by a combination of the following features: colonial morphology and pigmentation; production of coagulase, thermonuclease, acetone, β-galactosidase, phosphatase, and alpha toxin

(hemolysis); 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*.

2.5.7 Coagulase

The confirmation procedure most frequently used to establish the identity of *S. aureus* is the coagulase test. Coagulase is a substance that clots plasma of humans 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, pH of the medium and denigration. 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 (less than 2 min), and the results are more clearcut 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 Fig. 2.2.



Negative	No evidience of fibrin formation
1+ positive	Small unorganized clots
2+ positive	Small organized clot
3+ positive	Large organized clot
4+ positive	Entire content of tube coagulates and is not displaced when tube is inverted

Figure 2.2 Types of coagulase reaction.

2.5.8 Thermonuclease

Thermonuclease 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* sp. *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.

2.5.9 Ancillary tests

Additional tests in the identification of *S. aureus* include catalase anaerobic utilization of glucose and mannitol and lysostaphin sensitivity.

2.6 Detection of enterotoxins

The need to identify enterotoxins in foods encompasses basically two areas: (1) foods that have been incriminated in food-poisoning outbreaks; and (2) foods that are suspected of containing enterotoxin. In the former case, the identification of enterotoxin in foods is confirmation to support 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 are biological and serological testing.

2.6.1 Methods for toxin identification

Biological assays Prior to the advent of serological identification of toxins, all the toxins were identified by emetic responses in a monkey feeding assay. 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 h, and if vomiting occurs during the observation period, the sample is judged to contain toxin. While this animal assay is considered specific, a number of disadvantages exist. An alternative bioassay is through the IV injection of cats or kittens. However, other bacterial metabolites have been found to cause nonspecific emetic responses, although these nonspecific components can be neutralized or inactivated.

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 composition and biological activity, they can be differentiated based on serology. Five serological distinct types of enterotoxin have been characterized and designated as SEA, SEB, SEC (subtypes C_1, C_2, C_3), SED and SEE. Approximately 5.0% of the staphylococcal foodborne outbreaks are linked to an

unidentified toxin such as one or more of the newly-described enterotoxins with proven emetic activity SEG, SEH, and SEI all proven to exhibit emetic activity and the SE-like enterotoxin serotypes SElJ-SElU. Their existence can be demonstrated by biological tests and are not serologically related to the previously-established toxin serotypes. More recently, a new enterotoxin, SEH, was identified and partially characterized and a rapid method developed for its identification. However, antibodies to other enterotoxins and enterotoxin-like enterotoxins are not available in commercially-prepared kits. This has led investigators to use PCR methods to detect specific SE genes that potentially identify enterotoxigenic strains until a biological assay becomes available that demonstrates protein expression.

A number of methods employing polyclonal or monoclonal antibodies have been used to identify and measure enterotoxins. Methods developed earlier utilized precipitation and agglutination approaches, while more recently-developed methods employ tracer-labelled or tagging methods to increase assay sensitivity. Systems based on serological assays can, in general, be divided into a number of antigen–antibody reaction types: (1) gel immunodiffusion by direct precipitation or precipitation inhibition assays; (2) agglutination assays; and (3) tracer-labelled or tagged immunoassays. The most commonly-used earlier developed methods have been previously described in a general way, in reviews, and in stepwise procedural detail by a number of investigators. Earlier developed classical methods such as the microslide gel double-diffusion test have been described in detail in the FDA *Bacteriological Analytical Manual* and the American Public Health Association (APHA) *Compendium of Methods for the Microbiological Examination of Foods* (Vanderzant and Splittstoesser, 1992). Some commercially-available rapid methods for the identification of the enterotoxins are presented in Table 2.5.

Several enzyme-linked immunosorbent assay (ELISA) methods have been proposed for the identification of the 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 enterotoxin in samples is 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 (1997). Of the rapid methods proposed for the identification of staphylococcal enterotoxins in foods, only the microtiter plate polyvalent ELISA method has been studied exhaustively and is approved by the Association of Official Analytical Chemists (AOAC) International. 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 print-out data in approximately 80 min. Preliminary evaluation of this system shows that it is highly sensitive (≤1 ng/g) and, generally, specific. Other ELISA-based methods are dedicated to determining specific serotypes of

Table 2.5 Partial listing of commercially-available methods for the identification of staphylococcal enterotoxins

Kit name	Assay format	Company (Distributor)[b]
TECRA-SET[c]	ELISA, polyvalent[d] (A–E)[e]	TECRA 3M, St. Paul MN
TECRA-SET	ELISA, monovalent[f] (A–E)	TECRA 3M, St. Paul MN
SET-RPLA	Latex agglutination monovalent (A–D)	Unipath—Oxoid Division, Ogdensburg, NY
VIDAS-SET-2[c]	ELFA, polyvalent (A–E)	BioMérieux-Vitek, Hazelwood, MO
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, Darnstadt, Germany (BioTeck Instruments, Winooski, VT)
BV	ECL (SEA and SEB)	BioVeris
SMART	Immunoprecipitation (SEB)	New Horizon
BTA	Immunoprecipitation (SEB)	Alexeter Tech

[a] Abbreviations: SET, staphylococcal enterotoxin; RPLA, Reversed Passive Latex Agglutination; ELFA, enzyme-linked fluorescent immunoassay.
[b] Distributor.
[c] AOAC International status-adopted "Official First or Final" action.
[d] Does not distinguish between toxin serotypes.
[e] Identifiable toxin serotype.
[f] Distinguishes between toxin serotypes.
Source: Feng, 1998, *FDA Bacteriological Analytical Manual*, 8th edition (rev. A/1998), Gaithersburg, MD: AOAC International. Modified, in part, from Feng, 2007. Rapid methods for the detection of foodborne pathogens: current and next generation technologies, In: Doyle, M. P. and Beuchat, L. R., *Food Microbiology Fundamentals and Frontiers*, 3rd ed. Washington D.C.: ASM Press, pp. 911–934.

staphylococcal enterotoxins. Some of these monovalently configured assays are also listed in Table 2.5. 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.

2.6.2 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. 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 utilized to determine the enterotoxigenicity of suspect staphylococcal isolates from foods or other sources. Although commercial kits generally recommend broth media, these media 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 the enterotoxin gene exists in an isolate, although laboratory demonstration of toxin production is more direct and serves 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 other sources.

2.6.3 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 frequently the amounts may be larger. Toxin can be readily identified if the counts are or were at some time $\geq 10^6$ enterotoxigenic staphylococci/g of food. Such high counts are not acceptable. Marketable foods should contain no enterotoxin and should be readily 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 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.

In the food-poisoning episodes involving canned mushrooms, analysis of the product initially proved serologically negative, although there was 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. 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. 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.

2.7 Physical methods for destruction

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

Table 2.6 Leading factors attributed to staphylococcal foodborne outbreaks in the United States, 1988–1992

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: *CDC Surveillance Summaries*, Vol. 49, No. SS1, 2000.

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 stresses respectively by 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, if conditions are conducive, and continue to be a potential hazard to consumer health. The kinetics of sublethal injury and stressed cell rejuvenation has been reviewed and 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. 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. 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. Table 2.6 lists the leading factors attributed to staphylococcal foodborne outbreaks in the US.

To better understand the behaviour 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.

2.8 Prevention and control

Staphylococci exist ubiquitously in the environment. 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 potential that it will be contaminated. Not all strains of *S. aureus* and other species are enterotoxigenic, although a large percent (50–70%) may be. To prevent food-poisoning outbreaks, it is necessary to keep foods either refrigerated (below 10 °C) or hot (above 45 °C) to prevent proliferation of the organism to such numbers (10^5 cells/g) that are necessary for detectable toxin formation. Additionally, foods to be refrigerated should be placed 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 are critical. The leading factors that were attributed to staphylococcal food poisoning outbreaks in the United States during 1988–1992 are listed in Table 2.6.

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3 *Listeria monocytogenes*

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3.1 Introduction

As a leading cause of death due to foodborne illness, *Listeria monocytogenes* continues to cause sporadic cases and outbreaks of illness linked to consumption of food products. *Listeria monocytogenes* was first discovered more than 100 years ago, recognized primarily as an animal pathogen, but since 1981 its prominence as a foodborne pathogen has been extensively documented. To prevent foodborne listeriosis, it is necessary to understand the disease, susceptible persons, distribution of the organism within the environment, and behavior of the organism in foods. This chapter is designed to summarize current knowledge with respect to the foodborne role of *L. monocytogenes*.

3.1.1 Characteristics of *Listeria*

Listeria monocytogenes is a small (1.0–2.0 × 0.5 μm), Gram-positive, facultatively anaerobic, flagellated, rod-shaped bacterium, classified in the Firmicutes division. The organism can exist in an intracellular state within monocytes and neutrophils and is accordingly named because large numbers of monocytes are often found in the peripheral blood of monogastric animals infected by this organism. *Listeria* is recognized as a causative agent of the disease listeriosis, a zoonotic illness that affects both animals and humans.

Reports of organisms resembling *Listeria* first appeared in the scientific literature in 1891 when Hayem of France observed small, Gram-positive rod-shaped organisms in human tissue. This observation was repeated two years later (1893) by Henle, who was working in Germany. In 1911, Hulphers, in Sweden, reported the presence of Gram-positive rod-shaped organisms in the livers of rabbits and designated the organism *Bacillus hepatis*. The complete characterization of the organism now recognized to be *L. monocytogenes* was first achieved by Murray et al. (1926). These investigators, working at Cambridge University, observed infections of an animal colony caused by an etiologic agent that induced pronounced monocytosis and caused hepatic lesions. The name given to this agent was

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

Table 3.1 Main Characteristics of the Five most Thoroughly Characterized *Listeria* species

Characteristic	*L. monocytogenes*	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*	References
β-hemolysis	+	+	+	–	–	Farber and Peterkin, 1991
CAMP						Lovett, 1990
S. aureus	+	–	+	–	–	
Rhodococcus	–	+	–	–	–	
Fermentation of:						Lovett, 1990
Mannitol	–	–	–	–	–	
Xylose	–	+	+	–	+	
Rhamnose	+	–	–	±	±	
Pathogenic in humans	Yes	Very rare	Very rare	No	No	Busch, 1971; Rocourt et al., 1985, 1986

Bacterium monocytogenes. Pirie (1940), working in South Africa, isolated a small Gram-positive bacillus from the livers of gerbils that he designated *Listerella hepatolitica*, in honor of Lord Lister. In 1940, the genus–species designation *Listeria monocytogenes* was proposed for this organism. A detailed historical chronology can be found in the classic review article on *Listeria* and listeric infection, published by Gray and Killinger (1996).

Included within the genus *Listeria* are the species *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi* (Table 3.1), as well as the recently-characterized *L. rocourtiae* and *L. marthii*. Members of the genus *Listeria* can be differentiated by the following biochemical reactions: reduction of nitrates to nitrites; beta hemolysis; acid production from mannitol, L-rhamnose, and D-xylose; and the CAMP test (Table 3.1). *Listeria monocytogenes* and *L. innocua* are so closely related that within the 16S ribosomal ribonucleic acid (rRNA), only two of 1281 base pairs differ between the two species. Of the eight species within the genus *Listeria*, only *L. monocytogenes* is generally regarded as capable of causing illness in humans. Three reports of human infection caused by *L. ivanovii* exist in the scientific literature, along with one report of a case of human illness caused by *L. seeligeri*. *Listeria innocua* and *L. welshimeri* are not capable of causing illness. These nonpathogenic species are of interest from a food microbiology standpoint since they can serve as indicators for the potential presence of *L. monocytogenes*.

Listeria monocytogenes strains can be differentiated on the basis of serology and, to date, over 14 serotypes have been designated: l/2a, l/2b, l/2c, 3a, 3b, 3c, 4a, 4b, 4bX, 4c, 4d, 5, 6a, and 6b. Despite the widespread occurrence *of Listeria* in nature, only three serotypes (4b, l/2a, and l/2b) account for 96% of human infections reported in the United States. A similar survey of strains from cases of human listeriosis in Britain between 1967 and 1984 revealed 1/2, 3, and 4 as the predominant serogroups causing human infection. Serotyping has poor discriminating power and is therefore of limited value as a subtyping method when compared to more advanced methods of genetic analysis.

Pulsed-field gel electrophoresis (PFGE) has been the preferred method for subtyping of *L. monocytogenes* strains, offering a high degree of discrimination of *Listeria* strains as well as reproducibility. However, investigations of several outbreaks have revealed the PFGE limitations. Alternative subtyping techniques, such as ribotyping have been used with relative

effectiveness, but have never been as widely accepted as PFGE. Searching for a technique that could replace PFGE as the method of choice for strain typing, the use of multilocus variable number tandem repeat analysis (MLVA), multilocus sequence based typing (MLST) and matrix-assisted laser desorption/ionization time-of-flight based mass spectrometry (MALDI-TOF MS) have been actively evaluated with promising results. Rapid developments in whole genome sequencing appear to offer the most viable and powerful alternative to replace PFGE.

3.1.2 Distribution of *Listeria*

Listeria monocytogenes is very widely distributed in nature and can be readily isolated from soil, water, sewage, green plant material, decaying vegetation, and numerous species of birds and mammals, including humans. Cattle, sheep and goats are the domestic mammals most frequently afflicted by listeriosis A close relationship between onset of listeriosis in ruminants and feeding of contaminated silage has long been recognized. Palsson (1962) reported that in Iceland the relationship between silage feeding and onset of listeriosis was so strong that the disease has been referred to as 'votheysveiki', or silage sickness. Presence of *Listeria* in silage is strongly influenced by pH, and samples having a pH in the range of 5.0–6.0 or above are far more likely to be sources of *L. monocytogenes* than silage where the pH is below 5.0.

The most common disease syndrome of listeriosis in ruminants is encephalitis, leading to observations of nervous system involvement in cattle and sheep. Infected animals become disoriented and circle endlessly in one direction or another depending upon the direction in which their head is drooped. For this reason, listeriosis in ruminants is often referred to as 'circling disease'. Previous investigations have identified sheep as a major reservoir of *Listeria* in nature. In one study alone, 88% of tested sheep were identified as carriers of some member of the genus *Listeria*. Gray (1960) used serotyping techniques to demonstrate the relatedness of isolates obtained from listeric sheep and the oat silage they consumed.

More recent studies have used strain-specific ribotyping to support the link between on-farm sources of *Listeria* (silage) and subsequent contamination of dairy processing environments. Infected animals displaying symptoms of listeric infection may excrete *L. monocytogenes* in milk, blood and feces. High excretion rates of *L. monocytogenes* in milk from asymptomatic cows and goats have frequently been reported. Feeding silage to sheep and goats was strongly correlated with high prevalence of *L. monocytogenes* in animals as well as the processing environment. In dairy cow herds, however, the prevalence in feed bunks, water troughs, and bedding was almost twice the prevalence found in silage samples.

3.2 Listeriosis in humans

3.2.1 Disease characterization

Because of its ubiquity in the environment, humans frequently come into contact with *L. monocytogenes*. Exposure via contaminated food or other vehicles does not necessarily result in the development of infection. *Listeria monocytogenes* is frequently shed in the stools of healthy humans who otherwise show no signs of the illness. From several early studies, it has been estimated that as many as 5% of healthy humans harbor *L. monocytogenes* in their

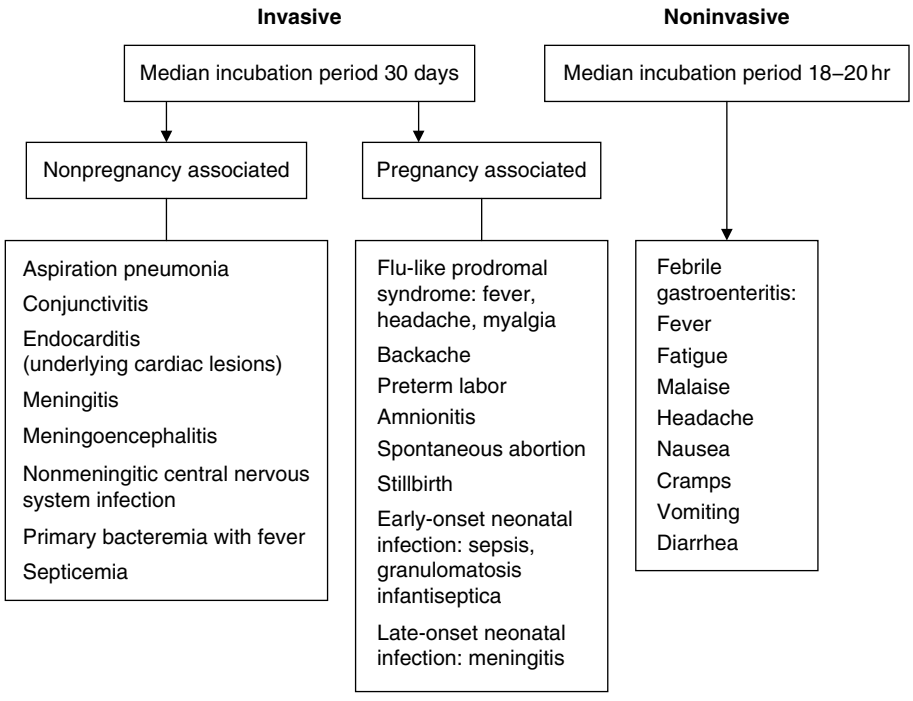

Figure 3.1 Listeriosis: disease characterization.

gastrointestinal tract. The first case of human listeriosis was described in 1929 and, since that time, listeriosis has been recognized as a rare but often fatal illness. In the most recent general morbidity and mortality estimate of foodborne disease in the US by the CDC, Scallan et al. (2011) confirmed that listeriosis is a relatively rare human illness with a total of 1660 cases per year. However, it remains a leading cause of death from a foodborne pathogen with approximately 255 deaths occurring annually. This illness has a case–fatality rate that ranges from 23 to 35%.

In adults, the disease listeriosis is characterized by two primary syndromes, an invasive form of the illness versus a noninvasive form (Fig. 3.1). Invasive illness is characterized by the onset of severe symptoms, including meningitis, septicemia, primary bacteremia, endocarditis, non-meningitic central nervous system infection, conjunctivitis, and flulike illness (fever, fatigue, malaise, nausea, cramps, vomiting, and diarrhea). The median incubation period for invasive illness prior to onset of symptoms is approximately 30 days. FoodNet monitoring data from 1998 indicated that *Listeria* infections resulted in a higher rate of hospitalization (95% of infected patients hospitalized compared to 21% for *Salmonella* infections) than any other pathogen and caused approximately half of all reported deaths. Gastrointestinal symptoms are observed in approximately one-third of documented cases of listeriosis.

A noninvasive form of listeriosis resulting in febrile gastroenteritis has been documented in several outbreaks (Fig. 3.1). Unlike the invasive form, the median incubation period prior to onset of symptoms is short, typically from 18 to 20 h. The frequency of febrile gastroenteritis as a result of *L. monocytogenes* infection is undetermined, as are host characteristics associated with this syndrome. The infectious dose needed to cause symptoms of febrile gastroenteritis is not known but has been shown to be higher than that associated with the invasive form of

Table 3.2 Underlying Patient Conditions Causing Predispositions to Listeriosis

Condition	References
Cancer (leukemia, lymphoma, hematological, pulmonary)	Fleming et al., 1985; Gellin and Broome, 1989; Linnan et al., 1988; Slutsker and Schuchat, 1999
Administration of steroids (corticosteroids)	Gellin and Broome, 1989; Slutsker and Schuchat, 1999
Treatment with cytotoxic drugs	
Renal transplant recipients, renal dialysis, chronic renal disease	
Human immunodeficiency virus (HIV), acquired immunodeficiency syndrome (AIDS)	Gellin and Broome, 1989; Slutsker and Schuchat, 1999
Pregnant women and neonates	Fleming et al., 1985; Gellin and Broome, 1989; Linnan et al., 1988; McLauchlin, 1990; Slutsker and Schuchat 1999
Age > 60 years	Gellin and Broome, 1989
Alcoholism, cirrhosis, liver disease, hepatitis	Fleming et al., 1985
Antacid, cimetidine use	Ho et al., 1986
Diabetes	Schuchat, 1992
Collagen vascular disease	Slutsker and Schuchat, 1999
Sarcoidosis	
Ulcerative colitis	
Aplastic anemia	
Intravenous drug abuse	
Conditions associated with iron overload	Mossey and Sondheimer, 1985

disease. When gastroenteritis due to listeriosis is suspected, clinicians and public health officials are advised to examine stool cultures for presence of *L. monocytogenes*.

Susceptible individuals typically have one or more underlying conditions that predispose them to acquiring listeriosis (Table 3.2). Humans shown to be at high risk for acquiring listeriosis include pregnant women, neonates, the elderly, organ transplant recipients, or those receiving immunosuppressive therapy. In the latter case, treatment of patients with corticosteroids or antimetabolites renders a suppressed immune system. Persons suffering from chronic disorders such as alcoholism, malignancy, diabetes, heart disease or acquired immunodeficiency syndrome (AIDS) have also been shown to be at risk. Additional underlying factors that have been reported in association with listeriosis include sarcoidosis, chronic otitis, collagen-vascular disease, idiopathic thrombocytopenic purpura, asthma, ulcerative colitis and aplastic anemia.

Age has been shown to be a predisposing factor in listeriosis. An 11% case–fatality rate is documented in persons age 40 or under; a 63% case–fatality rate is recorded for persons over age 60. Age-related reasons for increased incidence of listeriosis may include a decline of the immune system as a function of age, increased prevalence of immunosuppressive disorders, and increased dependence on immunosuppressive medications. Although the above-listed conditions may predispose patients to acquiring listeriosis, it should be noted that persons showing no apparent immunocompromising conditions have been shown to acquire listeriosis.

3.2.2 Listeriosis in immunocompromised hosts

Cell-mediated immunity (CMI) plays an important role in dictating the resistance or susceptibility of a human host to infection by *L. monocytogenes*. Listeriosis occurs most often in those persons with impaired CMI. CMI is dependent upon the activity of mononuclear

phagocytes as early response, nonspecific effectors, and specific T-cells as a secondary response to infection. Alteration of T-cell or macrophage function due to immunosuppression would result in an impairment of CMI, allowing a listeric infection to occur after the primary infection or after further contact with *Listeria*. Patients with cancer or undergoing treatment with steroids or cytotoxic drugs, pregnant women or neonates, renal transplant recipients, patients with AIDS, elderly or alcoholic patients, and those with diabetes are well known to have a greater likelihood for developing listeriosis. The risk of complications due to *L. monocytogenes* infection has long been recognized in CMI-compromised conditions such as Hodgkin's lymphoma and other hematological malignancies. North (1970) suppressed CMI of mice by administration of an antimitotic drug and induced a listeric infection after inoculation with *L. monocytogenes*. Golnazarian and collaborators showed that *L. monocytogenes* infectious dose for mice immunocompromised by administration of hydrocortisone acetate was more than100-fold less than for normal resistant mice.

During pregnancy, selective factors of CMI become depressed to prevent rejection of the fetus by the mother. However, depression of these selective factors may result in decreased maternal resistance to *L. monocytogenes* infections and thereby increase the maternal or fetal risk to onset of listeriosis. Such selective factors include shifts in levels of hormones or serum factors that affect lymphocyte or macrophage synthesis, activation, or function during pregnancy. Plasma levels of hydrocortisone increase during pregnancy to levels 3–7 times higher than those found in nonpregnant humans. Corticosteroids are known to suppress both lymphokine activation and phagocytic activity of macrophages. Low levels of immunoglobulin M (IgM) and decreased activity of the classic complement pathway during the neonatal period also occur and demonstrate the importance of opsonization in the immune response to *Listeria*.

In humans, listeriosis occurs most often during the third trimester of pregnancy. Three outcomes are normally followed: an asymptomatic maternal infection and a resulting infected infant; a severely ill mother who enters premature labor and delivers a stillborn or severely ill infant; or an unaffected fetus with death of the mother. In most perinatal cases of listeriosis, the mother is usually mildly affected, exhibiting flu-like symptoms, but neonatal morbidity and mortality are common. In early-onset neonatal listeriosis, transplacental infection results in a syndrome known as *granulomatosis infantisepticum*, a necrotic disease of the internal organs. Spontaneous abortion of the fetus and stillbirth of the neonate are most common, but if the fetus infected in utero is born alive, recovery is not likely. Late-onset listeriosis occurs several days after birth, and infants are generally full term and healthy at birth. Late-onset listeriosis is more likely than early-onset listeriosis to present as meningitis, and case–fatality rates are lower than for early-onset infection. Of late-onset cases reported in Britain during 1967–1985, 93% of cases presented evidence of central nervous system infection.

Buchdahl et al. (1990) described several cases in which complications due to listeriosis arose during the course of pregnancy. In one case, a mother at 32 weeks gestation developed a flu-like illness and developed irregular uterine contractions. Spontaneous membrane rupture occurred with release of meconium-stained amniotic fluid. The infant, delivered by caesarean section, was found to have blood and cerebrospinal fluid contaminated with *L. monocytogenes*. Although the infant survived, neurological handicap was evident. The mother recalled consumption of a soft-ripened French cheese nine days prior to delivery. Fortunately, perinatal listeriosis declined 63% between 1989 and 1997 in the United States due to peripartum use of the antibiotic ampicillin for group B *Streptococcus* prevention.

Listeriosis is well recognized as a complication of renal transplantation. Most patients become ill while they are receiving immunosuppressive therapy, which increases their susceptibility to listeriosis. Meningitis is recorded as the most common presentation of

listeriosis in renal transplant patients, and the fatality rate for listerial meningitis in this patient population is 38%. However, pneumonia due to *L. monocytogenes* has also been observed in renal patients, suggesting a possible respiratory route of transmission. In a study of healthy renal transplant recipients, fecal carriage of *L. monocytogenes* in eight of 37 patients was documented.

In a review of 83 cases of listeriosis in renal patients, Stamm and colleagues (1982) found that one-third of patients had been treated for acute rejection. Nieman and Lorber (1980) have reported that hemodialysis is not a predisposing factor for most patients in acquiring listeriosis. However, Mossey and Sondheimer (1985) observed four cases of *L. monocytogenes* bacteremia associated with long-term haemodialysis and transfusional iron overload. None of these patients were receiving immunosuppressive therapy. Many surveys document a higher incidence of listeriosis in the months from July to October, and the same seasonal variation has been reported for renal transplant recipients.

Patients with AIDS exhibit an impairment of T-cell-mediated immune response and therefore are at high risk for listeriosis. In early studies of the incidence of listeriosis in AIDS patients, *L. monocytogenes* was rarely implicated as an agent affecting persons with AIDS. Reasons given for this surprising finding included the fact that AIDS patients who displayed frequent gastrointestinal tract infections were given multiple courses of antibiotic therapy, thus decreasing exposure to *Listeria*. Five cases of listeriosis in Los Angeles County between January 1985 and March 1986 occurred in patients with AIDS. Prior or concurrent gastrointestinal illness was recorded in three of the patients, and four patients had no history of prior antibiotic administration. This and subsequent investigations have shown that, while listeriosis is a rare infection in patients exhibiting human immunodeficiency virus (HIV) infection, persons with AIDS have a 300- to 1000-fold increased risk of acquiring listeriosis compared with the general population. Persons with AIDS are therefore advised to refrain from ingestion of food items associated with listeriosis.

3.3 Pathogenesis

Listeria monocytogenes is an intracellular pathogen capable of invading epithelial enterocytes, macrophages and dendritic cells. The series of virulence factors involved in the attachment, invasion, growth and migration from cell to cell has been extensively characterized. Adhesion to host cells is the first step for invading individual cells that has recently been elucidated. *L. monocytogenes* synthesizes as many as eight different proteins and has three regulatory systems that allow the bacterial cells to adhere to the surface of epithelial cells. Some of the virulence factors that mediate this phenomenon include internalins (InlJ, InlF), adhesion proteins (Ami, DltA) and a fibronectin-binding protein (FpbA).

L. monocytogenes can enter host cells in two distinct ways: through active ingestion by phagocytic cells such as macrophages or through the production of specific gene products that control ingestion by normally non-phagocytic cells. Studies conducted by Gaillard et al. identified a surface protein of *L. monocytogenes*, internalin, which mediates bacterial invasion of epithelial cells. Once internalized, the life cycle of *L. monocytogenes* within both phagocytic and nonphagocytic cells is similar. The majority of virulence genes that produce products associated with the intracellular life cycle of *L. monocytogenes* reside on a region of the chromosome known as the *PrfA*-dependent gene cluster.

This *PrfA*-dependent gene cluster is comprised of the genes *prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB*. The *prfA* product is a positive regulatory factor, a 27-kDa regulatory factor that controls

all virulence genes of the virulence gene cluster. The *plcA* product is phosphatidylinositol-specific phospholipase C (PI-PLC), which contributes to vacuole escape in cells such as bone marrow-derived macrophages. The *plcB* gene encodes for a phosphatidylcholine-specific phospholipase C (PC-PLC) which, together with metalloprotease, the *mpl* gene product, enables listeriolysin O–independent escape of *L. monocytogenes* from primary vacuoles in human epithelial cells. Metalloprotease (*mpl*) permits bacterial movement from the cytosol to the host surfaces and the ensuing cell-to-cell spread. The *actA* gene locus is responsible for the accumulation of actin around *Listeria* in the cytosol. The lack of one of these determinants has been shown to interfere with the pathogenicity of *L. monocytogenes*.

The production of sulfhydryl-activated hemolysin, listeriolysin O (α-listeriolysin) is associated with the pathogenic potential of *L. monocytogenes*. Listeriolysin O is similar to streptolysin O and pneumolysin, and antigenic cross reactivity with these hemolysins as well as with hemolysins produced by *L. ivanovii* and *L. seeligeri* has been demonstrated. The virulence of *Listeria* species has also been associated with the ability to survive and grow intracellularly.

Gaillard et al. studied the role of hemolysin (listeriolysin O) in pathogenicity of *L. monocytogenes*. These investigators inactivated a genetic determinant for hemolysin production and obtained non-hemolytic mutants from hemolysin-producing strains. The loss of hemolysin production was shown to be associated with loss of virulence in a mouse model. Further studies by the same authors found that *L. monocytogenes* and *L. ivanovii* invaded a continuous gut epithelial cell line, whereas *L. seeligeri*, *L. innocua* and *L. welshimeri* did not. A non-hemolytic mutant of *L. monocytogenes* invaded these gut enterocytes at the same rate as the hemolytic wild type. This finding, which has been corroborated by others in a fibroblast 3T6 continuous cell line, demonstrates that listeriolysin O is not involved in invasion. An extracellular protein (p60) may be involved in the process of attachment and invasion of *L. monocytogenes*. Under heat shock conditions, listeriolysin is synthesized, whereas production of p60 no longer occurs. Protein p60 is found both as a major secreted protein and on the cell surface of all *L. monocytogenes* isolates. In addition, this protein possesses murein hydrolase and is involved in cell division. Rough mutants of *L. monocytogenes* that lack p60 form long chains of cells that fail to separate. These mutants also show reduced uptake by 3T6 fibroblast cells.

Factors other than hemolysin have been defined as essential virulence factors for *L. monocytogenes*. Hof and Rocourt found that a construct of a virulent *L. monocytogenes* EGD with selective blockade of phospholipase C production became avirulent. Tilney and Portnoy demonstrated that *L. monocytogenes* is capable of bypassing the humoral immune system by remaining in an intracellular state and spreading cell to cell. Following phagocytosis by host macrophages and escape from the phagocytic vacuole, *Listeria* species are coated with actin filaments, form a pseudopod, dissolve the phagocytic vacuolar membrane presumably by use of hemolysin, and repeat the cycle. It was postulated from this and other studies that once *Listeria* enters macrophages, listeriolysin O is needed to lyse phagosomes, thereby releasing *Listeria* into the cytoplasm so that it can multiply. *Listeria* species that lack hemolysin fail to grow in vivo because of the inability to dissolve the endosomal membrane and failure to escape from the endosome into the cytoplasm.

Cowart showed that hemolysin activity is stimulated in iron-deprived medium. The cytolytic activity of hemolysin is maximally expressed at pH 5.5. Therefore, when *L. monocytogenes* are engulfed in phagosomes that do not contain iron and have a pH value around 5.5, hemolysin production is maximized, allowing destruction of internal membranes surrounding them. An additional mechanism of intracellular survival is dependent upon the ability of

L. monocytogenes to resist killing by oxidizing agents produced by phagocytes. The production of superoxide dismutase (SOD) and catalase by *L. monocytogenes* has been a factor associated with intracellular survival. Bortolussi et al. demonstrated that resistance of *L. monocytogenes* to hydroxyl radical (OH) during the log phase of growth was due to the production of sufficient amounts of catalase to inactivate this product. Welch et al. found that catalase-negative strains of *L. monocytogenes* possessed at least twofold greater SOD activities than catalase-positive strains.

Not all strains of *L. monocytogenes* are pathogenic. Further, within *L. monocytogenes* strains, there may be particular serotypes that possess enhanced virulence potential. A survey conducted by Pinner et al. showed that foods containing *L. monocytogenes* serotype 4b were four times more likely to contain strains identical to patient strains than were foods containing serotypes l/2a or l/2b. These and other observations suggest that serotype 4b strains may have an enhanced capacity to cause human disease. Wiedmann et al. characterized 133 isolates of *L. monocytogenes* according to ribotype and virulence gene analysis. These authors first proposed that *L. monocytogenes* strains could be clustered into three distinct lineages. More recently, four lineages of *L. monocytogenes* strains were proposed by the same research group. In this recent classification, lineages I and II include almost all human pathogenic strains, and lineages III and IV encompass environmental and animal isolates that are largely nonpathogenic. Strains of lineage I ribotypes have been predominantly responsible for outbreaks and contribute to sporadic cases while lineage II strains are involved with sporadic cases and are frequently isolated from foods and the environment.

3.4 Foodborne transmission

3.4.1 Foodborne disease epidemics: North America

Listeria monocytogenes has emerged as a foodborne pathogen of major significance within the last three decades. Most of our knowledge of routes of foodborne transmission of *Listeria* has been gained through study of epidemiological data from outbreak investigations of human listeriosis. While outbreaks have occurred worldwide, this chapter will focus primarily on listeriosis in North America, where eight major outbreaks have occurred since 1979 (Table 3.3).

In 1979, listeriosis was diagnosed in at least 23 hospitalized patients in the Boston, Massachusetts, area. The vehicle of infection in this outbreak was linked to hospital food, and patients who had consumed lettuce, carrots, and radishes were more likely to contract the illness. Isolates from 20 of 23 cases were identified as serotype 4b. Symptoms reported by the afflicted patients included bacteremia or meningitis. Fifty percent of the patients involved in this outbreak were immunosuppressed because of cancer, chemotherapy, or steroid treatment. Curiously, 60% of the afflicted patients had reported the use of antacids or the antiulcer medication cimetidine. Cimetidine is a histamine-2 antagonist that blocks the H_2 effects of histamine, thereby decreasing gastric acid secretion, whereas antacids neutralize gastric acid. It was hypothesized that gastric acid neutralization following use of antacids or cimetidine predisposed humans to acquiring listeriosis as a result of ingestion of this organism via a foodborne vector. This finding was corroborated in later studies, where patients were more likely than controls to have used antacids, laxatives or H_2-blocking agents prior to onset of listeriosis.

In 1981, an outbreak of listeriosis occurred in the Maritime Provinces of Canada. The vehicle of transmission was identified as commercially-prepared coleslaw. Cabbages used

Table 3.3 Foodborne Listeriosis: North American Outbreaks, 1979–Present

		Cases					
Date and location	**Illness presentation**	**Number**	**Mortality**	**Food source**	**Edidemic serotype**	**Risk factors**	**References**
1979: Boston, MA	Bacteremia meningitis	23	15	Lettuce, carrots, radishes	4b	Cimetidine, antacid use	Ho et al., 1986
1981: Maritime Provinces, Canada	Meningitis, aspiration pneumonia, sepsis, abortion, stillbirth	41	41	Coleslaw	4b	Pregnancy	Schlech et al., 1983
1983: Boston, MA	Mengitis, septicaemia, death in utero	49	29	Pasteurized whole and 2% milk	4b	Cancer, alcoholism, corticosteroid therapy	Fleming et al., 1985
1985: Orange County, CA	Fever, vomiting, stillbirth gastroenteritis	142	33	Mexican-style cheese	4b	Pregnancy, cancer, steroid therapy, AIDS	Linnan et al., 1988
1994: Elizabeth, IL	Gastroenteritis with fever	45	0	Chocolate milk	1/2b	No chronic illness, pregnant female	Dalton et al., 1997
1998–1999: multistate	Severe febrile gastroenteritis	101	21 15 adult deaths, 6 miscarriages	Hot dogs	4b	Pregnancy, diabetes, kidney disease, lupus malignancy	CDC, 1999
2002	Fever, vomiting, stillbirth, gastroenteritis	54	11 8 adults, 6 foetal deaths	Sliced turkey deli	4b	Hematologic and solid malignancy, steroid use, AIDS, diabetes, liver disease	Gottlieb, et al., 2006
2008	Fever, gastroenteritis, meningitis	57	20	Sliced deli meats	1/2a	Elderly and hospitalized patients, mean age 74 years	PHAC, 2010
2011	Severe febrile gastroenteritis	147	33	Cantaloupes	1/2a, 1/2b	Elderly, median age 78 years	CDC, 2012(a)

Source: Ryser, E. T., Arimi, S. M., Bunduki, M. M.-C. and Donnelly, C. W. (1996) Recovery of different Listeriaribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. Appl Environ Microbiol 62, 1781–1787.

to prepare coleslaw were traced to a sheep farm where an outbreak of listeriosis had killed several sheep. Use of manure from infected sheep was suspected as a factor in this outbreak. Thirty-four cases of listeriosis in pregnant women resulted in spontaneous abortions, stillbirths, or live birth of ill infants. Seven nonpregnant adults who showed no evidence of immunosuppression had symptoms of meningitis, aspiration pneumonia and sepsis. The overall mortality rate for this outbreak was 41%. All patient isolates were identified as serotype 4b, and *L. monocytogenes* isolates from unopened packages of coleslaw were also identified as serotype 4b.

In 1983, 49 patients in Massachusetts were diagnosed with listeriosis. Epidemiological evidence pointed to a strong association between consumption of pasteurized whole and 2% milk and onset of the illness. Forty-two patients were characterized with underlying illnesses such as cancer or alcoholism, and several patients were undergoing corticosteroid therapy. Seven of the cases involved fetuses or infants. The overall case–fatality rate in this outbreak was 29%. Of 49 isolates available for serotyping, 32 were identified as serotype 4b. Despite numerous attempts, the epidemic serotype of *L. monocytogenes* responsible for this outbreak was never recovered from the incriminated milk.

In June 1985, Jalisco-brand Mexican-style cheese was implicated as the vehicle of infection in an outbreak of listeriosis in southern California. A total of 142 cases involving 93 pregnant women or their offspring and 49 non-pregnant immunocompromised adults were documented in Los Angeles County. Forty-eight deaths were recorded, accounting for a mortality rate of 33.8%. The majority of afflicted individuals (62%) were pregnant Hispanic women. Although an additional 160 cases occurred in other parts of California, for logistical reasons, the study reported by Linnan et al. was limited to Los Angeles County. In this outbreak, the cheese was most likely manufactured from a combination of raw as well as pasteurized milk, and the cheese plant that manufactured the incriminated cheese was found to harbor *Listeria* as an environmental contaminant. The epidemic strain in this outbreak was serotype 4b, and this serotype was recovered from unopened packages of Queso Fresco and Cotija Mexican-style cheese.

Dalton et al. reported an outbreak of listeriosis linked to consumption of chocolate milk served at a picnic during a Holstein cow show in Illinois in 1994. Forty-five individuals developed illness due to *L. monocytogenes*; however, unlike symptoms reported in previous outbreaks, illness in this outbreak was characterized by fever and gastroenteritis without progression to invasive disease. Additionally, none of the patients reported immune deficiency or chronic illness. One pregnant female patient delivered a healthy baby. This outbreak, in particular, illustrates how failure to adhere to good manufacturing practices can have severe human health consequences. In reviewing steps in the manufacture of this product, milk was pumped into a non-refrigerated holding tank where a breach in the lining may have allowed milk to leak into the insulating jacket and back into the product. Plant inspections revealed that sanitizing solution sprayers were severely clogged. The product was subsequently pumped into a filling machine over a 7-h period. Milk left the filler at 45 °F and was refrigerated but was transported for more than 2 h in an unrefrigerated truck the day before the picnic. The milk was refrigerated overnight. The next morning milk was placed in an unrefrigerated cooler and transported to the picnic. Most of the milk was consumed within the first hour of the picnic, but it remained available throughout the afternoon. Unopened cartons of milk contained *L. monocytogenes* at levels of 1.2×10^9 CFU/mL. The median dose of *Listeria* consumed by the afflicted individuals may have been as high as 2.9×10^{11} CFU/person. None of the samples taken from the holding tank yielded *Listeria*. However, isolates obtained from the floor drain and the valve connected to the chocolate milk pasteurizer

yielded *L. monocytogenes* serotype l/2b. Post-pasteurization contamination was implicated as the cause of this outbreak. Proctor et al. used PFGE to link four additional sporadic cases of invasive listeriosis to recalled 1% low-fat chocolate milk responsible for this outbreak.

During the period from August 1998 to March 1999, 101 cases of listeriosis were reported in 22 states. Twenty-one fatalities (15 adult deaths, six miscarriages) were recorded in this outbreak. The outbreak strain was identified as *L. monocytogenes* serotype 4b (E_0, E_1, E_2 PFGE pattern), and this rare strain was isolated from packages of hot dogs as well as environmental samples taken from the hot dog manufacturing plant. The start of the outbreak coincided with the removal of a large refrigeration unit near a hot dog packaging line. Plant records revealed an increase in the incidence of psychrophilic organisms from product contact surfaces coincident with removal of the refrigeration unit from the plant. Samples of hot dogs cultured quantitatively for *L. monocytogenes* serotype 4b revealed contamination at extremely low levels of <0.3 CFU/g, suggesting the possibility of enhanced virulence of this particular strain of *L. monocytogenes*.

A second major outbreak linked to deli meats occurred in the US from August to November 2002. In this event, as many as 54 listeriosis cases and eight deaths were caused by contaminated turkey meat products. Patients were reported in eight states of the country. A serotype 4b strain was also implicated, but this strain was isolated from the environment of one processing plant and from the turkey products of a second producing company. Other *L. monocytogenes* strains were isolated from the first plant and, similar to the previous outbreak, it seemed that the extensive contamination was related to a construction project in that plant. The plants were located at approximately 50 km from each other. This outbreak prompted the recall of more than 15 million kilograms of products between the two companies. This outbreak prompted the U.S. Department of Agriculture to issue new directives for testing and risk assessment of ready-to-eat meat and poultry plants.

One of the most recent outbreaks linked to RTE meats occurred in Canada in 2008 when several types of sliced deli meats were responsible for a total of 57 listeriosis cases and 20 deaths in different provinces. This outbreak was characterized for affecting elderly individuals that were in assisted living or in the hospital who were given unheated RTE sliced products. A strain belonging to serotype 1/2a was isolated from the patients as well as from at least 18 different meat samples. The meat products included ham, salami, pastrami, turkey breast and bologna, among a total of 23 types. The traceback investigation detected the same outbreak strain from the environment of the implicated processing plant, in particular from the slicing machine. This event illustrated the lethal consequences of poor equipment design combined with deficient sanitary practices. As a result of this outbreak the Canadian government prompted a thorough investigation.

One of the largest and most deadly listeriosis outbreaks occurred recently in the US as a result of contaminated cantaloupes. In the summer of 2011, as many as 147 people were afflicted by *L. monocytogenes* infections and 30 of those cases resulted in death. This major outbreak had several unique features: the first outbreak associated with a fresh fruit, the median age of patients was relatively high (78 years old) and as many as four different strains belonging to serotypes 1/2a and 1/2b were responsible for the infections. Patients were distributed among 28 states and 93% had reported eating cantaloupes within a month before the onset of symptoms. Traceback investigations identified a single farm responsible for the contaminated cantaloupes. FDA investigators detected the presence of all four outbreak strains in the packing facility of the farm resulting from lack of good sanitary practices. A major consequence of this outbreak has been the increased testing of fresh produce, resulting in larger numbers of fresh produce recalls in 2012.

3.4.2 Sporadic cases of listeriosis

Most cases of human listeriosis are not outbreak-related but rather occur as sporadic illnesses confined to a single individual. Ongoing and active disease surveillance has confirmed that most sporadic listeriosis cases are the result of foodborne transmission. From September 1986 to June 1987, the CDC conducted a population-based active surveillance for *L. monocytogenes* infections. This surveillance involved 154 patients from six regions of the United States (New Jersey, Missouri, Oklahoma, Tennessee, Washington, and Los Angeles). From these data, it was estimated that approximately 1700 cases of listeriosis occurred in the United States in 1986, for an annual incidence rate of 7.1 cases per million persons. Epidemiological evidence suggested that consumption of contaminated foods accounted for 30 of the 154 cases (20%) of listeriosis reported. Two foodborne sources were epidemiologically linked with onset of illness, these being uncooked hot dogs and undercooked chicken. As a result of this active surveillance, a recall of turkey franks commenced after being linked with the death of a patient in Oklahoma. *Listeria monocytogenes* serotype l/2a strains of identical isoenzyme types were isolated from the patient as well as unopened packages of turkey franks.

Between 1 November 1988, and 31 December 1990, the CDC conducted a second major case-control study in order to identify dietary risk factors for sporadic listeriosis. The population base in this active surveillance was in excess of 18 million persons distributed within five geographic regions of the United States. Cases were enrolled from patients identified through active surveillance. Underlying patient conditions included pregnancy, steroid therapy, cancer, renal dialysis, diabetes, HIV infection, liver disease and organ transplant recipients. Three hundred and one cases of listeriosis were confirmed in this study. Foods in refrigerators of patients were examined for presence of *Listeria*. Sixty-four percent of refrigerators (79 out of 123 examined) yielded an *L. monocytogenes* isolate. Of 2229 foods examined, 11% were positive for *L. monocytogenes*. Serotypes 4b, l/2a, and l/2b accounted for 95% of *L. monocytogenes* isolates recovered from foods. Of the *L. monocytogenes*-positive foods, 33% matched the patient isolates. Sixty-seven percent of dairy isolates matched patient strains, implicating dairy products as sources of *L. monocytogenes*. Specific dairy product sources included Mexican-style cheese, Feta cheese and commodity cheeses. Thirty-two percent of sporadic cases of listeriosis could be attributed to eating foods purchased from delicatessen counters, Mexican-style and feta cheeses, and undercooked chicken. Preliminary CDC data for 1991 suggested a decrease of 30–40% in the number of cases compared with 1989–1990. The annualized sporadic incidence for listeriosis was found to be 7.4 cases per million population, with an overall case–fatality rate of 23%. Serotypes l/2a (23%) and l/2b (36%) together accounted for 59% of the cases; serotype 4b was isolated from 37% of the patients.

Tappero et al. (1995) reported a 44% decrease in rates of invasive listeriosis and a 48% decrease in the numbers of deaths due to listeriosis in the United States from 1989 to 1993. There were 1092 cases of listeriosis reported in 1993, resulting in 248 deaths, for an overall annual incidence of 4.2 cases per million population. Case–fatality rates remained similar (25% in 1989 compared to 23% for 1993). The decreased incidence rate was attributed to food industry efforts, sustained prevention efforts, and continued active surveillance.

In 1996, the US FDA launched an active epidemiological system in several states or regions of the country referred as FoodNet. This system closely records any case of 10 different foodborne pathogens and includes *L. monocytogenes*. Starting in 2000, the system included a total of 10 states or regions in which public health authorities implemented active

surveillance. The number of total cases of *L. monocytogenes* in from 2003 to 2011 in the FoodNet system ranged from 114 to 149 for an average annual incidence of 0.28 cases per 100,000 people. While some of the cases could have been part of outbreaks, more than 95% were sporadic cases. The US government has targeted its National Health Objective for the year 2020 as 0.2 cases/100,000 persons for the FoodNet incidence reports.

3.5 Sources of *Listeria* in foods and food-processing environments

A host of unique properties possessed by *Listeria* render this a difficult organism to control in foods. *Listeria* can grow over a wide range of temperatures (–1.5 °C to 45–50 °C) and pH (4.3–9.6), survives freezing, and is relatively resistant to heat. Minimal water activity levels for growth of *L. monocytogenes* and *L. innocua* range from 0.90 to 0.97. Shahamat et al. reported survival of *L. monocytogenes* for 132 days at 4 °C in trypticase soy broth containing 25.5% NaCl. *Listeria* is a psychrotrophic pathogen, and growth at temperatures as low as –0.1 to –0.4 °C in chicken broth and pasteurized milk and –1.5 °C vacuum-packaged meat have been recorded.

The ability of *L. monocytogenes* to resist the heating temperatures used during milk pasteurization continues to be debated in the scientific literature. Fleming et al. in their studies of an outbreak of listeriosis in Boston, Massachusetts, concluded that "intrinsic contamination of the milk and survival of some organisms despite adequate pasteurization is both consistent with the results of this investigation and biologically plausible" (p. 407). Fleming et al. reached this conclusion based upon the fact that milk involved in the outbreak came from farms where outbreaks of listeriosis had occurred; there was no evidence of improper pasteurization or post-pasteurization contamination of the processed milk.

Comprehensive studies conducted by the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture along with Health and Welfare Canada have shown that *Listeria* is unable to survive normal conditions of milk pasteurization. Knabel et al. found that growing *L. monocytogenes* at 43 °C prior to heat inactivation caused an increase in thermotolerance, but a study conducted by Farber et al. demonstrated that even under worst-case scenario conditions, which included cultivation of *L. monocytogenes* populations at 43 °C prior to inactivation, pasteurization would render a 4.5- to 6.2-D (decimal reduction value) process. Lovett et al. and Beckers et al. estimated that extremely low levels of *L. monocytogenes* (0.5–1.0 *Listeria*/mL) exist in commercial bulk tank raw milk. Therefore, while populations of *L. monocytogenes* have been shown to survive minimum pasteurization treatments of 71.1 °C/16 s in various laboratory studies, survival under actual conditions of commercial milk pasteurization and processing is unlikely.

Listeria contamination of processed dairy products is most likely a function of post-pasteurization recontamination, and numerous surveys document the presence of *Listeria* within the dairy plant environment. Sources of *Listeria* within the dairy plant environment include floors in coolers, freezers and processing rooms, particularly entrances; cases and case washers; floor mats and foot baths; and the beds of paper fillers. Pritchard and colleagues, in a study of dairy processing facilities, found that those processing plants having a farm contiguous to the processing facilities had a significantly higher incidence of *Listeria* contamination than those farms without an on-site dairy farm. Arimi et al. used ribotype analysis to demonstrate the link between on-farm sources of *Listeria* contamination (dairy cattle, raw milk and silage) and subsequent contamination of dairy processing

environments. Raw milk is a well-recognized source of *Listeria*, and for this and numerous other microbiological reasons, consumption of raw milk should be avoided.

The heat tolerance of *L. monocytogenes* was an issue that resurfaced during the outbreak linked to deli meats in 2002. In this particular case, the presence of the same strain in both the raw product of a company and in the finished product of a second processing plant led to speculation that this pathogenic bacterium could have survived the cooking process. This hypothesis, however, could not be corroborated and recent studies have reported death kinetics of *L. monocytogenes* at temperatures of approximately 70 °C, which suggested that survival in a well-controlled cooking process would be very unlikely.

Studies by Ryser and Marth examined the fate of *L. monocytogenes* during the manufacture of Cheddar, Camembert and brick cheese. Rapid growth of *Listeria* to populations of 5×10^7 CFU/mL is observed in Camembert cheese, which has a pH that increases during ripening, thereby creating a favorable growth environment for *Listeria*. In contrast, *Listeria* populations show a marked decline in viable population levels during ripening of Cheddar cheese. However, population levels do not decline to undetectable levels. Current US regulations call for cheese made from raw or sub-pasteurized milk to be ripened at 1.7 °C (35 °F) for at least 60 days prior to sale. Ryser and Marth have shown that aging alone will not ensure the production of *Listeria*-free Cheddar cheese.

Genigeorgis et al. evaluated the ability of 24 types of market cheeses to support growth of *L. monocytogenes*. Cheeses able to support growth of *L. monocytogenes* included soft Hispanic-type cheeses, ricotta, Teleme, Brie, Camembert and cottage cheeses (pH range 4.9–7.7). Cheeses not supporting growth and that resulted in gradual death of *L. monocytogenes* included Cotija, cream, blue, Monterey Jack, Swiss, Cheddar, Colby, string, provolone, muenster, feta and Kasseri (pH range 4.3–5.6). A correlation was observed between growth of *Listeria* in cheeses having a pH greater than 5.5 and in cheeses manufactured without a starter culture.

The increased risk of Hispanic cheeses as vehicles for foodborne disease was first recognized in 1985, when 152 people contracted listeriosis from consumption of Mexican-style cheese that had been produced from unpasteurized milk. In 2001, another listeriosis outbreak that occurred in North Carolina was also caused by Mexican-style cheese produced illegally with raw milk; this affected a total of 13 patients. More recently, a string of outbreaks occurred from 2003 to 2007 in Texas from legally imported product from Mexico (Table 3.4). Despite the fact that most of these outbreaks have been caused by the use of unpasteurized milk, the presence of *Listeria monocytogenes* is still a major concern because environmental contamination can occur post-processing.

Table 3.4 Foodborne Listeriosis Cases Linked to Consumption of Queso Fresco in the US

Year	Serotype	No. of cases (fatalities/stillbirths)	Location	References
1985	4b	142 (48/30)	California	Linnan et al., 1988
2000	4b	12 (0/5)	North Carolina	MacDonald et al., 2005
2003	Non-typeable	6 (1/1)	Texas	Gaul, 2007
2004	Non-typeable	15 (3/1)	Texas and 7 other states	Gaul, 2007
2005	I. Non-typeable II. Unknown	I. 9 (n.a) II. 5 (n.a)	Texas and 7 other states	Gaul, 2007
2006	Non-typeable	8 (0/0)	Texas	Gaul, 2007
2007	Non-typeable	5 (0/0)	Texas	Gaul, 2007

The risk of *L. monocytogenes* in Hispanic fresh cheeses is based on its widespread occurrence in the environment of dairy plants and its ability to survive and even grow on these products. A longitudinal study reported that as many as 6% of cheeses and 11% of environmental samples from Hispanic fresh cheese plants that used pasteurized milk were positive for *L. monocytogenes*. In one of the first studies that investigated the ability of this pathogen to grow on fresh cheeses, Genigeorgis and coworkers indicated that *Listeria* could grow in commercial samples of Mexican-style fresh cheeses stored at refrigeration temperatures, and it could be present at significant levels after 30 days of storage. In another publication, the population of this bacterium in queso fresco increased from 10 to more than 1000 CFU/g in less than two weeks, and it remained at almost 10,000 CFU/g after 12 weeks of storage at 4 °C. These studies clearly indicated that Hispanic fresh cheeses can be a vehicle for transmission of *L. monocytogenes* and showed the importance of developing antimicrobial interventions post-pasteurization.

Ryser et al. examined the fate of *L. monocytogenes* during manufacture and storage of cottage cheese. *Listeria monocytogenes* survived in both creamed and uncreamed cottage cheese during 28 days of storage at refrigeration temperatures and was recovered in higher numbers from creamed (pH 5.32–5.45) versus uncreamed (pH 5.12–5.22) cottage cheese. Hicks and Lund examined the ability of *L. monocytogenes* to survive in creamed cottage cheese when stored at 4, 8, or 12 °C for 14 days. The organism survived but failed to increase in numbers during storage in product with a pH range of 5.06–4.53. Chen and Hotchkiss, however, found that *L. monocytogenes* grew in cottage cheese stored at 7 °C for 16 days or 4 °C for 63 days but would not grow under these conditions when modified CO_2 packaging conditions were used.

Conner and collaborators investigated the effects of temperature, NaCl, and pH on the growth of *L. monocytogenes* in cabbage. Results indicated that cabbage juice provided a good substrate for growth of *Listeria*. The organism was found to survive well at 5 °C even in the presence of 5% NaCl, and the organism could grow and tolerate a pH of less than 5.6. This study, together with the findings of Schlech et al. confirmed that cabbage can serve as a vector of transmission of *L. monocytogenes* to humans and demonstrates the potential for *L. monocytogenes* to persist and proliferate on vegetables and in brines used to ferment vegetables. Potatoes and radishes have been identified as sources of *L. monocytogenes* during retail food surveys (Heisick et al., 1989). Additional studies have confirmed growth and survival of *L. monocytogenes* on asparagus, broccoli, cauliflower, corn, green beans, lettuce and radishes (Berrange et al., 1989; Hughey et al., 1989).

The presence of *L. monocytogenes* in many fresh fruits and vegetables has been frequently reported in the literature. In 2012, as a result of the large outbreak linked to cantaloupes linked to a farm in Colorado in 2011, increased testing was implemented. From January to September, a record number of more than 20 recalls of fresh produce was reported by the FDA. These produce recalls included romaine lettuce, spinach, apples, salad mixes, cantaloupes, different varieties of sprouts, cut fruit salad, mushrooms and onions.

Ready-to-eat poultry products have been implicated as vectors of transmission of listeriosis to humans. Cooked-chilled chicken and turkey frankfurters were confirmed as vehicles of *Listeria* infection in England and the United States respectively during 1988 and 1989. A relatively small and first outbreak linked to turkey franks in the US prompted the first zero-tolerance policy for a foodborne pathogen in meats. As a result of this policy, significant research has been devoted to understanding the sources of contamination of RTE meats. Similar to dairy products, contamination of deli meats often occurs from poorly sanitized equipment and contact surfaces before the product is packaged. As a result of multistate

outbreaks of listeriosis associated with RTE products in 1998 and 2002, the USDA implemented stricter regulations to prevent and minimize post-processing contamination by promoting the use of post-lethality treatments and the use of antimicrobial ingredients.

Meat and poultry products including ground beef, pork sausage, cooked beef, roast beef, ham, bologna, bratwurst, sliced chicken and turkey have been identified as products that promote growth and can serve as sources of *L. monocytogenes*. Farber and Daley, however, found in naturally contaminated products, that when present initially on sliced ham, turkey breasts, wieners and paté at very low levels and stored at 4 °C, numbers of *L. monocytogenes* did not increase during storage. Extensive research conducted in RTE meats has resulted in multiple growth models on the ability of *L. monocytogenes* to grow in these products at refrigeration temperatures which have been used successfully for risk assessment. Some studies have reported that this pathogen can reach generation times of approximately one day at 4 °C in frankfurters. The USDA's Food Safety and Inspection Service (FSIS) microbiological monitoring data obtained from 1993 to 1999 identified hot dogs and luncheon meats as two products of concern that may serve as primary vehicles for foodborne transmission of *L. monocytogenes*. During those years, the FSIS reported that more than 3 and 5% of RTE sausages and luncheon meats respectively were contaminated with *L. monocytogenes*.

Raw poultry is a well-recognized source of *L. monocytogenes*, and numerous surveys have confirmed the presence of *L. monocytogenes* in retail poultry samples. Bailey et al. recovered *L. monocytogenes* from 23% of sampled broiler carcasses, the most prevalent serotype isolated being l/2b. Gilbert et al. confirmed the presence of *L. monocytogenes* in 12% of precooked RTE poultry products collected from the London-area retail establishments between mid-November 1988 and mid-January 1989. Survival of *L. monocytogenes* on chicken breasts processed by moist and dry heating methods has been reported.

Wenger et al. examined a turkey frank production facility in order to determine sources and incidence of contamination. *Listeria monocytogenes* was isolated from only two of 41 environmental samples from the plant, which included a cooler room floor and a conveyer belt attached to a peeler. Yet *L. monocytogenes* was isolated from six of seven retail lots of product produced over a 37-day production period. Product samples taken at the production line post peeler were more likely (12 of 14 samples positive) to be contaminated than samples from other production locations (two of 40 samples positive). Therefore, product contamination was found to occur at a single point during the peeling process prior to packaging of product.

Combinations of lactate and diacetate salts as antimicrobial ingredients have offered one of the most effective controls to inhibit the growth of *L. monocytogenes* in RTE meat products. The use of lactate/diacetate salts was first proposed by Schlyter and co-workers in 1993 when they treated turkey slurries with mixtures of sodium diacetate (SDA) and sodium lactate (SL) and observed effective growth inhibition of *L. monocytogenes*. After this initial report, multiple investigations were conducted to confirm the effectiveness of diacetate/lactate in meat products. Mbandi and Shelef reported that combinations of 2.5% SL and 0.2% SDA were bacteriostatic in ground beef. Additional work by different researchers confirmed that lactate/diacetate mixtures were able to inhibit *Listeria* growth in frankfurters, bratwurst, wieners, sliced ham and other meat products. The effectiveness of SL/SDA was extensively documented, and during the late 2000s their inclusion in a wide variety of RTE meats was adopted by most commercial brands. To this date, almost only those brands that carry the label of 'natural' or 'organic' do not include lactate/diacetate in their formulation.

Seafood is recognized as a source of *L. monocytogenes*. Weagant et al. documented the presence of *L. monocytogenes* in frozen seafood samples that included shrimp, crabmeat, lobster tail, fin fish and surimi-based products. Farber isolated *L. monocytogenes* from ready-to-eat shrimp, crab and smoked salmon, and further laboratory studies demonstrated growth at 4 °C of *L. monocytogenes* on cooked crabmeat, lobster, shrimp and smoked salmon. Jemmi, upon examination of 377 samples of smoked and marinated fish, found *L. monocytogenes* in 47 samples. A survey in Newfoundland also revealed the presence of *Listeria* in smoked seafood products. Crab and smoked fin fish accounted for the majority of seafood products involved in class 1 recalls during the 11-year period from 1987 to 1998 because of *L. monocytogenes* contamination.

3.6 Detection of *Listeria* in foods

3.6.1 Selective enrichment and enumeration

Detection of *L. monocytogenes* in food products or food-processing environments is accomplished by the use of a variety of standard or rapid microbiological procedures. Among the most widely used are protocols devised by the USDA-FSIS for the detection of *Listeria* in meat and poultry products and the FDA for the detection *of Listeria* in dairy products, fruits, vegetables and seafood products. A detection method widely used in Europe is the Netherlands Government Food Inspection Service (NGFIS), a method developed by Netten and others. In addition to traditional microbiological methods, there are a wide variety of rapid methods that have been recognized for regulatory screening.

In almost all food testing protocols, samples are usually mixed with an enrichment broth and allowed to incubate for 24–48 h. Following incubation, a portion of the enrichment mixture is either again mixed with an enrichment broth or plated onto the final isolation agar. Enrichment broths are usually nutritious liquid media that employ various antimicrobial agents to which *L. monocytogenes* is resistant. The most common antimicrobial agents include nalidixic acid, acriflavin and cycloheximide. Isolation agars include those used for direct plating, although less selective agars have also been used successfully. The FDA protocol employs buffered Listeria enrichment broth (BLEB) as the first step with a subsequent plating onto Oxford, PALCAM or MOX agar plates. In contrast, the current USDA method uses University of Vermont medium (UVM) as an initial step and a simultaneous transfer to MOX and modified BLEB as the second step after 24 h.

Numerous studies have been conducted to compare the efficacy of these and other widely-used detection protocols. Hayes et al. compared three enrichment methods for examination of foods obtained from the refrigerators of patients with active clinical cases of listeriosis. This study examined 2229 foods, of which 11% were positive for *L. monocytogenes*. A comparative evaluation of three microbiological procedures was conducted on 899 of the examined foods. The FDA procedure detected *L. monocytogenes* in 65% of the foods shown to be positive, while the USDA-FSIS and NGFIS procedures detected *L. monocytogenes* in 74% of foods shown to be positive. Thus, none of the widely-used conventional methods proved to be highly sensitive when used independently for analysis of *Listeria* contamination in foods. It was noted, however, that use of a combination of any two methods improved detectability from 65 to 74% (for individual protocols) and from 87 to 91% for combined protocols.

The USDA-FSIS verifies the adequacy of hazards analysis and critical control point (HACCP) systems used by meat and poultry processing facilities in part through the collection

and testing of ready-to-eat meat products for pathogenic bacteria such as *L. monocytogenes*. The use of HACCP strategies to effectively eliminate organisms such as *Listeria* from food-processing environments and, therefore, processed food products is predicated upon the use of sensitive, reliable, inclusive enrichment methods that facilitate the recovery of *Listeria*. However, since current procedures used by the USDA-FSIS to test for the presence of *Listeria* rely on the use of highly selective primary enrichment media, these media lack the sensitivity and reliability necessary to detect low level contamination of *Listeria* in food products. Low levels of *Listeria* existing initially in food products that undergo long periods of refrigerated storage can multiply to dangerous levels that can ultimately cause human illness. As the research cited above shows, existing regulatory procedures have only a 65–75% sensitivity and reliability rate. Simple modifications to existing regulatory protocols, such as those that utilize more than one primary enrichment broth, raise the sensitivity of detection to 90%.

Enumeration procedures described in the FDA's Bacteriological Analytical Manual include a choice of two methods: a most probable number (MPN) technique and a direct plating method. The MPN procedure is described as the most sensitive, detecting 100 CFU/g or less of *L. monocytogenes*. This procedure utilizes BLEB medium in a nine-tube series. The direct-plating method utilizes UVM as the diluent for homogenization of the sample, followed by direct plating onto modified Oxford medium (MOX). This method is described as being considerably less sensitive than the MPN method, possibly underestimating the actual number of cells present, particularly with respect to injured cells. Therefore, the direct-plating method would be utilized for samples anticipated to contain high levels of *L. monocytogenes*. Alternatively, the FDA protocol includes an MPN filter and DNA probe colony hybridization as additional enumeration methodologies. Utilization of chromogenic media greatly improves discrimination among *Listeria* species. A few commercial chromogenic agars offer the capability of discriminating *L. monocytogenes* from other species based on the identification of single virulence factors such as phosphatidylinositol-specific phospholipase C.

Several rapid commercial methods are available for the detection of *Listeria* and are based on enzyme-linked immunosorbent assay (ELISA) and DNA probe and amplification technologies. In 2012, according to AOAC International, there were over 45 different commercial kits as performance test methods. These procedures go beyond traditional identification methods through incorporation of genetic and immunological techniques to reduce identification time, and commercial kits are available for both. In fact, the FSIS protocol currently requires a PCR screening step parallel to the MOX identification from modified BLEB cultures. Rapid methods are faster than conventional methods and performance is similar. The main drawbacks to the rapid methods developed to date are cost, requirement for sufficient cell density to record positive results, and in certain instances inability to distinguish pathogenic from nonpathogenic species along with viable versus nonviable cells. Despite advances in *Listeria* detection methodology, all procedures developed to date lack sensitivity and reliability, and often positive samples escape detection.

3.6.2 Sublethal injury

In addition to missing low-level contamination due to *Listeria*, highly selective enrichment procedures do not account for recovery of sublethally injured *Listeria* that can exist within heated, frozen or acidified foods or heated, frozen and sanitized areas within food-processing environments. It is well recognized that *Listeria* can be injured as a result of exposure to a variety of processing treatments, including sublethal heating and freezing, drying, irradiation,

or exposure to chemicals (sanitizers, preservatives, acids). Under ideal conditions in food systems, injury is reversible and injured *Listeria* can repair sublethal damage. Refinement of existing *Listeria* recovery methods should consider the nutritional needs associated with specific genetic types, along with the physiological condition of *Listeria* isolates in foods.

Archer stated that the stresses to which bacteria in foods are subjected may result in increased expression of virulence in stressed pathogens and may promote adaptive mutations resulting in more virulent pathogens. As we employ processing procedures that tend to promote stress adaptation of organisms (extended refrigeration, cook/chill procedures, sous-vide), once repaired, we may generate organisms that become not only more adaptive to hostile environments but also more virulent. A host of genes control stress adaptation in *Listeria* through production of heat shock proteins, cold acclimating proteins, and acid tolerance response proteins. Hill and Gahan have shown that exposure of *L. monocytogenes* to pH 5.5 for a short period (30 min) can result in the subsequent survival of these cells to a normally lethal pH of 3.5. Bacteria, including foodborne pathogens, have developed a number of elaborate genetic systems that encode for the production of proteins which allow bacterial survival during lethal environmental challenges.

The acid tolerance response ATR response encodes for the ability to withstand lethal pH conditions following adaptation to sublethal pH in *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* and *Aeromonas hydrophila*. These mechanisms play a role in predicting the fate of pathogens in acidic foods. Roth and Donnelly assessed survival of acid-injured *Listeria* in acidic food products such as apple cider, yogurt, fresh coleslaw and salsa. Temperature was found to greatly dictate survival of *Listeria* in salsa. When stored at 30 °C, populations declined rapidly within three days. At 4 °C, populations persisted for up to 17 days of storage. In further studies, the efficacy of two different enrichment media for recovery of acid-injured *Listeria* from acidic foods was assessed using *Listeria* repair broth (LRB) and UVM. At time points where differences were detected, LRB detected the organism in 22 of 54 samples, compared with UVM, which detected only three of 54 contaminated samples.

Ryser and colleagues evaluated the ability of UVM and LRB to recover different strain-specific ribotypes of *L. monocytogenes* from meat and poultry products. Forty-five paired 25 g retail samples of ground beef, pork sausage, ground turkey and chicken were enriched in UVM and LRB (30 °C/24 h) followed by secondary enrichment in Fraser broth (35 °C/24 h) and plated on modified Oxford agar. A 3-h period of nonselective enrichment at 30 °C was used with LRB (with tested food) to enable repair of injured *Listeria* prior to addition of selective agents. Of 180 meat and poultry products tested, LRB identified 73.8% (133/180) and UVM 69.4% (124/180). Although there was not a statistically significant difference in these results, combining results from UVM and LRB enrichment improved recovery rates to 83.3%. These results demonstrate the enhanced recovery of *Listeria* through the use of LRB for repair/enrichment of samples in conjunction with the USDA-FSIS method.

After 24 h of incubation at 35 °C, *Listeria* colonies were biochemically confirmed and selected isolates were ribotyped using the automated Riboprinter Microbial Characterization System (E. I. DuPont), an automated system that distinguishes genetically unique strains. A total of 36 different *Listeria* strains comprising 16 *L. monocytogenes* (including four known clinical ribotypes), 12 *L. innocua* and 8 *L. welshimeri* ribotypes were identified from selected positive samples (15 samples of each product type, two UVM and two LRB isolates per sample). Twenty-six of 36 (3 *L. monocytogenes*) *Listeria* ribotypes were observed using both UVM and LRB; whereas three of 36 (1 *L. monocytogenes*) and seven of 36 (3 *L. monocytogenes*) *Listeria* ribotypes were observed using only UVM or LRB, respectively. Ground beef, pork sausage, ground turkey and chicken yielded 22 (8 *L. monocytogenes*),

Table 3.5 Ribotypes of *Listeria* spp. Recovered from Ten Samples of Raw Chicken Following Primary Enrichment in UVM or LRB and Secondary Enrichment in Fraser Broth

		Number of isolates	
Ribotype	***Listeria* spp.**	**UVM**	**LRB**
1-909-3	*L. innocua*	0	1
5-418-3	*L. moncytogenes*	2	0
5-415-4	*L. innocua*	4	0
5-413-2	*L. monocytogenes*	2	0
2-864-3	*L. welshimeri*	2	0
1-916-1[a]	*L. monocytogenes*	3	3
5-408-1	*L. monocytogenes*	2	0
1-909-4	*L. innocua*	5	5
1-910-7	*L. innocua*	0	1
5-426-1	*L. innocua*	0	1
1-923-1[a]	*L. monocytogenes*	0	3
5-408-4	*L. monocytogenes*	0	2
1-907-1[a]	*L. monocytogenes*	0	1
1-919-2	*L. monocytogenes*	0	1
1-864-7	*L. monocytogenes*	0	1
1-915-7	*L. monocytogenes*	0	1

21 (12 *L. monocytogenes*), 20 (9 *L. monocytogenes*), and 19 (11 *L. monocytogenes*) different *Listeria* ribotypes, respectively, with some *Listeria* ribotypes confined to a particular product. Many meat samples (pork sausage and ground beef) were found to harbour three or four different *Listeria* ribotypes in a single sample (Table 3.5). More importantly, striking differences in both the number and distribution of *Listeria* ribotypes were observed when 10 UVM and 10 LRB isolates from five samples of each product were ribotyped. When a third set of isolates was obtained using only one of the two primary enrichment media, UVM and LRB failed to detect *L. monocytogenes* (both clinical and nonclinical ribotypes) in two and four samples respectively (Table 3.5).

These findings stress the complex microbial ecology of *Listeria* in foods and the limitations of existing detection procedures to fully represent the total distribution of *Listeria* isolates in foods. Furthermore, two of the *L. monocytogenes* riboprints undetected using UVM were known clinical isolates of serotypes l/2a, both of which were responsible for sporadic and epidemic cases of human listeriosis in England and Scotland. These findings, combined with reports of *L. innocua* being able to outgrow *L. monocytogenes* in UVM media, suggest that genetically distinct strains of *L. monocytogenes* may vary somewhat in nutritional requirements or their ability to compete with other genetically distinct strains of *L. monocytogenes* or of other *Listeria* spp.

Characterization of *L. monocytogenes* isolates beyond the species level is primarily confined to epidemiological investigations where investigators attempt to confirm the vehicle of infection and conduct trace-back studies to a particular food-processing facility and/or environment. Methods for subtyping *Listeria* can be separated into two broad categories: (a) conventional methods that include serotyping (Schonberg et al., 1996), phage typing (Donnelly, 1998), and bacteriocin typing (Bannerman et al., 1996) and (b) molecular methods that encompass multilocus enzyme electrophoresis (MEE), chromosomal DNA restriction endonuclease analysis (REA) ribotyping, DNA macrorestriction analysis by PFGE, random amplification of polymorphic DNA (RAPD) by PCR, and DNA sequence-based subtyping.

In general, serotyping and phage typing are best suited as preliminary subtyping strategies, with phage typing now particularly popular in Europe for routine screening of isolates. Ribotyping and MEE lack sufficient discrimination to be used alone in epidemiological investigations and, with the exception of the RiboPrinter (a fully automated ribotyping system developed by Qualicon, Wilmington, DE), are also fairly labor intensive. Pulsed-field gel electrophoresis has been used in conjunction with phage typing since the late 1980s for routine screening of *Listeria* isolates in France. The CDC utilized the power of PFGE to establish PulseNet, a national network of public health laboratories that fingerprint foodborne bacteria using PFGE. Through use of standardized methods, genetic fingerprints can be compared with fingerprints maintained on a common database at the CDC to determine if bacteria isolated from ill persons originate from a common source. Use of PulseNet has been instrumental in identifying the common sources of outbreaks for more than 15 years.

With the advent of extremely advanced sequencing technologies, the potential of whole genome typing as an alternative to PFGE is considered by many scientists as quite feasible. Several companies have developed the next-generation sequencers that have the power to generate very reliable genomic information in a relatively short time at a relatively low cost. Some studies have demonstrated the feasibility of using single-nucleotide polymorphism (SNP) to be able to discriminate outbreak strains from sporadic cases. The main barrier to the replacement of PFGE as the method of choice for epidemiological investigations is the enormous database that has been accumulated in the last two decades. We face an interesting juxtaposition against the call for a relaxation of zero-tolerance standards by the food industry on the one hand, and availability of highly effective and discriminatory technology on the other hand, which, when coupled with epidemiological data, can effectively be used to trace back to contaminated food products causing listeriosis.

3.7 Conclusion

Despite reductions in disease incidence due to *L. monocytogenes*, this organism remains a leading cause of death due to a foodborne pathogen. Recent multistate outbreaks of illness and death highlight the need for renewed collaboration among industry, university and governmental agencies to control this dangerous but interesting foodborne pathogen. Improvements in testing and typing methods are also needed to ensure adequate sensitivity of detection of regulatory procedures used to identify and ultimately control *Listeria*.

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4 *Bacillus cereus*

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4.1 Introduction

Bacillus cereus belongs to the genus *Bacillus* comprising a taxonomically diverse group of Gram-positive, rod-shaped, endospore forming bacteria. The *Bacillus cereus* group, an informal term of a sub-division of the genus *Bacillus*, includes seven species: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus cytotoxicus*. The members of the *B. cereus* group are genetically highly homologous and share genetic elements that are specific to the group; however, they are differentiated by virulence factors, colony morphology, parasporal crystal inclusions, growth temperature and 16S rRNA sequence.

B. cereus is widely distributed in the environment and can be found in soil, sediments, dust, water and plants. Spores of *B. cereus* are highly resistant to adverse conditions such as heat, dehydration, acidity, radiation, disinfectants and cleaning agents. Due to the extensive distribution and the formation of resistant spores, *B. cereus* has been isolated from a variety of different types of foods. *B. cereus* produces a large number of extracellular substances potentially involved in pathogenesis, and the toxicity ranges from strains used as probiotics for humans to highly toxic strains responsible for food-related fatalities. *B. cereus* causes two different types of foodborne illness: the diarrhoeal type and the emetic type. The diarrhoeal type of foodborne illness is caused by enterotoxins produced during vegetative growth of *B. cereus* in the small intestine, whereas the emetic toxin is preformed in the food prior to ingestion. For both types the food involved has usually been heat-treated, and surviving spores are the source of the food poisoning.

In addition to being a well-known food-poisoning bacterium, *B. cereus* is an opportunistic pathogen and can cause a number of systemic and local infections such as endophthalmitis and septicaemia. Neonates, intravenous drug abusers, patients sustaining traumatic or surgical wounds and patients with indwelling catheters are predisposed for *B. cereus* non-gastrointestinal infections.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

4.2 Nature of illness

The two types of foodborne illness caused by *B. cereus* are due to very different types of toxins. Whereas the diarrheal type is caused by enterotoxins, the emetic disease is caused by a small cyclic peptide. The diarrhoeal syndrome is generally mild and self-limiting; the number of outbreaks of *B. cereus* foodborne illness is highly underestimated due to the relatively short duration of both types of illness (usually less than 24 hours), and that complete recovery is, with a few exceptions, rapid after the symptoms subside. The *B. cereus* diarrhoeal disease was first described after several outbreaks of diarrhoea in hospitals in Norway in 1947–1949. The diarrhoeal syndrome is caused by vegetative cells producing enterotoxins in the small intestine, where the vegetative cells are ingested as either viable cells or spores, or a combination of these two. The symptoms of diarrhoeal disease caused by *B. cereus* are abdominal pain and watery diarrhoea, occasionally accompanied by nausea and emesis, occurring six to 16 hours after ingestion of the contaminated food. *B. cereus* produces three different enterotoxins of protein nature. Two of them, Hbl (Haemolysin BL) and Nhe (Non-haemolyttic enterotoxin), are structurally-related three component toxins, while CytK (Cytotoxin K) is a single protein pore-former.

Hbl, consisting of the binding component (B) and two lytic components (L1 and L2), was the first to be identified and characterized. This toxin has dermonecrotic and vascular permeability activities, causes fluid accumulation in ligated rabbit ileal loops and contributes to *B. cereus* endophthalmitis. Experiments with purified toxin components have shown that all three components of Hbl are necessary for maximal toxic activity, in a ration close to 1:1:1. About 40 % of strains harbour the *hbl* genes.

The second three-component enterotoxin, Nhe, was first described after a large food-associated diarrhoeal outbreak in Norway in 1995. Nhe is a pore-forming toxin with structural and functional properties similar to the ClyA/SheA family of haemolysins in *E. coli*. All three components are necessary for optimal activity and Nhe is most active when the molar ratio between the three components NheA, NheB and NheC is about 10:10:1. The structure of Hbl component B has been determined by X-ray crystallography, and 3D homology models of NheB and NheC have been created based on this structure. The structures of the three toxin components are very similar, possessing an α-helix bundle and a unique subdomain containing a hydrophobic ß-hairpin. More than 99% of *B. cereus* strains are able to produce Nhe, although not all do so at 32–37 °C.

The third characterized *B. cereus* enterotoxin, cytotoxin K (CytK), was first described after a severe outbreak of foodborne illness in a nursing home in France in 1998. In this outbreak several people developed bloody diarrhoea and three persons died. *cytK* is present in about 40% of *B. cereus* strains. However, the strain causing the French outbreak was particularly virulent. Due to the genome sequence, it was proposed that the strain represents a novel bacterial species, *Bacillus cytotoxicus*. This species has just been recognized. CytK belongs to a family of β-barrel toxins and is similar to the β-toxin of *Clostridium perfringens*. It has been shown that CytK makes a pore of 7 Å in diameter, indicating that the toxin inserts into the membrane as a heptamer. CytK produced by *B. cytotoxicus* has been named CytK-1 and is about five times more toxic on epithelial cells than the other CytK (produced by *B. cereus* strains).

The emetic disease caused by *B. cereus* occures 0.5–6 h after ingestion of the contaminated food. The short period of time between ingestion of food and onset of illness indicates that this is an intoxication caused by a toxin preformed in the food. The *B. cereus* emetic

toxin, cereulide, is a cyclic dodecadepsipeptide and has a molecular mass of 1.2 kDa. Cereulide acts as a K+ ionophore, like valinomycin, and is produced by cereulide synthetase (Ces) via a nonribosomal peptide synthetase mechanism. Cereulide is resistant to heat, pH and proteolysis and it is not antigenic. The *B. cereus* emetic toxin triggers nausea and vomiting through vagus nerve afferents by binding to the 5-HT_3 receptor. A more severe activity by cereulide is the inhibition of fatty acid oxidation in the mitocondria and inhibition of human natural killer cells in the immune system. Mice injected intraperitoneally with cereulide develop a massive degradation of liver cells.

Most emetic patients recover within 6–24 hours; however, the toxin can be fatal. Since 1997, four cases of children and young adults dying of cereulide intoxication after ingesting reheated pasta or rice dishes have been reported. Postmortem findings in these cases have revealed liver damage and brain oedema, suggesting that cereulide can cause acute encephalopathy and liver failure.

4.3 Characteristics of the agent

The aerobic endospore-forming bacteria of the *B. cereus* group are large (cell width >0.9 µm). They produce central to terminal ellipsoid or cylindrical spores that do not distend the sporangia and they sporulate easily in most media after 1–3 days. *B. cereus* is a common soil saprophyte and is frequently found in raw material and ingredients used in the food industry such as vegetables, starch, spices (30% of samples with 10^2–10^5 cfu/g). Spreading from soil and grass to the udder of the cows and into raw milk, it is also a problem in milk and milk products. The problem is enhanced by the fact that *B. cereus* spores survive milk pasteurization. The *B. cereus* spore is more hydrophobic than any other *Bacillus* spp. spores, which enables it to adhere to several types of surfaces; hence, it is difficult to remove during cleaning, and it is a difficult target for disinfectants.

The members of the *B. cereus* group have highly similar 16S and 23S rRNA sequences, indicating that they have diverged from a common evolutionary line relatively recently. *B. cereus* group bacteria are differentiated by factors such as virulence, colony morphology, parasporal crystal inclusions and growth temperature (Table 4.1).

B. mycoides and *B. pseudomycoides* are phenotypically differentiated from *B. cereus* by rhizoidal colony shape on solid medium. *B. pseudomycoides* can only be differentiated from *B. mycoides* by fatty acid composition analyses or 16S RNA sequencing.

B. weihenstephanensis can be differentiated from *B. cereus* based on growth at temperatures below 7 °C and not at 43 °C or by identification of *cspA* (cold shock protein A gene) by PCR. Most emetic strains do not grow below 10 °C, and neither of them produces the Hbl toxin. However, a few strains of *B. weihenstephanensis* have been reported to produce

Table 4.1 Criteria to Differentiate Members of the *Bacillus cereus* Group

Species	Colony morphology	Hemolysis	Motility	Susceptible to penicillin	Parasporal crystal inclusion
B. cereus	White	+	+	–	–
B. anthracis	White	–	–	+	–
B. thuringiensis	White/grey	+	+	–	+
B. mycoides	Rhizoid	(+)	–	–	–

see Logan and De Vos, 1986.

cereulide, although not at temperatures below 8 °C. For most emetic strains cerulide is best produced at temperatures between 16 and 25 °C.

B. anthracis is closely related to the other species within the *B. cereus* group based on rRNA sequences, but it is unique regarding its highly virulent pathogenicity. Nonetheless, a *B. cereus* strain carrying a plasmid homologous to the *B. anthracis* toxin-encoding plasmid pXO1 has caused anthrax-like illness.

Complete genome sequencing of numerous *B. cereus* and *B. thuringiensis* strains have revealed that there is no taxonomical basis for separate species status; however, the name *B. thuringiensis* is retained for those strains that synthesize a crystalline inclusion (Cry protein) that is highly toxic to specific insects. The *cry* genes are usually located on plasmids and loss of the relevant plasmid makes the bacterium indistinguishable from *B. cereus*. *B. thuringiensis* produces the same enterotoxins as *B. cereus*, and can cause foodborne illness. This may develop into a serious problem, as spraying of this organism to protect crops against insect infestations has become a common practice in several countries. To ensure safe spraying of *B. thuringiensis*, the organism should not produce enterotoxins. Although all members of the *B. cereus* group harbour the genes for at least one of the enterotoxins (Nhe), some strains do not produce detectable amounts of the toxin(s).

The *B. cereus* diarrhoeal syndrome is generally mild and self-limiting, although three deaths were reported after a severe outbreak in France in 1998. The strain isolated from this outbreak is now regarded as the seventh member of the *B. cereus* group, *B. cytotoxicus*, and differs from other members of the *B. cereus* group by growing at higher temperatures (thermotolerant), not below 20 °C, and by producing a more toxic type CytK enterotoxin.

4.4 Epidemiology

B. cereus is isolated from a remarkable range of different foods and food ingredients, including rice, pasta, dairy products, spices, dried foods, meat products and vegetables. *B. cereus* has a double life both as a soil saprophyte and as a pathogen The major reservoir for *B. cereus* is soil, which has been reported to contain about 10^5–10^6 cells/spores per gram. From soil it is easily spread into foods through many routes. In dairy products, *B. cereus* is usually spread through contamination of the udders of cows during grazing or it may enter the dairy farm through bedding material or feed. However, the most common route into food products is probably through spices and different types of starch, which have been reported to contain 10^2–10^5 *B. cereus*/gram. Several other routes into food production plants or restaurants are also possible, but it is how the foods are treated during production and handling that is most important in order to prevent foodborne outbreaks due to *B. cereus*. It is almost impossible to completely prevent the presence of *B. cereus* in many food products but, with proper knowledge, growth to numbers that can result in food poisoning can be prevented. At least 10^3 (usually $>10^4$) *B. cereus*/g food is necessary to cause foodborne illness, and growth to at least 10^5/g is necessary for emetic strains to cause intoxication by production of cereulide.

4.5 Detection of organism

B. cereus in foods can be isolated and enumerated by selective agars that utilize the fact that *B. cereus* is mannitol-negative, resistant to certain concentrations of Polymyxin and produces insoluble degradation products from egg yolk lecithin. In addition, the use of blood agar plates is supportive with the huge haemolysis zones observed after growth of most strains.

Three commercial immunoassays are available for detection of *B. cereus* enterotoxins. The BCET-RPLA Toxin Detection Kit from Oxoid measures the presence of the HblC component whereas the Tecra Bacillus Diarrhoeal Enterotoxin kit (3 M Tecra) detects the NheA component. The new Duopath Cereus Enterotoxins from Merck KGaA/EMD Chemicals is an immunological screening and confirmation test for simultaneous detection of both Hbl and Nhe. No commercial kit is available for detection of CytK.

Detection of cereulide is difficult and several methods, such as monkey feeding tests, cell culture assays and boar sperm motility tests, have been employed. Although none of these specifically detects cereulide, the boar sperm assay is easily performed and is well suited for screening purposes. Detection of cereulide can be done conclusively by HPLC-MS and LC-MS, although these are costly, specialized analyses.

PCR targeting the nonribosomal peptide synthetase genes (*ces*) responsible for the cereulide production or *cytK* can be used for detection of respective genes; this will, however, not give any information about expression of the corresponding toxins.

4.6 Physical methods for destruction

Vegetative *B. cereus* cells are killed by a few seconds at 72 °C (pasteurization). Since *B. cereus* in not very fond of competition, fermented products will usually not contain *B. cereus* due to the fermentative flora. Spores of *B. cereus* are usually quite heat stable, and can survive cooking for several minutes, although there are huge differences in stability among strains and among spores produced under different conditions, with variation in D values at 100 °C ranging from 1–8 minutes. The most stable spores are those that are newly made at high growth temperatures. The temperatures required for killing all spores in a product will reduce organoleptic and nutritional quality radically. Therefore a double heat treatment can be used to control spores in foods. Spores are activated by a mild heating at about 70 °C followed by cooling and germination, then a second heat treatment at about 90 °C is applied which will kill the germinated cells. The double heat treatment will reduce the spore content considerably without giving the product an over-cooked appearance. In recent years, high pressure processing has become increasingly important for preservation of food and may be used as an alternative to heat treatment.

4.7 Prevention and control

Prevention and control of *B. cereus* is relatively easy, apart from in the dairy industry, where it is causing major problems. There are also a growing number of precooked long-life products on the market that are difficult to produce completely free from *B. cereus* spores. Rapid cooling and proper reheating (>60–70 °C) of cooked food is essential if not consumed immediately. *B. cereus* can also be controlled through pH (<4.8). Emetic strains are relatively common in starchy food products such as rice and pasta, and these products must either be eaten hot or stored below 8 °C to prevent cereulide production (stops at about 10–12 °C). Even pizza stored for extended periods at room temperature may support growth of surviving spores and cause both types of *B. cereus* foodborne disease. In products stored below 6–8 °C *B. weihenstephanensis* will dominate; however,

this species within the *B. cereus* group is probably unable to cause human infection since it grows slowly at 37 °C and hardly produces toxins at this temperature.

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5 *Clostridium perfringens*

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5.1 Introduction

The genus *Clostridum* consists of a diverse group of bacteria that do not grow in the presence of oxygen and have the ability to form heat-resistant endospores. Many of these anaerobes are pathogenic for both human and animals. Historically, it is best known as the predominant cause of gas gangrene in wound infections. The advent of antibiotics has greatly reduced the incidence of such infections.

The bacterial pathogen *Clostridium perfringens* is the most prolific toxin-producing species within the clostridial group. The toxins are responsible for a wide variety of human and veterinary diseases, many of which can be lethal. *Clostridium perfringens* causes two quite different human diseases that can be transmitted by food: one a common form of foodborne illness and the other necrotic enteritis (pig-bel), which is found only rarely. The foodborne illness caused by *C. perfringens* is among the most common caused by consumption of contaminated food. Although the association of *C. perfringens* with foodborne illness was first proposed about 100 years ago, it was not until the 1960s and 1970s that conclusive evidence accumulated showing that an enterotoxin is associated with sporulation of the organism in the intestine of ill individuals.

The bacterium possesses several attributes that contribute significantly to its ability to cause foodborne illness: (1) a ubiquitous distribution throughout the natural environment, giving it ample opportunity to contaminate foods; (2) the ability to form heat-resistant spores, allowing it to survive incomplete cooking of foods or improper sterilization; (3) the ability to grow quickly in foods, allowing it to reach the high levels necessary for food poisoning; and (4) the ability to produce an intestinally active enterotoxin, responsible of the characteristic gastrointestinal symptoms of *C. perfringens* food poisoning.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

5.2 Nature of illness in animals and humans

Clostridium perfringens enterotoxin (CPE) is traditionally recognized as a virulence factor responsible for the diarrheal and cramping symptoms associated with *C. perfringens* type A food poisoning. This toxin is a single 34-kDa polypeptide with an isoelectric point of 4.3, a 309 amino acid sequence, and a unique mechanism of action. It is produced intracellularly during the sporulation of the bacterium, and it is released together with the mature spore. Genetic studies indicate that the *cpe* gene can be either chromosomal or plasmid borne (see below). Most food-poisoning isolates carry the *cpe* gene on their chromosome while the gene is episomal in CPE-associated, non-foodborne human gastrointestinal disease isolates. Molecular epidemiological surveys suggest that only a low percentage (4–6%) of all *C. perfringens* isolates carry the gene. The role of CPE in the physiology of the bacterial cell is unknown.

Foodborne illness is produced 8–24 h after the ingestion of food contaminated with large numbers of vegetative bacteria ($>10^5$ ent$^+$ CFU/g). Many of the ingested cells may die when exposed to stomach acidity, but if the food vehicle is sufficiently contaminated, some vegetative cells survive passage through the stomach and enter the small intestine, where they multiply, sporulate, and produce CPE. The possible role of ingested sporulating cells or preformed enterotoxin is unlikely, since studies with volunteers indicate that the amount of ingested CPE necessary to produce symptoms would require cell numbers that would impart adverse sensory qualities to such foods.

Once the CPE, a pore-forming toxin, is released into the small intestine, the following sequence of events occur: (1) CPE binding to a 50-kDa protein receptor, forming a small complex of 90 kDa; (2) the development of a postbinding physical change to this small complex; that could represent either the insertion of CPE into the membrane or a conformational change to the small complex; (3) an interaction between this physically changed small complex and a 70-kDa membrane protein, forming a large 160-kDa complex; and (4) the initiation of a series of biochemical events that alter the normal permeability of brush border membranes in the epithelial cells of the small intestine. This CPE-induced permeability change becomes cytotoxic and causes localized tissue damage (Fig. 5.1), which leads to a breakdown in normal fluid and electrolyte transport properties and hence to diarrhea. Treatment of CPE with trypsin increases its activity at least twofold, suggesting a possible role for the intestinal enzyme in cases of human illness.

The main symptoms of *C. perfringens* food poisoning include diarrhea and severe abdominal pain. Nausea is less common, and fever and vomiting are unusual. Death is uncommon but has occurred in debilitated or institutionalized individuals, especially the elderly. The cases are self-limited and antibiotic therapy is not recommended.

Animal carcasses and cuts of meat can become contaminated with *C. perfringens* from soil, animal feces, or handling during slaughtering and processing. Many organisms that compete with *C. perfringens* are killed when meat and poultry are cooked, but *C. perfringens* spores may be difficult to eliminate. Heat activation of spores during the cooking process would facilitate germination when the temperature becomes favorable for growth. Also, during cooking E_h values drop to levels that favor subsequent multiplication of *C. perfringens*. Temperature abuse can occur during improper cooling, for example at room temperature or by refrigeration of large portions that cool slowly or by improper holding temperatures. In such cases bacteria commence multiplication. If such foods are served without being reheated to a temperature sufficient to kill vegetative forms of *C. perfringens*, illness may

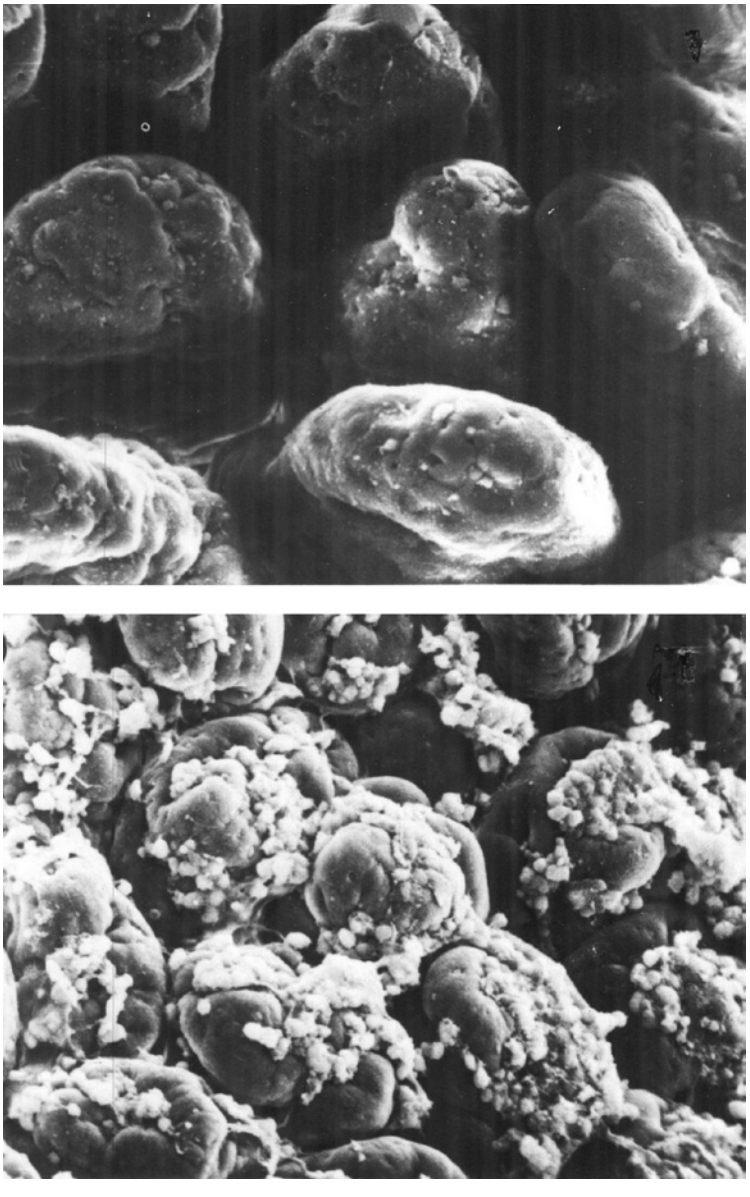

Figure 5.1 Effect of *C. perfringens* enterotoxin on rabbit villi: (top) control; (bottom) treated with enterotoxin for 90 min showing collapsed villus tips and protruding structures at the surface of epithelial cells. From McDonel, J., Change, L., Pounds, J. and Duncan, C. The effects of *Clostridium perfringens* enterotoxin on rat and rabbit ileum. *Lab Investig* **39**, 210–218. Lippincot Williams & Wilkins, Baltimore MD, 1978.

result. Therefore, when foodborne outbreaks occur, one or more of the following events usually have occurred: (a) improper cooling; (b) improper hot holding; (c) preparation of food a day or more in advance; or (d) inadequate reheating.

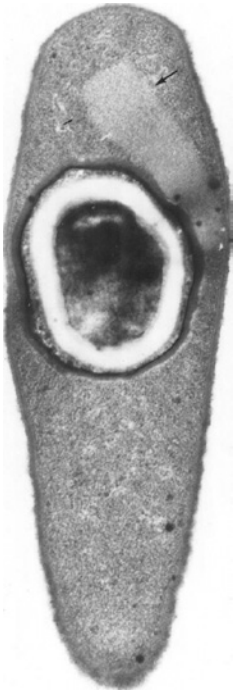

Figure 5.2 Sporulating cell of *C. perfringens*. The enterotoxin is released along with the spore upon lysis of the mother cell.

5.3 Characteristics of agent

Clostridium perfringens was first described as *Bacillus aerogenes* in 1892 and later called *Clostridium welchii*. It is an anaerobic, gram positive, rod-shaped, encapsulated, spore-forming bacterium (Fig. 5.2). More than 100 restriction sites and almost 100 genetic loci have been located on the genome. Thus far, the complete genome sequence of three Type A strains have been published.

This organism is found in soil, water, air, and foods. The virulence of the microorganism results from its ability to produce numerous protein toxins (Table 5.1). It has been classified into five types (A–E) based on the production of extracellular toxins and hydrolytic enzymes, including lecithinase, hemolysins, hyaluronidase, collagenase, deoxyribonuclease (DNAse), and amylase. These allow it to scavenge required nutrients from its environment.

Foodborne illness is apparently caused only by type A strains. *Clostridium perfringens* types C and D also produce enterotoxin. However, there are no data implicating these strains in foodborne illness.

Types B, C, and D primarily occur in the intestines of animals and only occasionally in humans. The strains of these types have also been isolated from soils in areas where enteritis by the organisms was affecting a significant number of animals and humans (Table 5.2). The pathogenicity of type E strains is not clear and has seldom been isolated.

Table 5.1 Toxins Produced by *C. perfringens*

***C. perfringens* type**	**Toxins produced**										
	α	β	ε	ι	δ	θ	κ	λ	μ	ν	**CPE**
A	+++	–	–	–	–	+	+	–	+	+	+
B	+	++	+	–	+	+	+	+	+	+	+
C	+	++	–	–	+	+	+	–	+	+	+
D	+	–	++	–	–	+	+	+	+	+	+
E	+	–	–	+	–	+	+	+	–	+	+

Table 5.2 Disease Caused by *C. perfringens*

***C. perfringens* type**	**Disease produced**
A	Gas gangrene (clostridial myonecrosis); food poisoning; necrotic enteritis of infants; necrotic enteritis of poultry; enterotoxemia in cattle and lamb; possibly colitis
B	Lamb dysentery; enterotoxemia of sheep, foals and goats; hemorrhagic enteritis in neonatal calves and foals
C	Enterotoxemia of sheep (struck); necrotic enteritis in animals; human enteritis necroticans (pig-bel); acute enterotoxemia in adult sheep
D	Enterotoxemia of sheep (pulpy kidney disease), lamb, and calves
E	Enterotoxemia of rabbits; canine and porcine enteritis

Another toxin, NetB, has been more recently associated with necrotic enteritis in chickens, which has emerged as a significant economic issue in the poultry industry.

Alpha toxin is produced by all five types and is a phospholipase C that can hydrolyze lecithin to phosphorylcholine and diglyceride. This toxin is believed to be a major factor responsible for the tissue pathology of the organism. The major lethal effects associated with this toxin are gas gangrene (clostridial myonecrosis) in humans and necrotic enteritis and enterotoxemia in animals.

Two toxins are known to be active in the human gastrointestinal tract, the β toxin and the CPE. The β toxin is produced by types B and C strains and is responsible for the lesions associated with necrotic enteritis also. The CPE toxin is more important in human illness.

Clostridium perfringens is different from many other clostridia in that it is nonmotile and in vitro forms spores only in specialized culture media. The organism is fermentative and grows rapidly in media containing carbohydrates. Under these conditions, it produces copious amounts of H_2 and CO_2, which help to maintain an anaerobic environment. Owing to its rapid growth and relative aerotolerance, compared to other anaerobes, *C. perfringens* is relatively easy to work with in the laboratory.

C. perfringens is not particularly tolerant of low A_w or extremes of pH or curing salts, but is more tolerant of elevated E_h than most anaerobes, explaining the role of cooked meat and poultry as vehicles in outbreaks.

The location of the gene responsible for CPE (*cpe*) may be located on a plasmid or chromosome. Most food poisoning isolates to date have been shown to carry *cpe* on the chromosome while most non-foodborne gastrointestinal disease isolates show a plasmid location. Vegetative cells of chromosomally-located *cpe* isolates survive heating better than cells of plasmid *cpe*

isolates, also show higher $D_{4°C}$ and $D_{-20°C}$ values, and have a higher maximum and minimum growth temperature, traditionally listed as between 15 and 50 °C. Spores of chromosomally-located *cpe* are also more resistant, showing an approximately 60-fold higher $D_{100°C}$ values compared to spores from isolates with *cpe* on a plasmid or lacking *cpe*. These characteristics may explain the strong association of chromosomal *cpe* isolates with foodborne outbreaks.

5.4 Epidemiology

Meat and poultry are the most common food vehicles for *C. perfringens* type A food poisoning with beef being the vehicle in nearly 30% of all outbreaks. Turkey and chicken together account for another 15%. Fish are not commonly involved in food-poisoning outbreaks because of this organism.

In the US foodborne outbreaks due to *C. perfringens* are not tracked by the Center for Disease Control Food Net active surveillance program and its omission undoubtedly results in an underestimation of annual cases and outbreaks. Nevertheless, foodborne illness caused by this organism is estimated to be second only to *Salmonella* in the annual number of cases caused by bacterial agents. The number of individuals affected per outbreak is typically between 40 and 70. However, like other agents of foodborne illness, it is widely under-reported in part because of the anaerobic conditions required for its cultivation and the perceived mild nature of the illness.

As mentioned above, the most common vehicles are meat and poultry-based products, spreading from intestinal contents during slaughter. The presence of this organism in the spore state on herbs and spices (as well as in meat and poultry) can create a hazard since they can survive the cooling process and resume growth in temperature-abused or poorly cooled foods, resulting in the large number of cells required for an outbreak. The organism's fastidious growth requirements are easily met in meat and poultry items.

Temperature abuse of cooked foods is invariably involved in outbreaks, reflecting the organism's relatively high optimum growth temperature [43–46 °C (110–115 °F)]. This and its spore-forming ability are the two outstanding characteristics contributing to its etiological role in foodborne illness.

Food service establishments such as restaurants, hospitals, prisons, schools, and caterers are the most likely sites for acquiring the illness.

The potential for rapid growth of *C. perfringens* during cooling is reflected in the USDA-FSIS compliance guidelines for chilling of thermally processed meat and poultry stating that chilling of uncured meat and poultry products from 54.4 °C to 26.7 °C (130° to 80 °F) should take no longer than 1.5 h and cooling from 26.7 °C to 4.4 °C (40 °F) should take no longer than 5 h. Predictive models have been developed for growth during cooling of uncured meat, the latter accessible via www.combase.cc.

5.5 Detection of organism

Requirements for implicating *C. perfringens* in an outbreak include one of the following: (a) $>10^6$ ent$^+$ spores/g feces from ill individuals; (b) $>10^5$ ent$^+$ cells/g of incriminated food; or (c) direct detection of CPE in outbreak stools. Previous recommendations for identifying common serotypes in implicated foods and stools have not been widely adopted because many isolates cannot be serotyped. All responsible strains are assumed to be type A.

In North America and the United Kingdom the recommended selective plating medium for enumerating *C. perfringens* from food and feces is tryptose–sulfite–cycloserine (TSC) agar, which is commercially available. The selective antibiotic cycloserine must be added separately. Normal stool samples typically contain 10^3–10^4 *C. perfringens* spores/g. Stool samples are heated before dilution and plating. Colonies appear black on this medium. Typically 10 are confirmed using lactose–gelatin and motility nitrate medium. Details of the procedure are described in the U.S. FDA Bacteriological Analytical Manual and by the International Organization for Standardization (ISO).

Certain otherwise healthy elderly individuals may carry high spore levels of *C. perfringens*, limiting the elevated spore count as an implicating criterion. This has promoted the direct detection of CPE in outbreak stools as a useful technique for establishing this organism as the etiological agent. Several enzyme-linked immunosorbent assay (ELISA) procedures that have been developed for this purpose have sensitivities of several nanograms per gram of feces. Stools from healthy individuals contain undetectable levels of CPE compared with outbreak stools, which contain >1 μg/g. A reversed passive latex agglutination (RPLA) with similar sensitivity has been developed. Both RPLA and ELISA assay kits are commercially available.

Detection of CPE in laboratory cultures requires the sporulation of such isolates. Many isolates sporulate in vitro only reluctantly, and numerous media have been proposed for this purpose. Because of such difficulties, molecular methods to detect the CPE have been developed. These have been useful since the *cpe* gene (chromosomal and episomal) is highly conserved. The PCR has been widely adopted in determining the presence of *cpe*. Multiplex PCR methods have also been developed that permit the detection of CPE as well as the four extracellular toxins used in toxigenic typing of the organism and have proven useful in research as well as in epidemiological and veterinary studies. In the case of outbreaks the presence of the α–toxin is typically determined along with the presence of *cpe*. As all outbreaks are due to Type A strains there is no need for toxigenic typing.

Subtyping methods have included pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), ribotyping, and other methods. However, a disadvantage of these techniques is that most require pure cultures and require specialized training typically associated with research and reference laboratories.

5.6 Physical methods for destruction

As with most foodborne pathogens, pasteurization temperatures [72 °C (161 °F)] for 5–10 min and routine cooking procedures readily inactivate vegetative cells of this organism. Of far greater concern is the heat resistance of their spores, which varies depending on the strain. This led to the designation of strains as being 'heat resistant' or 'heat sensitive.' For example, in one study the decimal reduction values at 95 °C (D_{95}) for the former were between 17.6 and 63 min compared with between 1.3 and 2.8 min for the latter. Not surprisingly, the so-called heat-resistant strains were more often associated with cases of foodborne illness. However, determination of this phenotypic characteristic is no longer sought for purposes of laboratory confirmation.

Extrinsic factors that may affect spore heat resistance include sporulation temperatures and the menstrum in which spores are heated. The *D* values for a number of strains have been summarized elsewhere. These two groups also vary in their radiation resistance. Not surprisingly, prior irradiation has a sensitizing effect on spores that are subsequently heated.

Unlike vegetative cells of other species, *C. perfringens* is unusually sensitive to refrigerated and frozen storage. Because of this, food samples to be tested for *C. perfringens* vegetative cells should be analyzed immediately or otherwise refrigerated and tested as soon as possible but not frozen. For storage of more than 48 h, samples should be treated with a glycerol salt solution to give a 10% final concentration of glycerol and stored frozen at –60 °C until analysis. By contrast, spores, for example in fecal samples, are unaffected by frozen storage at –20 °C. Unlike *Staphylococcus aureus*, enterotoxin CPE is not detected preformed in foods implicated in outbreaks. Nevertheless, studies have demonstrated that it is relatively heat sensitive with substantial inactivation occurring within a couple of minutes at 60 °C (140 °F).

5.7 Prevention and control

Clostridium perfringens is commonly found in retail meat and poultry at levels of perhaps 10–100 g^{-1}. As mentioned above, most are enterotoxin negative. It is not reasonable to expect such products to be free of this organism. Rather, the goal is to prevent multiplication to levels sufficient to cause illness. If present as spores, the organism can survive cooking procedures and is able to multiply rapidly between 37 and 45 °C (98 and 112 °F). In virtually all outbreaks, the most significant contributing factor is the failure to properly cool previously-cooked foods, especially when prepared in large portions. Cooking procedures also drive off oxygen, promoting anaerobic conditions. Cooked, chilled foods should be reheated to a minimal internal temperature of 75 °C before serving in order to inactivate vegetative cells. The standard dictum of holding cooked foods outside the range of 5 °C (40 °F) to 60 °C (140 °F) will effectively prevent the growth of this organism in hazardous foods.

All humans carry the organism in the large intestine, predominantly enterotoxin-negative strains. Regardless, it is not possible to prevent carriers from handling food. Hence preventative measures depend largely of food service workers' knowledge of proper food preparation and storage techniques, in particular temperature control.

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6 *Clostridium botulinum*

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6.1 Introduction

Studies of botulism started after an outbreak in Germany in 1793 associated with 'blood sausage'. *Clostridium botulinum* was isolated first by van Ermengem in 1897 from raw salted ham implicated in an outbreak in Belgium that affected 23 people of whom three died. Since then, botulism has been shown to occur in many animals and birds. The disease results from production by the bacteria of a powerful neurotoxin. Different isolates of *C. botulinum* produce toxins that differ antigenically, and seven toxin types (A–G) have been differentiated. Botulism in humans is almost always caused by strains producing toxin types A, B or E and occasionally by toxin type F. On the basis of their properties, strains of *C. botulinum* have been placed in different groups (Table 6.1). The majority of strains produce toxin of a single antigenic type, but some strains form two types of toxin, usually a major amount of one toxin and a minor amount of another. Such strains have been designated Af (indicating the major and minor toxin, respectively), Bf, Ab, and Ba. Some strains possess the genes for the production of two types of neurotoxin but only form one active toxin; for example, type A(B) strains possess genes for type A and type B neurotoxin but only form active type A toxin. Rare isolates of *C. butyricum* and *C. baratii* form botulinum neurotoxin. The neurotoxins act primarily at peripheral, cholinergic synapses, where they block the release of the neurotransmitter, acetylcholine. Botulism in humans is caused predominantly by *C. botulinum* Groups I and II (Table 6.1), and this chapter will concentrate on these bacteria. Botulinum toxin has many medical applications in the treatment of neurological conditions and is also used very commonly for cosmetic purposes.

6.2 Botulism

Several types of botulism have been described (Table 6.2).

In *foodborne botulism*, symptoms may occur within 12–36 h or as long as eight days after consumption of food containing botulinum neurotoxin. The initial symptoms may be nausea

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

Table 6.1 *Clostridium botulinum*. Groups and Neurotoxins Formed

Group	Neurotoxin formed	Main species affected
I Proteolytic	A	Humans, horses
	B	Humans, cattle, horses
	F	Humans
II Non-proteolytic	B	Humans
	E	Humans, fish, birds
	F	Humans
III	C	Birds, farmed chicken and pheasants, horses
	D	Cattle, sheep
IV	G	Not known

Table 6.2 Types of Botulism

Type	First recognized	Cause
Foodborne botulism	1897	Consumption of food in which bacteria have multiplied and formed toxin.
Infant botulism	1976	Affects infants under 12 months old. Follows ingestion of spores that become established in the intestine giving vegetative bacteria that form toxin.
Wound botulism	1943	Trauma and contamination of wound with spores that germinate and form toxin. Injection of contaminated drug, e.g. heroin.
Adult infectious botulism	1986	Can affect adults after surgery to gastrointestinal tract or with a condition such as inflammatory bowel disease. Follows ingestion of spores that become established in the intestine giving vegetative bacteria that form toxin.
Iatrogenic botulism	1997	May follow therapeutic or cosmetic use of botulinum toxin.
Inhalation botulism	1963	Follows breathing of liquid aerosol of botulinum toxin.

and vomiting; it is not clear whether these symptoms are caused by the toxin, by other products of *C. botulinum* or other products of spoilage. Initially, the toxin causes symptoms such as double vision, inability to focus, drooping eyelids (ptosis), dry mouth, difficulty in speaking clearly (dysphonia) and inability to swallow (dysphagia). Increasing failure of muscles occurs and death can result from failure of the muscles needed for breathing or of the heart muscles. Sometimes, botulism has been misdiagnosed as another disease such as Guillain-Barré syndrome or myasthenia gravis.

Infant botulism, which was first recognized in the USA, occurs in infants less than one year old, most often in infants less than 35 weeks old. As few as 10–100 spores can cause infection. Following ingestion, the spores germinate and the organism can become established in the intestine of young infants, probably because the normal intestinal flora has not yet been established sufficiently to prevent this colonization. The infant suffers constipation, generalized weakness and progressive paralysis, and other neurological symptoms. As many as 6×10^8 colony-forming units (CFU) of *C. botulinum* per gram can be present in the faeces of a child with infant botulism. In several cases *C. botulinum* spores have been detected in specimens from infants whose death had been attributed to sudden infant death syndrome

(SIDS). In the USA, in a few patients with infant botulism the histories and symptoms are indistinguishable from those of typical cases of SIDS, and the age distribution of infant botulism is identical to that of SIDS.

Wound botulism cases have followed traumatic injuries and infected surgical wounds. Infection of the wound is followed by growth of *C. botulinum* and neurotoxin formation. Wound botulism is a particular risk for heroin users who inject subcutaneously or intramuscularly, and contaminated batches of heroin have probably caused outbreaks in several countries.

Cases of *adult infectious botulism* are associated with intestinal abnormality or surgery and/or with antibiotic treatment. In most adults, the intestinal flora prevents the establishment of any *C. botulinum* spores that have been ingested. Few cases of adult infectious botulism have been reported, either because it occurs rarely or because it is under-recognized.

Cases of *iatrogenic botulism* have been reported after the therapeutic or cosmetic use of botulinum toxin. Some cases have followed the cosmetic use of highly concentrated and unlicensed toxin.

Inhalation botulism has occurred in laboratory workers following inhalation of toxin and is reported in patients following inhalation of spores in cocaine. Release of aerosolized toxin as a weapon has been attempted.

The most frequent type of botulism reported in the USA is infant botulism (Fig. 6.1). Treatment of botulism very often requires respiratory support. In the USA, intravenous injection of heptavalent (anti A–G) antitoxin is recommended as soon as possible after diagnosis. The antitoxin will neutralize circulating toxin but not toxin bound to, or within, nerve cells. It is prepared from horse serum and carries a risk of hypersensitivity reactions. The USA Centers for Disease Control and Prevention (CDC) has released this antitoxin for treatment of botulism in outbreaks in several countries. Human-derived botulism immune globulin (BabyBIG) is approved by the US FDA, and since 2005 has been made available for treatment of infant botulism types A and B inside and outside the USA.

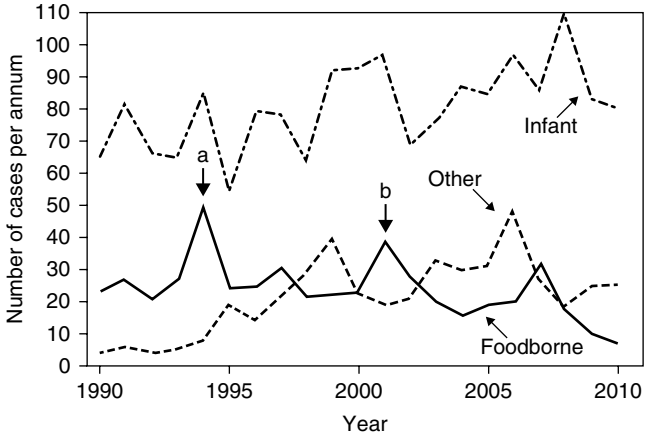

Figure 6.1 Numbers of reported cases of foodborne, infant, and other (wound and unspecified) botulism in the USA, 1990–2010. Peak **a** includes outbreak caused by baked potatoes added to yoghurt, peak **b** includes outbreak caused by chilli sauce. Compiled from reports by the Centers for Disease Control and Prevention.

Table 6.3 Properties of Bacteria that Form Botulinum Neurotoxin[a]

	***C.botulinum* Group I, proteolytic**	***C.botulinum* Group II, non-proteolytic**	***C.botulinum* Group III**	***C.botulinum* Group IV**	***C.baratii***	***C.butyricum***
Toxins formed	A,B,F	B,E,F	C,D	G	F	E
Proteolysis[b]	+	–	–	+	–	–
Liquefaction of gelatin	+	+	+	+	–	–
Lipase	+	+	+	–	–	–
Optimum growth temperature[a]	37°–42°C	30°–35°C	40°C	37°C	30°–45°C	30°–37°C
Minimum temperature for growth	10°–12°C	3°C	15°C		10°–15°C	12°C
Minimum pH for growth	4.6	5.0	5.1			
Minimum NaCl to prevent growth	10%	5%		6.5%		
Minimum a_w for growth, humectant: NaCl/glycerol	0.94/0.93	0.97/0.94				
Spore heat resistance[c]	$D_{121°C}$ = 0.21 min	$D_{82.2°C}$ = 2.4/231 min[d]	$D_{104°C}$ = 0.9 min	$D_{104°C}$ = 1.1 min		$D_{100°C}$ = <0.1 min
Similar nontoxigenic organisms	*C.sporogenes*	No species name given	*C.novyi*	*C.subterminale*	typical *C.baratii* strains	typical *C.butyricum* strains

[a] modified from Lund and Peck (2000) and Peck (in press)

[b] proteolysis denotes the ability to degrade native proteins such as coagulated egg white, cooked meat particles and casein: Groups I–IV of *C.botulinum* can degrade the derived protein, gelatin

[c] spore heat-resistance determined in phosphate buffer pH7.0

[d] D value without/with lysozyme during recovery

6.3 Properties of *Clostridium botulinum* and botulinum neurotoxins

6.3.1 *Clostridium botulinum*

On the basis of differences in their physiology and genetic relatedness, the four groups of *C. botulinum* are sufficiently different to be classified as different species (Table 6.3). Nevertheless, the groups have not been renamed because of the practical importance of neurotoxin formation. Some proteolytic strains and non-proteolytic strains produce similar type B or type F toxin. Very rarely, strains of *C. butyricum* have been isolated that form type E toxin and strains of *C. baratii* that form type F toxin; apart from their ability to form botulinum toxin these strains are very similar to nontoxigenic strains of the species.

Spores of *C. botulinum* can survive for long periods in air and can germinate in the presence of oxygen, but vegetative cells are sensitive to oxygen and gradually die. The concentration of oxygen present and the resulting oxidation-reduction potential can therefore have a controlling effect on survival and growth of the bacterium in the environment, in foods, and during experiments. In foods and other environments that appear to be aerobic, microaerobic or anaerobic conditions may be present or may result from the metabolism of aerobic and facultative microorganisms and allow growth of *C. botulinum*.

Group I, proteolytic *C. botulinum* Spores of Group I *C. botulinum* have much greater heat resistance than that of Group II strains. These proteolytic strains are of major concern in determination of the heat-processing given to canned, low-acid foods (foods with a pH higher than 4.5 or 4.6). The minimum temperature allowing growth is 10–12 °C, so that growth is prevented by effective refrigeration.

Group II, non-proteolytic *C. botulinum* Heating spores of non-proteolytic *C. botulinum* at about 80 °C results in sublethal damage by inactivating enzymes involved in germination. Lysozyme can enable germination of the sublethally damaged spores and subsequent growth of the organism. Enzymes with lysozyme activity are present in many foods; thus the apparent heat-resistance of spores in foods can depend on the presence or absence of enzymes with lysozyme activity. Usually the presence of lysozyme does not increase the estimated heat-resistance of spores of Group I strains. Group II strains can multiply at temperatures as low as 3 °C, making these bacteria of concern in foods that are produced using a mild heat treatment and are intended to have an extended shelf life at refrigeration temperatures.

6.3.2 Botulinum neurotoxins

There are seven types of botulinum neurotoxin (A–G) and a number of subtypes (e.g. A1–A5 and several subtypes of B, E and F), with different amino acid sequences. The botulinum neurotoxins are formed initially as single-chain polypeptides with a molecular weight of about 150 kDa and a relatively low toxicity. In the case of proteolytic *C. botulinum* the single-chain form is usually cleaved by a clostridial protease to give a dichain, highly toxic form. In non-proteolytic *C. botulinum* the single-chain form is not activated by clostridial proteases, usually, but by unidentified proteases in the host cells. The dichain form consists of a light chain (L) 50 kDa Zn^{2+} metalloproteinase and a heavy chain (H) 100 kDa polypeptide, linked by a disulphide bond. Group I, proteolytic *C. botulinum* generally

Table 6.4 *Clostridium botulinum* Neurotoxins and Toxin Complexes

Complex	Approximate size	Components	Toxin types
S 'derivative toxin'	150 kDa (7S)[a]	Single chain	A,B,C,D,E,F,G
M 'progenitor toxin'	300 kDa (12S)	S + non-toxic non-haemagglutinin	A,B,C,D,E,F,G
L 'progenitor toxin'	450–500 kDa (16S)	M + haemagglutinin	A,B,C,D,G
LL	900 kDa (19S)	Probably a dimer of L.	A

[a] Sedimentation coefficient, S = Svedberg unit
Source: Modified from B. M. Lund and M. W. Peck (2000) *Clostridium botulinum*. In: B. M. Lund, T. C. Baird-Parker, and G. W. Gould (Eds) *The Microbiological Safety and Quality of Food*, pp. 1057–1109. Gaithersburg, MD: Aspen.

form the dichain molecule in media and in foods, but Group II, nonproteolytic, *C. botulinum* form the single- chain molecule and it is necessary to treat with trypsin in order to demonstrate the active toxin. The neurotoxins act primarily at peripheral, cholinergic synapses, where they block the release of the neurotransmitter acetylcholine. The heavy chain of the toxin is involved in binding and entry into the motor neuron, and the light chain acts as an endopeptidase enzyme, degrading neuron proteins involved in the release of acetylcholine. Botulism symptoms can be caused by consumption of food containing as little as 30–100 ng of toxin, and it is estimated that consumption of about 10 mg or 5 µl of foods has caused botulism.

In foods and in culture media the toxin is usually present as a complex (M or L complex) that is much more stable than the derivative toxin (Table 6.4). The composition of the food or culture medium in which growth occurs can affect the size of the toxin complex formed. The stability of the toxin complex may be sufficient to prevent or reduce breakdown of the toxin in the stomach.

In *C. botulinum* Groups I and II the neurotoxin genes are located on the chromosome or on a plasmid. In *C. botulinum* Groups III and IV the neurotoxin genes are present on a bacteriophage and a plasmid, respectively.

The primary concern in preventing foodborne botulism is to control food production so that growth of *C. botulinum* and toxin formation do not occur. Toxin in food can be inactivated fairly readily by heating; the temperature and time needed will depend on the composition of the food. Heating food at 79 °C for 20 min or at 85 °C for 5 min has been reported to inactivate about 10^5 mouse intraperitoneal median lethal doses (LD_{50}) /g and these treatments have been suggested as a guideline. Heating of food by consumers cannot be regarded as a safety factor, however, and an adequate protection factor against growth and toxin formation by *C. botulinum* must be provided in foods as they are supplied to the consumer.

6.4 Detection and isolation

The hazardous nature of proteolytic *C. botulinum* and non-proteolytic *C. botulinum*, and the extreme potency of the botulinum neurotoxins require that all practical work is restricted to containment laboratories that afford a high degree of worker protection. In many countries, the number of laboratories that work with *C. botulinum* and its neurotoxins is also restricted by security considerations.

6.4.1 Enrichment

Detection and isolation of the bacteria often involvs enrichment from the sample. Enrichment media that are used include cooked meat medium, cooked meat medium plus glucose, chopped meat medium plus glucose and starch, or TPGY medium (trypticase peptone, bacto peptone, D-glucose, yeast extract, cysteine HCl, pH 7.4) to which trypsin may be added to activate toxin. At least two tubes of enrichment medium should be inoculated, one of which is heated to select for spores, the other is unheated to allow detection of vegetative *C. botulinum*. Heating at 75 °–80 °C for 10–15 min can be used in isolation of proteolytic *C. botulinum* but for non-proteolytic *C. botulinum*, with less heat-resistant spores, less severe heat treatment, e.g., at 60 °C for 30 min, is suitable. Treatment with ethanol before enrichment can also be used to eliminate competing vegetative bacteria. The addition of trypsin to TPGY medium may inactivate bacteriocins produced by related clostridia and active against *C. botulinum*. The addition of lysozyme to the enrichment medium may increase the recovery of heat-damaged spores, particularly those of non-proteolytic *C. botulinum*. Incubation of the enrichment cultures at 35 °C is suitable for Group I *C. botulinum* but a lower temperature, e.g. 12°–26 °C, is more selective for Group II *C. botulinum*. It is important that the enrichment media are strictly anaerobic, and the redox dye resazurin can be used as an indicator. It is essential to demonstrate the efficiency of the method(s) used; this can be achieved by showing that low numbers of spores of known strains can be recovered following addition to test samples. An extended period of incubation (seven days or longer) should be allowed for growth and neurotoxin formation, particularly for enrichments at low temperature.

Enumeration is usually made by a most-probable number (MPN) method in which a series of dilutions of the sample is inoculated into the enrichment media.

6.4.2 Isolation

After the incubation a portion of the enriched culture can be used to test for botulinum neurotoxin or the encoding gene (see below).

In order to isolate the organisms a portion of the enrichment culture can be plated onto a selective agar medium (e.g., *C. botulinum* Isolation [CBI] agar, Botulinum Selective Medium [BSM]) or a nonselective medium [e.g., egg-yolk agar]). Egg yolk is included in these plating media because colonies of proteolytic *C. botulinum* and non-proteolytic *C. botulinum* have a typical appearance associated with their lipase activity. After incubation, well-separated and typical colonies should each be streaked to plates of a nonselective agar medium (e.g., egg-yolk agar) incubated aerobically and anaerobically. After incubation, this process may need to be repeated until no growth is found on the aerobic plate and well-separated colonies typical of *C. botulinum* are found on the anaerobic plate. Repeated plating of isolated colonies may be needed to ensure a pure culture.

6.4.3 Tests for neurotoxin genes

Direct tests on samples or tests on enrichment cultures can be made using a multiplex PCR method that enables simultaneous detection of types A, B, E and F neurotoxin genes in a single reaction. Gel electrophoresis is used to confirm the size of the product. The specificity of the PCR can be confirmed by sequencing the product. Four European laboratories have recently contributed to a ring trial of a real-time PCR assay for detection and typing of

botulinum neurotoxin genes. Both multiplex PCR and real-time PCR methods have been proposed as international standards.

6.4.4 Tests for toxin

The 'gold standard' method for the detection, identification and quantification of botulinum neurotoxins is still the mouse test. This involves intraperitoneal injection, into mice, of a small volume of microbial enrichment culture or other material. The method can be made specific by the use of antisera, and by careful observation for typical botulism symptoms. The method can also be made quantitative by injection of series of diluted samples. The mouse test measures the biological activity of the neurotoxin, is extremely sensitive (5–10 pg of neurotoxin), should detect previously unknown neurotoxins, and is repeatable and reproducible. Limitations of the mouse test include the need to wait several days before a sample can be judged negative, the need for skilled personnel, and the high cost of maintaining animal facilities. The international move to minimize the use of animals in research has led to the development of alternative methods. Nonlethal animal methods have been developed and the use of human neuron cells is being explored. Several immunochemical methods have been developed that are cheaper, quicker, and easier to use than the mouse test, and in some cases have equal sensitivity and specificity. Limitations of these methods may include failure to detect unknown neurotoxins or neurotoxin subtypes, detection of biologically inactive neurotoxin, cross reactivity, and some tests require complex and expensive amplification systems. Tests have been developed also that measure the highly-specific endopeptidase activity of botulinum neurotoxin light chains (but not the activity of heavy chains).

6.4.5 Characterization of isolates

Phenotypic methods are useful in the characterization and differentiation of strains. Proteolytic and non-proteolytic strains forming type B or type F neurotoxin can be distinguished by testing for proteolysis, e.g., by plating on a medium containing milk, and by testing for ability to grow at 8°–10 °C. PCR-based methods can also distinguish between proteolytic and non-proteolytic *C. botulinum*.

Molecular methods used for typing include ribotyping and more discriminatory methods including pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), variable number tandem repeats (VNTR), multi locus sequence typing (MLST) and DNA microarrays which can distinguish between closely-related strains of proteolytic *C. botulinum* and those of non-proteolytic *C. botulinum*. Whole genome sequencing is becoming affordable and rapid and provides excellent strain discrimination. More than a dozen strains of *C. botulinum* have been sequenced and in the future this is likely to be the molecular method of choice for characterization of strains of C. botulinum.

6.5 Epidemiology

6.5.1 Incidence of *C. botulinum* in the environment and in foods

In considering reports of the incidence of *C. botulinum* the possible limitations of the methodology for detection and isolation need to be taken into account. It is not practical to review

the methodology used in reports cited here, but surveys show that there can be a relatively high incidence in some environmental samples and foods. The number of clostridia in soils can be up to 10^4–10^6/g but the number of *C. botulinum* is usually much lower. *C. botulinum* has been detected in soils and sediments worldwide. Types A and B have been reported frequently in inland soils, often in low numbers (<200/kg) but 25,000 Type A spores/kg of soil were reported in a district of China. In coastal soils, and in sediments from lakes and coastal waters in North America, Europe, Russia and Japan a high prevalence of Type E spores has been found.

Most types of food are liable to carry *C. botulinum* spores. Raw and processed fish are frequently contaminated; in temperate regions type E strains usually predominate whereas in tropical regions type A, C, D and F strains also occur. In fresh fish from coastal regions of northern France type B was, unusually, the main type detected. In meat and meat products the reported incidence of *C. botulinum* is usually lower than that in fish, from 0% in some meats to 73% in some samples of vacuum-packed bacon, and the main toxin types are A and B. The reported incidence in vegetables varies from 0–43%, mainly type A or B strains.

Samples of honey from various parts of the world including the USA, Argentina, Brazil, Japan, Italy, Sicily, Finland, Denmark, Norway and Sweden have been reported to contain *C. botulinum* spores. In many samples of honey the concentration of spores may be <40 spores/kg, but some honey associated with infant botulism contained approximately 5–80 *C. botulinum* spores/g.

6.5.2 Incidence of foodborne botulism

The reported incidence of botulism depends on the quality of the local reporting and surveillance system. In some countries, the lack of facilities to detect the toxin, and the lack of botulinum antitoxin for treatment, may limit conclusive diagnosis, and the true incidence of botulism may be unknown. Reported incidence in some countries is shown in Table 6.5. In the European Union in 2008 and 2009, one of the highest reported rates of botulism was 0.12 per 100,000 population in Romania. The majority of cases were associated with consumption of home-prepared meat (pork) products, mainly raw sausages and smoked, dried meat, and were due to type B toxin. In Georgia, the median annual incidence increased to 0.9 per 100,000 in 1990 and declined to 0.2 per 100,000 in 1998. The high incidence was attributed to economic collapse when the country gained independence, resulting in increased home-preservation of food. Large outbreaks of foodborne botulism have been reported from Thailand, China and Rwanda. The largest reported outbreak since 1982 occurred in Algeria in 1998 when 340 cases and 37 deaths due to *C. botulinum* type A were attributed to commercially-produced cachir. This was made from raw chicken and other meat, corn flour, olives and spices; it resembled corned beef and had the shape of salami. While the majority of reported cases of botulism in the USA are infant botulism, the majority of cases reported in England and Wales in recent years have been wound botulism.

6.5.2.1 *Foodborne botulism outbreaks associated with proteolytic* C. botulinum

Examples of outbreaks are shown in Table 6.6. The low fatality rate was probably due partly to the ready availability of antitoxin. For example, in the outbreak in Thailand associated with bamboo shoots vials of antitoxin were provided from the USA, the UK and Japan, and also purchased from a Canadian company.

Table 6.5 Reported Botulism in Different Countries

Country	Period	Total cases	Average per year	Total cases 2008	Total cases 2009
Argentina	1980–2004	84	3		
Belgium	1982–2000	32	2		
Canada	1971–2005	439	13		
China	1958–1983	4377	175		
Denmark	1984–2000	18	1	1	0
France	1971–2003	1286	39	8	29
Georgia	1980–2002	879	40		
Germany	1983–2000	376	22	10	5
Italy	1979–2000	750	34	23	32
Japan	1951–1998	530	11		
Poland	1971–2000	9219	307	22	31
Romania	2003–2008	210	35	26	37
Spain	1971–1998	277	10	5	12
Sweden	1969–2000	13	1		
UK	1971–2006	38	1	4[ab] (0, 0, 4)[c]	21[ab] (0, 2, 19)[c]
USA	1971–2008	1029	27	145[b] (17,109, 19)[c]	118[b] (10, 83, 25)[c]

Note: Based on data from Peck (in press); ECDC, 2011; CDC, 2011; HPA, 2011
[a] = data for England and Wales
[b] = total cases
[c] = foodborne botulism, infant botulism, other botulism
Unless specified otherwise, the numbers may include all types of botulism. For example, in England and Wales in 2008/2009 the majority of cases were associated with wound botulism, there were two reported cases of infant botulism and no reported cases of foodborne botulism.

Table 6.6 Examples of Outbreaks of Foodborne Botulism Probably Caused by Proteolytic *C.botulinum*

Outbreak	Food	Cases (deaths)	Toxin type	Probable cause
1985, Canada	Restaurant-prepared, chopped garlic in soybean oil	36 (0)	B	Dried garlic rehydrated, immersed in oil. No lethal treatment, not refrigerated
1989, UK	Commercial hazelnut yoghurt	27 (1)	B	Canned hazelnut conserve inadequately heated and added to yoghurt
1994, USA	Restaurant-prepared dips made with baked potatoes	30 (0)	A	Potatoes wrapped in foil, baked, then stored for several days at ambient temperature
1996, Italy	Commercial mascarpone cheese used by three families for home-prepared tiramisu	8 (1)	A	*C.botulinum* type A spores and toxin found in some retail samples. No heat-inactivation or inhibitory conditions, inadequate refrigeration.
1998, Algeria	Commercial cachir	340 (37)	A	Unknown
1998, Argentina	Small-scale company produced Argentine meat roll (*matambre*)	9 (0)	A	Insufficient heating, inadequate refrigeration
2000, France	Home-canned asparagus	9 (0)	B	Inadequate heat treatment.

Table 6.6 (*Continued*)

Outbreak	Food	Cases (deaths)	Toxin type	Probable cause
2001, USA	Commercial frozen chilli sauce purchased from salvage store	16 (0)	A	No heat-inactivation, inadequate refrigeration
2004, Italy	Probably green olives preserved using salt	28 (0)	B[a]	No lethal heat treatment, no controlled inhibitory conditions
2004/5, USA (2 outbreaks)	Illicit prison-made alcohol, made with potatoes	5 (0)	A	Mild heat treatment, maintenance at ambient temperature
2005, Turkey	Home-made suzme (condensed yoghurt)	10 (2)	A	Condensed yoghurt filled into plastic jars and buried in earth for two months. Yoghurt in one jar had been in contact with soil
2006, Thailand	Home-canned bamboo shoots	209 (0)	A	Inadequate heat treatment
2006, USA, Canada	Commercial pasteurized carrot juice	6 (1)	A	Safety relied on refrigeration only; refrigeration inadequate
2007, China	Commercially-produced sausages	66 (0)	A	Production unknown, no refrigeration
2007, USA	Commercially-canned chilli sauce	8 (0)	A	Deficient canning process
2008, Turkey (Dutch tourists)	Probably home-packed, unprocessed black olives	8 (0)	B[a]	Unknown
2008, France	Commercial chicken enchiladas	2 (0)	A	Product pasteurized, probably stored at room temperature for two weeks. Reheated by microwaving
2010, Italy Unlinked cases	Commercial products, artichoke preserve; cream of vegetable soup.	2 (0)	B[a]	Products pasteurized. Labelling of preserve indicated a shelf life of two years, refrigeration not specified. Labelling of soup indicated shelf life of 45 days, refrigeration suggested.
2011, USA Unlinked cases	Commercial potato soup	2 (0)	A	Product pasteurized, stored without refrigeration
2011, France	Commercial product, artisan producer. Ground green olive paste	9 (0)	A	Inadequate heat treatment
2011, Finland	Commercial product from Italy. Olives stuffed with almonds	2 (1)	B	Product pasteurized. No inhibitory conditions reported
2011, Scotland	Commercial product. Mild curry sauce	3 (0)	A	Probable increase in pH of product due to growth of other microbes

[a] proteolytic *C.botulinum* type B suspected

Outbreaks associated with inadequate heat treatment of canned or bottled foods

The largest reported outbreak in the UK, in 1989, was caused by the commercial underprocessing of canned, low-acid hazelnut conserve, which was then added to yoghurt. Cans of the conserve had blown well before the outbreak.

The outbreak in USA in 2007, caused by commercially-canned chilli sauce, was the first botulism outbreak associated with a commercial canning facility in the USA for 30 years. Following the outbreak, FDA officials tested 17 swollen cans of the chilli sauce produced in the same set of retorts as the cans associated with the botulism cases. Sixteen of the 17 cans were positive for botulinum toxin type A. Home-canned or bottled vegetables are frequently underprocessed and have caused many outbreaks; they are the most important single cause of foodborne outbreaks in the USA except Alaska. The outbreak in Thailand in 2006, caused by home-canned bamboo shoots, followed previous outbreaks affecting small numbers of patients, which alerted physicians to the symptoms and resulted in a rapid response. The food was served at a religious festival. A total of 209 persons were affected, 134 were hospitalized and 42 needed mechanical ventilation; treatment of patients with antitoxin was probably life-saving.

Outbreaks associated with pasteurization and inadequate refrigeration of foods

Several of the outbreaks in Table 6.6 were caused by foods that received a heat treatment that was sublethal for proteolytic *C. botulinum* and whose safety relied on refrigeration. For example, in the outbreak in Argentina in 1998 the meat roll (*matambre*) was prepared by a small-scale commercial producer in his own home. The ingredients included beef, carrots, hard-boiled eggs, salt, spices and potato flour. The meat rolls were vacuum-packaged and immersed in water heated to 78 °–80 °C for approximately four hours to ensure an internal temperature of 68 °C, then cooled and stored in a refrigerator before sale to supermarkets or to consumers. Refrigeration by the producer and by the seller was inadequate. One patient needed mechanical ventilation and all patients were treated with trivalent botulinum antitoxin.

The restaurant-associated outbreak in the USA in 1994 was associated with potato-based dips and followed previous outbreaks associated with potatoes. The potatoes had been wrapped in aluminium foil and baked in an oven set at 250 °C, but because the foil retained moisture the temperature at the surface of the potato did not exceed 100 °C. The foil-wrapped potatoes were maintained at ambient temperature for sufficient time to allow toxin production.

The outbreak in the USA and Canada in 2006 associated with commercially-produced pasteurized carrot juice was notable for the severity of the illness and the extremely high concentration of toxin found in the serum of one patient. The carrot juice consumed by one patient contained 100,000 mouse intraperitoneal lethal doses per ml, the highest concentration reported in a food associated with an outbreak. Five of the patients were treated with antitoxin, one was not because toxaemia was not present at diagnosis. One year after the onset of illness two patients had been discharged, two were in rehabilitation (one ventilator-dependent), one was hospitalized and ventilator-dependent and one died 90 days after the onset of illness. The carrot juice had a pH of 6.8 and contained no barrier other than refrigeration to prevent growth of *C. botulinum*. The bottles were labelled ‘keep chilled’ and ‘keep refrigerated’ in very small print. The fact that the causative bacterium was *C. botulinum* type A, indicates that at some stage the bottles had been maintained at a temperature

above 10 °C. Following this outbreak the FDA recommended that there is a need for control measures for low-acid juice that would ensure that *C. botulinum* growth and toxin production would not occur if the juice was kept unrefrigerated during distribution or by consumers. This could be achieved by methods such as acidification of the juice to a pH of 4.6 or below, or thermal treatment by an F_o3 process (see Section 6.6).

Two unlinked cases in Italy in 2010 were caused by commercial artichoke preserve and commercial cream of vegetable soup. Both products were pasteurized and had a pH of 5.7–5.72 and water activity of 0.941–0.947. The preserve was labelled with a shelf life of two years and refrigeration was not recommended; the soup was labelled with a shelf life of 45 days and although refrigeration was suggested some storage at higher temperature had occurred.

Two separate cases in the USA in 2011 were caused by potato soups that received a heat treatment not sufficient to inactivate proteolytic *C. botulinum*, and were inadequately refrigerated. The FDA recommendation relating to juices appears to merit application to low-acid soups also. In some cases the direction to store foods under refrigeration was stated to be in small print on the packaging and not easily visible to the consumer.

Outbreaks associated with unheated foods, including those associated with vegetables and herbs in oil or water

An outbreak in Italy in 2004 was associated with consumption of olives that had been soaked in salt water for 35 days after which the olives were decanted into jars and salt water was replaced with fresh water. Neither the amount of salt used nor the pH was controlled at any stage. The pH of a jar of olives prepared at the same time as those associated with the outbreak was 6.2. The product must have been sufficiently anaerobic to allow growth of *C. botulinum* in the absence of inhibitory conditions.

An outbreak in Canada in 1985 was associated with consumption of sandwiches made with garlic-buttered bread. The garlic butter was made from bottled, chopped garlic in soybean oil. The sun-dried garlic had been rehydrated without additives and the bottles of garlic in oil had been stored in a restaurant for eight months unrefrigerated, despite being labelled with instructions to refrigerate. Bottles of garlic in oil from the same production lot as that implicated had a mean pH of 5.4 (range 4.6 to 5.7). A further outbreak associated with garlic in oil from the same processor occurred in 1989 and affected three people. Following this outbreak the FDA ordered companies to stop making garlic-in-oil mixes that are only protected by refrigeration and advised the addition of microbial inhibitors or acidifying agents. Despite this warning, home-bottled garlic in oil has been associated with further outbreaks and home-prepared mushrooms, eggplant, and aubergine stored in oil have been associated with botulism. In Italy between 1994 and 1998 a high proportion of cases of botulism were caused by home-made vegetable preserves in oil or water.

6.5.2.2 *Foodborne botulism outbreaks associated with non-proteolytic* C. botulinum

The majority of reported outbreaks due to non-proteolytic *C. botulinum* have been associated with fish products and involved type E strains (Table 6.7).

The outbreak in Egypt in 1991 was the largest reported outbreak of foodborne botulism due to type E toxin. This was caused by consumption of a traditional food prepared by salting uneviscerated mullet fish. Traditionally, the fish were obtained from the Red Sea or from salt lakes, but more recently had been obtained from fish farms. The fish were stored for

between several hours and one day to allow swelling and putrefaction before they were salted, and stored for between two weeks and one year. Some time would elapse before sufficient salt penetrated into the gut of the fish to inhibit *C. botulinum*, and maintenance of the fish at temperatures above 3–5 °C would allow any non-proteolytic *C. botulinum* spores in the gut of the fish to germinate, resulting in growth and toxin formation before the salt concentration became inhibitory. Several other outbreaks involved raw or home-salted fish that was refrigerated inadequately.

Table 6.7 Examples of Outbreaks of Foodborne Botulism Caused by Non-Proteolytic *C.botulinum*

Outbreak	Food	Cases (deaths)	Toxin type	Probable cause
1991, Egypt	Commercial, uneviscerated, salted fish	>91 (18)	E	Fish allowed to putrefy before salting
1997, Argentina	Home-cured ham	6 (0)	E	Suggested that spores originated from seasoning; no lethal treatment, inadequate refrigeration.
1998, Germany	Commercially-smoked, vacuum-packed fish	4 (0)	E	Suspected temperature abuse
1998, France	Commercial, frozen, vacuum-packed scallops	1 (0)	E	Temperature abuse
1998, France	Commercial, frozen, vacuum-packed prawns	1 (0)	E	Temperature abuse
2001, Canada, two outbreaks	Home-made, fermented salmon roe	4 (0)	E	Unsafe process
2002, USA	Raw 'muktuk' (skin and blubber from beluga whale, stored in sealed plastic bags)	12 (0)	E	Growth and toxin production in sealed bag
2003, Germany	Home-salted, air-dried fish	3 (0)	E	Fish gutted, salted in brine, dried, no refrigeration
2003, Norway	Home-made 'rakfisk'	4 (0)	E	Inadequate salt and inadequate refrigeration
2004 Germany	Commercial vacuum-packed, smoked salmon	1 (0)	E	Consumed after 'use-by' date
2005, USA	Home-prepared, uneviscerated, salted fish	5 (0)	E	Uneviscerated fish with salt placed in sealed plastic bag and left at ambient temperature for ~1 month
2006, Iran	Traditional soup ('Ashmast')	11 (0)	E	Soup included spinach that had been stored in an airtight container
2006, Finland	Commercial vacuum-packed, smoked whitefish	1 (0)	E	Probable temperature abuse
2009, France	Commercial vacuum-packed, hot-smoked whitefish	3 (0)	E	Probable temperature abuse

The heat treatments in commercial processes for hot smoking fish do not usually give sufficient inactivation of *C. botulinum* type E spores, and safety depends on the salt content and storage below 3 °C. Abuse of temperature control or time of storage probably contributed to several of the cases in Table 6.7.

There are few recent reported outbreaks attributed to non-proteolytic *C. botulinum* type B. In Germany and France many outbreaks of botulism have been traced to raw hams dry-salted domestically. There is evidence that most of these cases were caused by non-proteolytic *C. botulinum* type B. Two outbreaks in Iceland in 1981 and 1983, associated with blood sausages, were also caused by non-proteolytic *C. botulinum* type B. In Poland in 1990, the majority of cases were associated with consumption of home-prepared meat products and type B toxin. There is a need to differentiate between cases due proteolytic *C. botulinum* type B and those due to non-proteolytic *C. botulinum* type B strains in order to understand and prevent these cases.

6.5.3 Infant botulism

Since the first recognition of the disease in the USA infant botulism has been reported in many countries (Table 6.8). The great majority of cases have been caused by spores of proteolytic *C. botulinum* forming type A or type B toxin. A few have been caused by non-proteolytic *C. botulinum* type E or *C. butyricum* producing type E toxin or by *C. baratii* producing type F toxin. Several cases have been caused by strains that formed two types of toxin.

Environmental dust inhaled or swallowed by the infant appears to be the source of the spores in many cases. Honey is the only proven food source of spores linked to cases of infant botulism. In Europe up until 2002, more than half the reported cases of infant botulism had a history of ingestion of honey. In most cases, samples of honey were not available for microbiological testing, but in five cases where samples were available *C. botulinum* of the same toxin type was isolated from the infant and from honey. In the UK between August 2009 and July 2011, five unlinked cases of infant botulism were reported, each associated with ingestion of honey. In two of these cases the same toxin type of *C. botulinum* was isolated from the faeces of the infant and from the honey implicated. In California, a decline has occurred in the proportion of infant botulism cases with a history of honey ingestion, from 39.7% in the 1970s to 4.7% in the 2000s. This may be due to the familiarity of physicians in California with the disease after treating more than 1000 cases and diagnosis of cases unlinked to honey, and to the publication of advice that honey should not be given to infants.

Table 6.8 Examples of reported Global Occurrence of Infant Botulism

Country	Time period	No. of cases
United States	1976–2006	2419
Argentina	1982–2006	410
Australia	1978–2006	32
Italy	1984–2008	29
Canada	1979–2006	27
Japan	1986–2007	24
United Kingdom	1985–2011	13
Spain	1985–2007	11
France	1983–2009	7

Data from Fenicia and Anniballi (2009), King et al. (2010), Dr K. Grant pers. comm. (2011)

Surveys of honey in many countries have demonstrated the presence of *C. botulinum* spores in between 2% and 24% of samples. Some honey associated with cases of infant botulism contained 5–80 spores/g. The infectious dose is estimated as 10–100 spores, thus the ingestion of a very small quantity of honey may lead to illness. Spores are unable to germinate and form vegetative bacteria and toxin in honey, but they can probably survive for several years, and heating honey sufficiently to inactivate the spores would damage the product. A study of honey production in Finland indicated that 10% of samples of extracted honey contained *C. botulinum* and that soil-derived spores may be carried into the hive by bees.

In Spanish-speaking communities, particularly in Argentina, infusions of herbs such as chamomile, anise, mint and linden are often given to infants to treat colic or as a beverage. Strains forming botulinum toxin have been reported in chamomile and linden and two cases of infant botulism in Spain associated with use of infusion of chamomile have been reported.

Infant botulism is rare but also probably under-recognized. Recognition depends on awareness of infant botulism by pediatricians and specialist departments.

6.6 Prevention and control

The ability of spores of *C. botulinum* to survive heat treatment and the effect of temperature, pH, salt concentration and other factors on growth of the bacteria (Table 6.3) determine the methods used to prevent survival and growth of *C. botulinum* in foods (Table 6.9). Control can be expressed by the Protection Factor, Pr, which is the number of decimal reductions in the probability of growth and toxin production. This can be the sum of the number of 10-fold reductions in viable spores caused by lethal treatments, Ds, and the number of reductions in the probability of growth caused by inhibition, In,

$$\mathrm{Pr} = \mathrm{Ds} + \mathrm{In}.$$

Category 1

The term *low-acid foods* is applied to foods with a pH higher than 4.5, or 4.6 in the United Kingdom and United States, respectively; these foods could allow growth of *C. botulinum*. The heat treatment used during canning of these foods resulted from experiments by Esty and Meyer (1922), in which the heat resistance of large numbers of spores of over 100 strains of *C. botulinum* was tested. This led to the calculation that inactivation of about 10^{12} of the most heat-resistant spores of *C. botulinum* would require heating at 121.1 °C (250 °F) for 2.52 min giving a 12D process, or Pr = 12. Thus, if one viable spore of *C. botulinum* was present in each of 10^{12} cans before heating, one of those 10^{12} cans would contain one viable spore after the heat treatment. Treatment for one min at 121.1 °C is designated as an F_o1 process, and for low-acid, canned foods, except those containing curing salts, processing to an F_o3 as a minimum value is used to inactivate *C. botulinum*. Spoilage organisms with a greater heat resistance than *C. botulinum* are liable to be present in foods and a higher F_o treatment is needed commonly to achieve 'commercial sterility'. The use of this standard has ensured the usual safety of commercially-canned foods, and botulism incidents associated with commercial canned food are primarily the result of failure to deliver the specified process. In addition to the use of an adequate heat treatment, precautions are needed to prevent post-processing contamination of canned foods by access of bacteria through the seams of cans during cooling.

Table 6.9 Examples of Main Factors used to Control *C.botulinum* in foods

Category	Examples of foods	Main controlling factors
Shelf-stable foods		
1	Low-acid canned foods, pH >4.5 (UK) or pH >4.6 (US)	Heat process>F_o3
2	Shelf-stable, canned cured meats	Heat process<F_o3, NaCl, nitrite
3	Canned, acid foods, pH <4.5 (UK) or <4.6 (US) e.g., many types of canned fruit and vegetables	pH, heat process<F_o3
4	Raw salted and salt-cured meats, e.g., salt pork, salt bacon, salt hams, dry-cured hams, and bacon	a_w (NaCl+drying) +/– nitrite. Refrigeration below 5 °C during salting
5	Fermented sausages, e.g., summer sausage, pepperoni, acidulated sausage.	pH, a_w (NaCl+drying), nitrite or nitrate
Perishable foods		
6	Vacuum-packed raw meats, vacuum- or modified atmosphere-packed fish	Strict control of refrigeration
7	Perishable, cooked, cured meats, paté, pressed ham, frankfurters	Mild heat treatment, NaCl, nitrite, refrigeration
8	Salted fish, lightly and semi-preserved fish products, hot- smoked fish	NaCl, refrigeration, nitrite in many products, mild heat treatment, in some cases sorbate, benzoate, nitrate or smoke
9	Some processed cheese products, caviar	a_w (NaCl and other components) pH, refrigeration of some products, mild heat treatment, in some cases sorbate
10	Vacuum- and modified atmosphere-packed, chilled foods, ready meals	Heat treatment, pH, NaCl, a_w, refrigeration, limited shelf life.
11	Vegetables or herbs immersed in oil or water	pH adjusted to <4.6; refrigeration.

a_w = water activity

The process criterion of heating at 121 °C for at least 3 min (F_o3), or an equivalent process, is used for thermal processing of shelf-stable, low-acid foods. In general, the use of new methods, such as pressure-assisted thermal sterilization, for production of such foods will require an equivalent Pr. Spoilage organisms with greater resistance than *C. botulinum* spores to combined pressure and heat treatment may necessitate the use of increased treatment.

Category 2

Shelf-stable, canned cured meats receive a minimal heat process that does not inactivate *C. botulinum* spores, but some injury occurs. The combination of nitrite and salt prevents outgrowth from the injured spores. Pr for these foods has been estimated as ~6.6–8.

Category 3

The great majority of evidence shows that *C. botulinum* does not multiply in 'acid' foods with a pH <4.5–4.6. Canned acid foods can be given a heat treatment much lower than an F_o3 process, and any spores of *C. botulinum* are liable to survive in these foods. A few cases of botulism have resulted after survival and multiplication

of other microorganisms in an acid canned food has raised the pH and allowed growth and toxin formation by surviving *C. botulinum*.

Category 4

The safety of raw, dry-salted products relies on maintenance below 5 °C until the salt content has established an internal a_w below 0.96 (Table 6.9).

Category 5

Fermented sausages rely on a combination of reduced pH (4.6–5.3), resulting from fermentation of added sugar by lactic acid bacteria, and reduced a_w (<0.95) sometimes combined with use of nitrite/nitrate and sometimes smoking.

Category 6

Outbreaks of botulism have occurred in Inuit populations in Alaska and Northern Canada resulting from consumption of putrid raw meat of marine animals. Fish products may pose a greater risk than meat products because of the extent of contamination with *C. botulinum* spores, and safety relies on strict control of refrigeration.

Category 7

The safety of perishable, cooked cured meats depends on a combination of factors. In canned Bologna-style sausage (3% brine, pH 6.0, cooked to a core temperature of 76 °C), containing 83 mg $NaNO_2$/kg after storage for six months at 5 °C the Pr was estimated as >8, but in a product containing no $NaNO_2$ and stored at 8 °C the Pr was estimated as 4.6.

Category 8

Salted, uneviscerated raw fish have caused several outbreaks of botulism. Careful evisceration before preservation will probably remove the main source of spores and help to ensure penetration of salt throughout the fish. In hot smoking the heat treatment is not sufficient to inactivate spores of non-proteolytic *C. botulinum*, although they may be damaged. The presence of salt, nitrite and other factors and refrigeration are required for safety.

Category 9

An outbreak of botulism in Argentina was associated with a processed cheese spread in which onions were included. Outbreaks associated with caviar have been reported from several countries.

Category 10

This includes prepared foods sealed in air-tight containers, as well as vacuum- and modified atmosphere-packed foods. The safety of foods in this category usually depends on a mild heat treatment combined with refrigeration and other preservative factors. The cooking will not inactivate spores of proteolytic *C. botulinum* and may only damage spores of non-proteolytic

C. botulinum. Storage of these foods at 3 °C or lower would prevent growth of *C. botulinum*, but maintenance at ≤3 °C is only possible in a dedicated, in-house system such as is specified in the UK Department of Health Guidelines on Cook-Chill and Cook-Freeze systems. In the UK, the Food Standards Agency has advised that, *in addition to chill temperatures* (specified ≤8 °C as in England and Wales), which should be maintained throughout the food chain, the following factors should be used *singly or in combination* to prevent growth and toxin production by non-proteolytic *C. botulinum* in prepared chill foods with an assigned shelf life of more than 10 days. The shelf life will begin as soon as the controlling factor(s) have been first applied:

- a heat treatment at 90 °C for 10 minutes or equivalent lethality (the product should be heated in the sealed final pack, or after heating the product should be packed under conditions that minimize microbiological contamination)
- a pH of 5 or less throughout the food and throughout all components of complex foods
- a minimum salt level of 3.5% in the aqueous phase throughout the food and throughout all components of complex foods
- a water activity of 0.97 or less throughout the food and throughout all components of complex foods
- a combination of heat and preservative factors which can be shown consistently to prevent growth and toxin production by non-proteolytic *C. botulinum* throughout storage.

A heat treatment at 90 °C for 10 min combined with storage at <8 °C for up to 42 days gave Pr = 6 for non-proteolytic *C. botulinum*.

Category 11

Chopped garlic and olives, immersed in oil or water and kept at ambient temperature have caused several outbreaks of botulism, the low-acid pH and exclusion of oxygen having enabled growth of *C. botulinum*. The FDA directs that acidification of vegetables or herbs immersed in oil should be used by commercial producers to inhibit *C. botulinum*, but this cannot be relied on in the home environment. Storage at or below 3 °C would be effective, but storage at such temperatures cannot be relied on in the home. Storage of vegetables or herbs in oil in the home by freezing the product has been recommended for assurance of safety.

6.7 Conclusion

Diagnosis and reporting of botulism, particularly infant botulism, is limited in some parts of the world. Improved methods for detection of neurotoxin and increased availability of antitoxin and of human botulism immune globulin should help to improve diagnosis and treatment.

Many outbreaks and cases of botulism are caused by failure to implement controls that are known to prevent foodborne botulism. Dissemination of information about control methods to industry, and to the general public where home preservation of food is practised, continues to be needed.

Moves to reduce the content of salt and preservative factors in foods or to extend shelf lives require assessment of any increase in risk due to *C. botulinum*. With the introduction of new types of food and new methods of production there is a need to ensure that levels of safety that are presently built into existing foods are applied in new products.

In parts of the world infant botulism is liable to be under-diagnosed and under-reported, and a greater awareness of this condition by medical staff is needed.

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7 *Salmonella*

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7.1 Introduction

Salmonella is a Gram-negative, non-spore forming bacillus and facultative anaerobe that grows well between 35 and 37 °C. George Gaffky first isolated the typhoid bacillus which is now known as *Salmonella* Typhi (Typhi) in 1884 and, later, Theobald Smith isolated Choleraesuis from pig's intestine while searching for the cause of hog-cholera in 1885. *Salmonella* was first named after Daniel Elmer Salmon who was Smith's director and the administrator of the United States Department of Agriculture (USDA). After the initial identification of the organism, over 2600 different *Salmonella* serotypes have been isolated, and while only a minority of these have been linked as the causative organism for illness in both animals and humans the majority are capable of causing infection in humans. Non-typhoidal salmonellosis continues to be one of the leading foodborne illnesses causing hospitalization.

7.2 Taxonomy and typing methodology

The genus *Salmonella* is a member of the family *Enterobactericeae*. These organisms are able to ferment glucose, reduce nitrates to nitrites and are oxidase negative. The most common way to classify *Salmonella* is by serotyping, in which the bacteria are distinguished by differences in their somatic (O) and flagellar (H) surface antigens using the Kauffmann-White scheme. There are two major species of *Salmonella*: *S. enterica* and *S. bongori*. Over 99% of the serotypes are grouped into the species *S. enterica*, which is divided into six subspecies: *enterica* (I), *salmonae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houteae* (IV) and *indica* (VI; what was subspecies V, is now *S. bongori*, a separate species). While serotypes are no longer used for speciation, they maintain a vital place in classifying *Salmonella* and are typically used as the main descriptor of isolates, especially in reporting of isolates collected during investigations.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

Additionally, isolates within a particular serotype can be further subdivided using molecular subtyping methods or by phage typing. Phage typing relies on the ability of specific bacteriophages to infect bacteria with complementary surface receptors. Phage can attach to the receptors, infect and lyse the bacterium which appears as a zone of clearing (plaque) within a lawn of bacteria on a media plate. Phage types are determined based on which specific typing phages are able to lyse the bacteria. Phage typing has been used to further differentiate *S*. Enteritidis isolates from different geographical locations. *S*. Enteritidis phage type (PT) 4 was most commonly detected in Europe, while PT 8 was found to be more prevalent in the US.

The most important molecular typing methods used to characterize *Salmonella* are pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and multiple locus variable number of tandem repeat analysis (MLVA). PFGE uses the selective ability of rare-cutting restriction enzymes, primarily XbaI or AvrII (BlnI) for *Salmonella*, to digest the bacterial genome into a limited number of restriction fragments. These restriction fragments are separated using specialized electrophoresis conditions in which the field polarity is varied during the run to facilitate the separation of large DNA fragments (>800 kb). Differences in the microbial genomes can lead to variability in the location and number of restriction sites or in the size of DNA fragments which are detected as banding profile differences following electrophoresis. The restriction profiles are compared to one another to predict the relatedness among isolates. PFGE is often considered the 'gold-standard' molecular typing method for *Salmonella*.

As access to high-quality DNA sequencing became more readily available, newer typing techniques have attempted to utilize DNA sequencing to genotype *Salmonella*. MLST relies on sequencing multiple loci in the *Salmonella* genome and comparing the DNA sequences to identify differences among the sequences analyzed. The loci that are generally sequenced are housekeeping genes, which are typically present in all members of the particular species and are not subject to strong selective pressure that can lead to rapid sequence changes. These genes do need to display some level of genetic diversity within the population, yet have very conserved regions for PCR primer binding needed to amplify the locus prior to sequencing. For *Salmonella*, the most widely-used set of loci are *thrA*, *purE*, *sucA*, *hisD*, *aroC*, *hemD*, and *dnaN*. The sequencing results of these loci can be input into a *Salmonella* MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica) and specific allele types will be determined for each loci. These allele types are combined to determine the sequence type (ST) for a particular strain of *Salmonella*. The different allelic profiles and STs for each isolate can be compared to one another to predict the relatedness of the different strains.

Another subtyping method that has been utilized for certain *Salmonella* is MLVA, which involves sets of repetitive elements located throughout the bacterial genome to characterize isolates. Each of the repetitive loci, termed tandem repeats, consists of identical repeated sequences ranging from a few to greater than 100 base pairs in size that are adjacent to each other in the genome and repeated multiple times. The numbers of these direct repeats are often polymorphic in less related strains, thus allowing for differentiation among strains. In the process termed variable number of tandem repeat (VNTR) analysis, PCR primers are designed for the regions flanking the tandem repeats, which allow the repeat region to be amplified. Differences in the number of repeats can be detected following PCR amplification which allows for comparison among strains. Often fluorescently labeled primers are used to amplify the VNTR loci so that the reactions can be separated with an automated DNA sequencer to facilitate efficient analysis. With MLVA, multiple VNTR are

analyzed and compared among strains to identify potential relationships among isolates. MLVA has been used to subtype members of serovars Enteritidis and Typhimurium that are clonal with PFGE.

7.3 *Salmonella* pathogenesis

7.3.1 Etiology

Numerous *Salmonella* serotypes have the ability to induce localized gastroenteritis in humans and some domestic animals but the range of *Salmonella* infections in different hosts is dependent both on pathogen virulence mechanisms and host resistance and immunity capabilities. Some serotypes have relatively narrow host ranges including *S*. Typhi and *S*. Paratyphi A, which are associated with human infections while *S*. Gallinarum is associated with poultry. Others such as *S*. Choleraesuis and *S*. Dublin also have limited host ranges, primarily swine and cattle respectively, but are also known to cause infections in humans from sources such as raw milk. *Salmonella* serotypes Typhimurium and Heidelberg have broad host ranges and can colonize and infect a wide range of animal hosts. For *S*. Typhimurium, the human infections are believed to be mostly caused by the transmission through contaminated food such as poultry products. *S*. Enteritidis has been most closely identified with chicken eggs and egg products and this may be linked to its apparent affinity for infection and invasion of reproductive tissues in susceptible laying hens followed by internal contamination of shell eggs. However, there is a sufficiently wide range of salmonellosis cases originating from nonpoultry and nonfood sources such as domestic mammalian and reptile pets that precludes any attempts to assign quantitative levels for individual sources. Once humans ingest an infectious dose of *Salmonella*, organisms are able to colonize and multiply in the small intestine and invade and survive in intestinal tissues leading to an inflammatory reaction by the host. In certain cases, *Salmonella* can enter macrophages or other immune cells and be transported to the liver, spleen or mesenteric lymph nodes where they can cause more severe illness. Young children (age under 10), the elderly and immuno-compromised individuals are more susceptible to salmonellosis. In general, salmonellosis is associated with three major types of illness – gastroenteritis, septicemia and typhoid fever – and can be associated with sequela following the acute phases of disease.

Gastroenteritis is the predominant form of salmonellosis associated with the consumption of contaminated foods. Symptoms of gastroenteritis typically appear within 12 to 72 hours following ingestion, but may take as long as a week. The most common symptoms typically start with nausea and vomiting and can develop to diarrhea, abdominal pain and fever. The illness is most often self-limiting with complete resolution in 4–7 days without antimicrobial therapy and may only require administration of fluid and electrolyte replacement. In rare cases, the illness can be fatal due to prolonged excretion and subsequent dehydration.

Septicemia or bacteremia associated with salmonellosis typically develops from gastroenteritis when organisms invade and survive in host tissues and immune cells and enters the blood stream. The pathogen can be transmitted throughout the body and infect organs such as lung, brain, liver and kidney causing a life-threatening infection. The symptoms include fever, elevated heart and respiratory rate, pain in the thorax and abdomen, chills and anorexia. These more severe infections are typically treated with antimicrobial therapy to limit the infection. Systemic infections may occur in humans who are vulnerable either because of altered gastrointestinal tract physiology due to changes in microflora after

antibiotic treatment or various disease states such as parasitism, carcinoma or viral immunosuppression. In some cases, these infections can lead to more chronic conditions such as arthritis or endocartitis and prolonged antibiotic therapy, which are commonly associated with serotypes *S*. Typhimurium, *S*. Dublin or *S*. Choleraesuis.

Enteric or typhoid fever is a systemic invasive disease caused by *S*. Typhi and *S*. Paratyphi strains and is a common life-threatening disease in developing countries. The infectious dose can be fewer than 1000 cells, which is lower than that associated with gastroenteritis. While gastroenteritis exhibits an incubation time between 12 and 72 hours, enteric fever occurs over a much longer time period of anywhere from seven to 28 days, and the victim can take up to eight weeks to recover. Common symptoms are malaise, nausea and abdominal pain in the early stages and high fever and diarrhea in the late stages. The bacteria can translocate the gut, survive and multiply in gut lymphoid follicles, mesenteric lymph nodes and liver and spleen endothelial tissues causing bacteremia.

Sequelae can occur following *Salmonella* infections. In one study from Canada following an outbreak caused by *S*. Typhimurium, 27 of 423 (6%) infected individuals developed reactive arthritis (ReA), with many exhibiting symptoms that persisted for at least five years. A number of different joints can be affected by ReA including the knees, shoulders, elbows, hands, feet and back. Other studies have found even higher rates of ReA following outbreak associated cases of salmonellosis caused by serotypes *S*. Enteritidis and *S*. Typhimurium.

Treatment of salmonellosis mostly relies on supportive therapy and antimicrobial treatment for more severe cases of disease. The preferred first lines of antimicrobial therapy are the fluoroquinolones or extended spectrum cephalosporins, such as ceftriaxone. Alternative antimicrobial therapies include chloramphenicol, trimethoprim/sulfasoxizole or doxycycline. Unfortunately, an increasing number of *Salmonella* strains have developed resistance to these antibiotics and antimicrobial resistance is a growing concern for effective treatment, especially in developing countries where typhoid fever is common. An example of prominent antimicrobial resistance concerns were the pandemic *S*. Typhimurium definitive type 104 (DT104) strains, which were identified to cause severe gastrointestinal infection following consumption of contaminated food and were typically resistant to at least five antibiotics, including ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. The severity of infection may also be a consequence of exposing the indigenous microflora to antibiotics. There is experimental evidence from mouse models that administration of antibiotics can not only modify the composition of the indigenous intestinal microflora before *S*. Typhimurium is introduced but can also lead to increased susceptibility to colonization, greater postinfection compositional alterations of the intestinal microflora and a more pronounced intestinal pathology. During recovery from salmonellosis, *Salmonella* mainly persists in the feces and are eventually excreted; however, in some cases, the organism remains and the host becomes a chronic carrier causing a significant public health hazard to individuals exposed to the carrier.

7.3.2 Pathogenesis – pathways and mechanisms

The main route of infection is orally, with the infectious dose estimates ranging from approximately 30 to more than 10^9 colony forming units (CFU). This variability in infectious doses is associated with the contaminated food matrix and the genetics of the organism. Once ingested, *Salmonella* must be able to survive the low pH of the stomach. *Salmonella* employ numerous acid shock proteins in an acid tolerance response (ATR) to facilitate survival in the low pH environment of the stomach and the intestinal tract. Some

of the major regulatory proteins include RpoS σ-factor, PhoPQ and Fur, which play a role in the expression of cellular regulatory proteins, transcription and translation factors, molecular chaperones, envelope proteins and fimbriae that are important components of the ATR.

Once in the gastrointestinal tract (GIT), particularly in the lower regions, *Salmonella* must also be able to survive the presence of high concentrations of short chain fatty acids (SCFA) generated by fermentation of the indigenous microflora of the GIT. Kwon and co-workers were able to demonstrate that *S*. Typhimurium survived and could express acid tolerance mechanisms when exposed to SCFA at neutral pH and in concentrations similar to those found in the GIT. There appears to be potential differences in responses to SCFA as *S*. Enteritidis can adapt to propionate while *S*. Kentucky differs in its acid sensitivity when compared to other *Salmonella* serotypes. The presence of SCFA may also impact *Salmonella* pathogenesis. A series of gene fusion and tissue culture studies demonstrated that growth phase, type of SCFA and pH level all could not only influence the extent of both attachment to and invasion of HEp2 cells by *S*. Typhimurium but alter expression of specific genes involved in virulence regulation and expression. Further studies demonstrated that some SCFA increased *Salmonella* virulence gene expression whilst other SCFA repressed expression. More in-depth genetic analysis is needed to sort out the interactions that occur metabolically in *Salmonella* versus its virulence responses, particularly since *Salmonella* under certain conditions such as anaerobic growth can actually produce fermentation acids, suggesting that some of the resistance to SCFA may in fact be related to protection from their own end products.

Salmonella that survive the stomach are able to colonize multiple sites in the gastrointestinal tract. Attachment to the intestinal epithelium is facilitated by fimbriae or pili on the surface of the bacterial cell. There are multiple types of *Salmonella* fimbriae that may play a role in colonization by interacting with different surface receptors on the intestinal epithelial cells to facilitate attachment to the small intestine. Although the development and progression of *Salmonella* infections is dependent on various host and serotype factors, those factors influencing this specificity are not well understood. In general, *Salmonella* adhere to the intestinal epithelial cells of the host using fimbriae and trigger cytoskeletal rearrangements of the epithelial cells. The rearrangement leads to membrane ruffling and the subsequent internalization of the *Salmonella* in the host epithelial cells.

Salmonella can migrate from the luminal to basal membrane of the cell where they can interact with the host macrophage, become internalized in a vacuole, and their respective virulence genes activated for survival and replication of the internalized bacterial cell. Invasive *Salmonella* infections are often associated with the ability of the organisms to express virulence associated genes that enable survival in macrophages and dendritic cells following colonization and invasion of intestinal epithelial cells. Many of these *Salmonella* virulence genes are located in different *Salmonella* Pathogenicity Islands (SPI) on the chromosome as distinct genomic clusters which are classified as SPIs 1 through 5 for *S. enterica* serovars although some *Salmonella* such as *S*. Typhi may possess additional SPIs. Among the SPIs, SPI1 and SPI2 are involved in colonization/invasion of the host tissues and macrophage survival, SPI3 encodes for macrophage survival and a specific magnesium transporter along with adhesion associated with intestinal persistence, and SPI4 is believed to encode for proteins that mediate adherence to epithelial cells in a wide range of hosts. SPI1 and SPI2 encode factors that are essential for *Salmonella* survival and multiplication in macrophages and other phagocytic cells and SPI5 encodes for fluid secretion including effector proteins that are secreted by the SPI1 and/or SPI2 Type III secretion systems (T3SS). SPI1 encodes T3SSs which are proteins that allow the transfer of virulence factors

directly into host cells through a needle-like organelle which helps facilitate the invasion and infection. There are several other genes located in the SPIs that function as virulence genes for the *Salmonella* infection.

Following attachment to host cells, *Salmonella* express the SPI1 T3SS that facilitates cellular uptake and invasion of host intestinal tissues. *Salmonella* can reside in the vacuoles and express SPI2-encoded T3SS and SPI2-affiliated effector proteins that aid in avoiding bacterial killing by the normal phagocytic processes used by immune cells for pathogen elimination. The bacterial cells surviving in the macrophages can be transported to other parts of the body and lead to systemic infections.

SPI1 also includes transcriptional regulators such as *hilA* (hyperinvasion locus), *hilC*, *hilD* and others. The *hilA* gene activates the invasion related genes in response to environmental conditions such as pH, osmolarity, oxygen tension and low nutrients. These environmental conditions may play important roles in the interaction between the GI tract microflora and *Salmonella*'s ability to colonize. An example of this is laying hens that are undergoing extensive dietary restrictions used historically by the egg industry for inducing molt to shut down egg production. The resulting extreme nutrient limiting conditions not only alter the GIT microflora and enhance invasiveness of *S*. Enteritidis but can be directly linked with increased expression of *hilA*. However, when molt diets with fermentable substrates are provided, dramatic shifts in GIT populations no longer occur during molt and *S*. Enteritidis' ability to colonize the GI tract and become invasive is curtailed.

Certain T3SS associated proteins appear to play an important role in the development of inflammation and the alteration of ion balances within cells leading to fluid secretion into the intestinal lumen and diarrhea commonly associated with gastroenteritis. This may have significance for *Salmonella* to successfully compete with the GIT microflora. More specifically, it has been suggested that acute inflammation may provide growth advantages to *Salmonella* against the indigenous microflora of the GIT. Once *Salmonella* cross the intestinal epithelium and survive within macrophages, the resulting interactions lead to generation of cytokines and other host proteins associated with the intestinal inflammation response. Using a mouse colitis model, it was demonstrated that inflammation triggered by *S*. Typhimurium led to a shift in the intestinal microbiota and suppression of their growth. It was concluded that inflammation not only did not inhibit *Salmonella* but actually may be necessary for overcoming a fully-developed GIT microflora that normally would serve as a barrier to *Salmonella* colonization. This result was consistent with mouse studies conducted previously where it was concluded that *Salmonella* virulence factors associated with local host GIT mucosal responses were responsible for temporal alterations in the microbial composition. How these GIT microbial shifts occur mechanistically remains elusive, although it has been demonstrated more recently that reactive oxygen produced during inflammation may in fact combine with luminal thiosulphate to form the terminal electron acceptor tetrathionate which *Salmonella* can use for respiration to outgrow anaerobically fermenting GIT microflora.

7.4 Epidemiology

7.4.1 Incidence

According to the CDC, *Salmonella* is the most common bacterial foodborne infection reported, with 17.6 illnesses per 100,000 persons, 2290 hospitalizations and 29 deaths. *Salmonella* infections mainly affect the young, with an annual incidence rate of 121.7/100,000

infants compared to 10.2/100,000 for adult women and 8.8/100,000 for adult men. Therefore, the incidence in the pediatric populations is 10-fold higher than in adults. The overall incidence rate for salmonellosis has not significantly decreased since the beginning of the FoodNet surveillance program in 1996. The most commonly-detected serotypes causing human infections in the US are serotypes *S*. Enteritidis, *S*. Typhimurium, *S*. Newport, *S*. Heidelberg and *S*. Javiana and those most associated with outbreaks are serotypes *S*. Enteritidis, *S*. Typhimurium, *S*. Newport, *S*. Heidelberg, *S*. Montevideo and *S*. Braenderup.

In 2009, *S*. *enterica* serovar *S*. Enteritidis, *S*. Typhimurium, *S*. Newport, *S*. Javiana and *S*. Heidelberg caused approximately 50% of the human illness. *S*. Typhimurium was the most common cause of illness until the mid-1980s in the US when *S*. Enteritidis became more prominent. *S*. Typhimurium is still a major concern in animal meats and dairy products. However, outbreaks by *S*. Enteritidis began rising to similar levels as *S*. Typhimurium in the late 1980s due to an increase in egg-related outbreaks.

S. Heidelberg and *S*. Javiana have also been consistently identified in the past decade as two of the more frequently-occurring serovars in meat animals. In addition to being one of the most commonly-detected isolates in turkey and chickens, *S*. Heidelberg is also a major public health concern because it is not only invasive but also resistant to antimicrobials. In-depth examination of *S*. Heidelberg strains indicates that antimicrobial resistance is not only common among isolates but resistance to multiple antimicrobials occurs by carrying large plasmids which are transferable among genetically diverse strains. *S*. Javiana infections appear to occur primarily in the Southeastern United States and have frequently been associated with certain amphibian species that correspond to these regions. This animal reservoir corresponds with the seasonality of *S*. Javiana-associated outbreaks peaking between July and September. There also appear to be some parallels between outbreaks, periods when rainfall levels were at their highest and the increased frequency of frogs and toads appearing during these times. Illness related to *S*. Kentucky has emerged recently, although it is still not considered a major public health issue. However, *S*. Kentucky has been recently shown to possess virulent ColV plasmids commonly found in avian pathogenic *Escherichia coli* which are believed to impart competitive advantages for colonization in chickens. The hypothesis is that this could also be a public health risk since the appearance of virulent ColV plasmids may serve as evidence for horizontal gene transfer between *S*. Kentucky and enteric bacteria.

7.4.2 Vehicles

Outbreaks continue to be attributable to poultry consumption but outbreaks related to ready-to-eat (RTE) products and fresh produce are becoming increasingly important sources as well. *Salmonella* outbreaks linked to RTE products can be attributed to poor post-processing handling. The major food-safety risk factor associated with RTE products such as vegetables is that consumers eat the products without further cooking. Hence, the USDA-FSIS has updated information on the *Salmonella* compliance guide for small plants producing RTE meats. Recent large-scale outbreaks have been associated with contaminated peanut butter causing 628 illnesses in 47 states (during 2006–2007) and German chocolates resulting in 439 infections of mostly young children in several European countries. Other types of RTE products such as snack foods and frozen potpies have been associated with illnesses due to *Salmonella* contamination. RTE products containing high fat ingredients, such as cheese, chocolate and egg-based products, are more likely to be associated with human infections due to their protective effects on pathogen survival. Fresh produce-associated

Salmonella outbreaks include one traced to tomatoes, 1400 illnesses in 2008 caused by jalapeno and serrano peppers from Mexico, and contaminated tomatoes that caused 510 illnesses (2002) and 72 illnesses (2005).

Human-to-human transmission of *S*. Typhi associated with poor hygienic practices of food workers has led to cases of typhoid fever. One quite well-known case was 'Typhoid Mary' who was a cook in the US and long-term carrier of *S*. Typhi, exposing thousands of people to enteric fever between 1901 and 1914. In 1981, an outbreak caused by *S*. Typhi caused 80 illnesses due to contaminated meats. However, for the most part, infections in the US have been rare due to effective control of public hygiene. There are still a number of cases of *S*. Typhi infections occurring in developing countries due to challenges in hygiene and sanitation practices as well as emergence of antibiotic resistant strains and continued presence of human carriers.

7.4.3 Reservoirs

Salmonella can be easily spread through the natural environment, including water and soil, and transmitted through domestic and wild animals, birds, rodents and humans. *Salmonella* foodborne contamination can occur from raw poultry meat, but contamination can originate from a wide variety of food products and by different *Salmonella* serotypes. Although primarily considered an intestinal organism, *Salmonella* has been found in different environments impacted by fecal contamination. Studies have shown that *Salmonella* can survive for several months in feces and manure and over a year in chicken fluff and dust. Animal feeds and feed ingredients have also been implicated as potential sources of *Salmonella* for food animals at various stages of production. Feed represents a potentially important source since it has been shown that *Salmonella* can survive for months in feeds, despite the relatively low water activity, and does not seem to depend on protein composition or conventional versus organic feed ingredients. Recent studies have also indicated that neither plant-based nor meat and bone supplements in poultry feeds influence *S*. Heidelberg environmental contamination from experimentally infected birds. The level of *Salmonella* contamination in feeds may be underestimated as well. It has been demonstrated that the presence of antimicrobials in animal feeds could influence recovery of *Salmonella* in cultural media leading to underestimates of the level of contamination. Feed mills remain a problematic area for feed contamination as well because cleaning and chemical treatment of commercial feed mill equipment and feed fail to remove endemic *Salmonella* serovars that could be recovered from finished feed products and corresponding poultry flocks. Part of the difficulty in removing *Salmonella* from these environments is that they may be able to persist via formation of biofilms. It has also been realized that, although thermal processes such as pelleting should eliminate *Salmonella* in the feed mill, recontamination after feed processing can still occur.

Routes of environmental transmission can vary as well. Sources of *Salmonella* for food production and processing are commonly associated with the local environment but the original contamination of these sites is likely to have been introduced by some sort of vector. Agricultural buildings may become contaminated with *Salmonella* from livestock shedding, exposure to contaminated feed, water, and animal wastes and through rodents, wild animals, birds and insects which are known potential carriers of *Salmonella*. Other routes include the movement of pathogens via aerosols which can occur not only among animals in housing but from off – site locales as well. Contaminated agricultural environments can lead to transmission of future cohorts of animals that enter the agricultural environment, perpetuating

contamination problems. These *Salmonella*-infected animals can lead to contaminated meat and poultry during processing, which serves as potential vehicles of human salmonellosis. The relationship between the presence of *Salmonella* in the live animal production environment and carcass contamination can be fairly complex. For example, it has been demonstrated that *Salmonella*-contaminated poultry carcasses could be associated with the grow-out environment, particularly the poultry house litter at the time of harvest and prior to flock placement. For fresh vegetables, environmental exposure at pre-harvest can be critical as well. For example, if irrigation water is contaminated with animal feces it can lead to the contamination of fresh produce. Proper handling during all phases of produce production and retail has also been shown to be critical to minimize *Salmonella* contamination.

7.5 Detection of *Salmonella*

7.5.1 Cultural methods

Given the disease-causing potential of *S. enterica* and subsequent impact on commerce, there is a vested interest among the industry and public health officials for rapid and accurate detection of *Salmonella* from sampled sources. Accurate detection that reflects true contamination levels requires representative and uniform sampling methodologies has been summarized by others in recent reviews, and generally involves consideration of a multitude of factors including appropriate sample preparation, transportation and storage, choice and frequency of sampling sites and quality experimental design. For some food products such as liquid eggs, sampling protocols are fairly standardized but others such as animal feeds, animals and animal housing sampling approaches still lack uniformity.

Cultural-based methodology for *Salmonella* usually has four basic steps outlined previously: (1) pre-enrichment via a nonselective medium to ensure recovery and growth of injured bacterial cells; (2) enrichment via a selective medium that favors exclusive growth of *Salmonella* versus non-*Salmonella*; (3) isolation of individual colonies on selective agar; and (4) confirmation. Identification and confirmation can be achieved in a variety of ways, including biochemical tests, immunological typing and molecular-based methods. Although the basic procedural steps are fairly intuitive, there is a wide range of media formulation variations, modifications in incubation times and differences in incubation temperatures which have been extensively reviewed. Typically, the recovery of *Salmonella* involves suspension of a food sample in a pre-enrichment broth such as buffered peptone water (BPW) or trypticase soy broth (TSB) at 37 °C for 18 hours to resuscitate stressed or damaged cells. An additional (modified) pre-enrichment step using Rappaport-Vassiladis soy broth (RVS) or Mueller-Kauffman tetrathionate-novobiocin (MKTTn) broths may be necessary in the case of certain food products that contain inhibitory compounds. This may require an additional 24-hour incubation at 41.5 °C (for RVS) or 37 °C (for MKTTn). The enriched suspensions are plated on at least two chromogenic agar plates such as brilliant green, Hektoen enteric agar, xylose lysine desoxycholate agar, xylose lysine tergitol agar or bismuth sulfite agar, which are selective and differential for the growth of *Salmonella* at 37 °C for 24 hours. Typically, *Salmonella* colonies are then subcultured on a specific medium (triple sugar iron agar and lysine iron agar), and finally confirmed by serological testing. A detailed description of the U.S. Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM) procedure for identifying *Salmonella* in various food products is available on the FDA website (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/Bacteriological

AnalyticalManualBAM/ucm070149.htm). These culturing methods have been optimized and used in different combinations to detect low levels of *Salmonella* from the sampled sources. The International Organization of Standards has also published a method (ISO 6579:2002) for detection of *Salmonella* in food and feed: (http://www.iso.org/iso/catalogue_detail.htm?csnumber=29315).

7.5.2 Rapid methods

Depending on the food matrix and logistics of testing, it can take anywhere from four to seven days or longer for detection and confirmation of *Salmonella* using traditional methods based on growth of isolates followed by identification and confirmation tests. Because of the length of time it takes culture methods to detect and confirm the presence of *Salmonella* in a sampled product, there is a greater demand for alternate rapid screening methods and kits for detection of *Salmonella* in food, feed and environmental samples (http://safefood.wsu.edu/wsud2.html; http://www.foodhaccp.com/d2.html). Rapid methods have been broadly grouped previously into four types of technologies: (1) miniaturized identification systems; (2) assay modifications and specialized media; (3) antibody-based methods; and (4) DNA-based approaches. *Salmonella* rapid methods are often based on enhanced culturing techniques combined with nonculture-based detection methods. These include: nucleic acid-based detection (PCR, real-time PCR and their variations); immunological detection (ELISA-based assays incorporating fluorescent detection; lateral flow immunodiffusion, bioluminescent enzyme separation and enzyme-linked immunomagnetic chemiluminescensce), flow cytometry, biosensors, and microarrays. Some methods have been automated to screen a large number of test samples in a relatively short amount of time. Almost all of these rapid methods require an enrichment step to increase the number of bacteria in the sample preparations. The enriched sample is subsequently used for testing for the presence of *Salmonella* employing the methods (or kits based on the methods) described previously instead of traditional culture methods. There are approximately 30 commercially-available methods for *Salmonella* detection that have been validated by the AOAC International (http://www.aoac.org/testkits/testedmethods.html).

Traditionally, nucleic acid and PCR-based detection methods in particular have offered the advantage of increased sensitivity and more rapid response time. Since then there has been extensive development of nucleic acid-based assays. It has been suggested previously that the advent of the automated DNA sequencer and Basic Local Alignment Search Tool (BLAST) software accelerated this development due to the ability to compare large sequence data sets. Consequently, combining multiple primers (multiplex) for simultaneous detection of several gene targets with *Salmonella* high throughput and real time PCR assay approaches have been developed to not only differentiate *Salmonella* from non-*Salmonella* isolates but distinguish among individual *Salmonella* serovars. Application of reverse transcriptase PCR for detection of RNA avoids the false positives associated with detectable DNA remaining after bacterial death and thus allows for quantifying viable *Salmonella* cells in environmental samples and/or assessment of individual gene responses under these environmental conditions. When using these quantitative PCR approaches it is critical to overcome sample matrix interference along with routine confirmation and validation practices not just for presence of the organism but for accurate gene expression assessment. As the need for multiple gene targets in *Salmonella* detection systems grows, DNA microarrays – either as amplicon or oligonucleotide based-arrays – are being employed more frequently for comparative genetic analysis, gene detection and quantification.

7.6 Prevention and control

7.6.1 Pre-harvest

Salmonella can not only be ubiquitously found in the environment and in a wide range of food products but can grow/survive in foods and bacteriological media within a wide temperature range (2–54 °C), survive low (4.5) and high pH (9.5), but not water activities below 0.93. Hence, prevention and control of *Salmonella* is warranted in the entire chain of the farm-to-the-fork continuum. Pre-harvest control and intervention measures have been broadly categorized previously as those targeted towards environmental reduction and those that decrease and/or eliminate pathogen loads on animals and plants. Environmental interventions for *Salmonella*, including a myriad of management practices, facility sanitation protocols, feed and water treatments, and egg disinfectants, have been extensively reviewed elsewhere.

S. Typhimurium, *S*. Enteritidis and a number of other serotypes can asymptomatically colonize the intestinal tract of adult farm animals; thus it can be difficult to identify carriers. This is important because, even when the number of *Salmonella*-positive birds arriving in a processing plant is low, there are still numerous potential sites within the processing plant where cross-contamination can occur that can result in a greater incidence of contaminated carcasses leaving the plant. Consequently, control measures at the processing plant still represent an effective means of reducing *Salmonella* contamination in the final products. However, more integrated approaches that also include reduction of *Salmonella* in live animals are now being applied. Reducing and/or eliminating *Salmonella* in animals and plants on farms has been pursued for a number of years with varying degrees of success. Generally, interventions are designed to either prevent initial colonization by the pathogen or eliminate already-established pathogens. Preventing and/or eliminating *Salmonella* GIT colonization in farm animals involves either administration of some biological agent which is active specifically against *Salmonella* or indirectly via an additive that generates a hostile host GIT environment.

Preventative biological agents include dietary manipulations, prebiotics, probiotics, and immunological agents. Most of these have been explored as single agents or various combinations for creating multiple barriers to GIT *Salmonella* establishment. Many of these involve enhancing the indigenous GIT microflora's ability to serve as a barrier against incoming *Salmonella*. Some are as simple as changing the diet in such a manner to either retain or help establish protective GIT microflora. For example, high fiber sources such as alfalfa added to diets of laying hens undergoing stress during molt induction have been shown to retain the fermentation activity and decrease *S*. Enteritidis colonization. More specific dietary amendments include prebiotics which are nondigestible compounds such as fructooligosaccharides (FOS) that can be specifically utilized by GIT bacteria known to be beneficial to the host. Prebiotics are also known to be effective for controlling pathogens; for example, supplementation of alfalfa diets with FOS has been shown to retain GIT microbial fermentation and limit *S*. Enteritidis infection in laying hens during molt induction.

Probiotics are mixtures of viable microorganisms that, when administered to an animal, become established in some fashion to elicit detectable benefits to the host. Although probiotics have been shown to be effective in preventing *Salmonella* colonization, particularly in poultry, consistency continues to be problematic suggesting that much remains to be determined on mechanisms. For example, in poultry it appears that complex microbial

mixtures of probiotic cultures retaining the integrity of the original inoculated probiotic consortium are required for limiting *Salmonella* in the bird. As molecular tools continue to advance, the ability to characterize establishment and interactions of microbial populations in different regions of the GIT should help to improve the consistency of these cultures to prevent *Salmonella* establishment. Immunological approaches for preventing *Salmonella* include passive immunization by oral antibody administration or triggering the immune system via vaccination. Specific egg yolk antibodies can be generated against the primary foodborne *Salmonella* serotypes but appear to undergo denaturation and degradation during passage through the GIT, rendering them ineffective when included in broiler diets. *Salmonella* vaccination programs have been generally more successful, although vaccine serovar specificity limits effectiveness for broad spectrum *Salmonella* protection.

The options for eliminating already-established *Salmonella* in food animal GIT systems is much more limited. Traditionally, antibiotic therapy was considered a possibility for young chicks where *Salmonella* serotypes could produce mortalities but concerns over development of resistance to antibiotics of clinical importance has discouraged this practice. A wide range of antimicrobial proteins, organic acids, botanicals and plant derivatives have been examined for *in vivo* application but most are either fairly broad spectrum (and therefore do not necessarily target pathogens such as *Salmonella* exclusively) or are not easily scaled up for commercialization. *Salmonella*-specific bacteriophage offer a very specific biological approach for directly attacking resident *Salmonella* in the GIT but *in vivo* results have been inconclusive due to the complexity of the GIT system, the ability of *Salmonella* to become phage resistant over time and, in many cases, being too serotype/strain specific.

7.6.2 Post-harvest

Exposure of food products during processing to *Salmonella* can, not only be due to incoming contamination, but contact with resident populations within the plant through pathways such as chilling and washing with contaminated water during vegetable processing. Consequently, control measures need to not only decrease contamination of the food product but minimize cross-contamination from other points of origin during processing. Pathogen control in vegetable processing can be particularly challenging due to the potential for internalization into the plant tissue when warm produce is submerged into cooler water. Although a variety of sanitizers have been used for fresh vegetables, chlorine remains the most common post-harvest treatment for decontamination. Strategies for development of more specific and effective biological control measures such as *Salmonella* bacteriophage, microflora antagonistic to *Salmonella* and bacteriocins have been explored.

Contamination of *Salmonella* can occur in the poultry and meat production facilities, during transportation from the farms to the processing plants, and cross-contamination in the processing plants. Employment of the Pathogen Reduction/Hazard Analysis of Critical Control Point program since the mid-1990s by the USDA Food Safety and Inspection Service (USDA-FSIS) has contributed to the decrease in frequency of *Salmonella* carcass contamination. Several physical and chemical treatments, such as air scrubbing, heat, irradiation, ozone, chlorine, organic acids (lactic, acetic, citric, malic, propionic acids among others) and chemicals (phosphates, quaternary ammonium salts, glutaraldehyde, sorbates, and Tween 80 among others) have been employed and/or examined for decontamination of *Salmonella* and other foodborne pathogens on fresh meat and poultry products. More

specific biological agents such as bacteriophage and bacteriocins have also been employed for limiting pathogens, including *Salmonella*, on carcasses.

Concerns have risen over the continued use of single intervention methods and the emergence of pathogen resistance, and more interest has been directed toward employment of multiple interventions also referred to as multiple hurdles. However, even when multiple hurdles are administered there is still concern that cross-protection can occur whereby sublethal doses of one antimicrobial triggers resistance to another seemingly unrelated antimicrobial. For example, *S*.Typhimurium adapted to SCFA at neutral pH was more resistant to extreme conditions such as high pH, high osmolarity, and reactive oxygen. Such results suggest that triggering of shared genes encoding resistance to multiple interventions occurs in *Salmonella* and transcriptome analysis of *Salmonella* responses to predict which anti-*Salmonella* compounds or treatments could be used to identify the least amount of overlap occurring among genes. The utility of this approach has been demonstrated by identifying the physiological and genetic responses to synergistic inhibition of *Salmonella* synergism of thermal treatment in combination with organic acids.

Regardless of the intervention strategies that are used in pre- and post-harvest control of *Salmonella*, it is imperative that the consumer be aware of the risks involved in consumption of uncooked or undercooked foods. In order to prevent salmonellosis, the CDC advises that: consumers should avoid eating raw or undercooked eggs, poultry or meat; cook foods to the recommended temperatures; avoid cross-contamination of foods by separating uncooked meats from cooked and/or RTE packaged foods; and promptly refrigerating leftover foods (http://www.cdc.gov/salmonella/general/prevention.html). The USDA-FSIS recommends cooking all fish, beef, pork, lamb and veal steaks, chops and roasts to an internal temperature of 62 °C (145 °F) and poultry to 74 °C (165 °F). (http://www.fsis.usda.gov/factsheets/salmonella_questions_&_answers/). Consumers should also be aware of personal hygiene, such as washing hands and using sanitary conditions in the kitchen while preparing foods. A combination of pre- and post-harvest intervention strategies can minimize the risk of human exposure to *Salmonella*, thereby reducing the medical and productivity costs associated with salmonellosis.

7.7 Conclusions

Foodborne salmonellosis continues to be a public health issue for the food industry despite the extensive efforts made to limit and control dissemination during production of food. In addition, new food safety challenges face the food industry including the rise of organic and natural food products which are more limited in the range of antimicrobials that can be used for control during pre- and post-harvest production. What makes *Salmonella* so difficult to control in all of these production systems is its ability to not only survive in a multitude of environments, but exist in a non-pathogenic physiological state leading to asymptomatic carrier hosts. Consequently, tracking and monitoring *Salmonella* remains difficult and outbreaks can still occur without warning. However, advances in genetic techniques such as high throughput sequencing have made it much easier to differentiate specific strains and trace back to the point of origin. Likewise, improvements in genetic information analyses now make it possible to delineate the more intricate details of pathogenesis mechanisms and achieve a better understanding of how pathogens interact with specific host cells. As these results become more clear, applications such as the development of better *Salmonella* vaccines and antimicrobials designed to more specifically target *Salmonella* can become possible.

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8 *Shigella* species

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8.1 Introduction

Shigella species remain a formidable foodborne pathogen worldwide. In 1999, 164 to 200 million people in developed and developing countries had diarrheal illnesses caused by shigellae with an estimated number of 1.1 million deaths. Recently, the Centers for Disease Control and Prevention (CDC) reported that in Asia, there are nearly 125 million cases of shigellosis with approximately 14,000 deaths, much lower than the original estimate. The decrease in numbers may be attributed to health and nutrition-related interventions.

This pathogen was first identified as a separate etiological agent of dysentery from amebic dysentery by Kiyoshi Shiga in 1898. Three additional serologically and pathogenically similar species were isolated subsequently, and in 1950, a new genus, *Shigella*, was adopted by the International Association of Microbiologists. The four species that comprise the genus *Shigella* are based on serological and biochemical characteristics as well as clinical presentations: *Shigella dysenteriae* (Group A; 15 serotypes), *Shigella flexneri* (Group B; 14 serotypes and subserotypes), *Shigella boydii* (Group C; 19 serotypes) and *Shigella sonnei* (1 serotype). Originally designated as *Bacillus dysenteriae*, the prototypical *Shigella* species was renamed to *Shigella dysenterieae*. This pathogen has been associated with epidemic outbreaks in areas of the developing world encompassing Africa, Asia and Latin America. *S. dysenteriae*, particularly type 1, causes the most severe form of bacillary dysentery, due to in part that only this *Shigella* serotype carries the genetic information for the shiga toxin (see Fig. 8.1).

The traditional classification of *Shigella* as its own separate genus has been challenged during the past decade, particularly with the advent of whole genome sequencing. Comparative genome sequences and sequences from specific conserved genes indicate that the four *Shigella* species evolved from different ancestral lineages of *Escherichia coli*. In these studies, it has been postulated that the genome of *Shigella* has undergone two major events: the acquisition of a large (180–220 kilobase pairs) virulence plasmid and the loss of different genetic functions due either to deletions of segments of DNA from the genome or base pair changes mutating the gene to a pseudogene.

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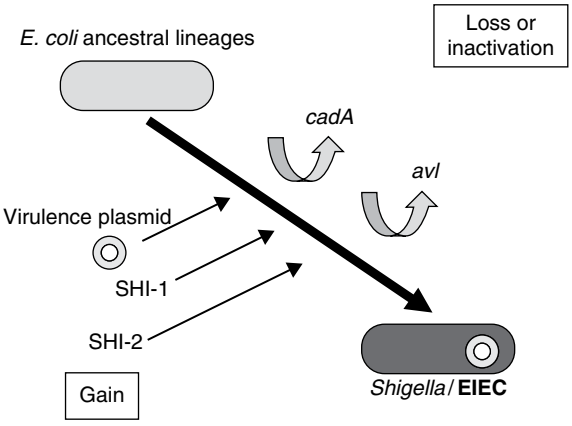

Figure 8.1 Hypothetical pathway of the evolution of *Shigella* species and EIEC from ancestral *E. coli* lineages. Acquistion of the virulence plasmid as well as several pathogenic islands are noted in addition to the loss of specific genetic loci that appear to enhance pathogenesis of *Shigella* species.

The changes in genomes could reflect the adaptation of *Shigella* from its original environmental habitats to its present niche, humans. In some instances, loss of gene function overcomes the inhibitory activity of a virulence gene product. Such is the case with *cadA*, in which cadavidine adversely affects the activity of a *Shigella* enterotoxin. Significant changes in the *Shigella* genome can also arise from bacteriophage-mediated gene acquisition and by insertion sequences which can not only lead to loss of gene function but, in some cases, result in chromosomal rearrangement.

Enteroinvasive *E. coli* (EIEC) carries the same virulence genes as does *Shigella* and has characteristics unlike *E. coli* K-12; EIEC is unable to utilize lactose (some EIEC may be late lactose fermenters), is lysine decarboxylase negative, and non-motile, which are characteristics more aligned with *Shigella*. Furthermore, EIEC share O-antigenic epitopes with some *Shigella* species and cause the same disease, bacillary dysentery. As for its evolutionary lineage, it appears that EIEC arose independently of each *Shigella* species, evolving from a different *E. coli* ancestor. These pathogens may represent an intermediate stage of convergent evolution of these *E. coli* lines that would lead to similar virulence capability of the other *Shigella* species.

As with other enteric pathogens, *Shigella* species have a broad arsenal of genetic determinants that affect their pathogenesis and,, subsequently, the host immune response. Elucidating the pathogenesis of *Shigella* at the molecular level began in the early1960s with the identification and isolation of a virulence plasmid in *S. flexneri*. The pathogenic islands associated with the ability of *Shigella* species to invade human colonic epithelial cells were eventually delineated at the genetic level. Thirty-one genes comprise two divergent operons that encode for *Shigella* effectors and the Type Three Secretory System, the latter acting as a conduit to shuttle the internally-synthesized bacterial effectors into the host milieu which are primarily responsible for the invasion of the bacterial cells into the host. Briefly, *Shigella* species invade the epithelial cells after initial uptake by M cells, part of the lymphoid system. The pathogens are then transcytosed through the M cell until they encounter macrophages and dendritic cells. *Shigella* species are able to lyse macrophages, stimulate a host inflammatory response, and are released at the basolateral membrane of the colonic epithelial layer. At this point, shigellae invade adjacent epithelial cells, multiply intracellularly and move to neighboring cells intracellularly via actin polymerization, using this process as a means of mobility.

8.2 Nature of illness in humans

Shigellosis, or bacillary dysentery, the disease caused by *Shigella*, ranges from mild watery diarrhea to a severe form, dysentery. Most patients exhibit abdominal pain in addition to diarrhea, and others symptoms include cramps, tenesmus, malaise, and fever. In the severe dysenteric stage, approximately 40% of infected individuals will have bloody diarrhea with mucoid cells. *Shigella sonnei*, the most prevalent shigellae isolated in developed countries, produces the mildest form of shigellosis. The most severe disease, dysentery, can be caused by all four species but *S. dysenteriae*, and only type 1, is the etiological agent for the sequellae, hemolytic uremic syndrome (HUS). This syndrome is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. *S. dysenteriae* is typically found in developing countries and associated with the highest number of deaths. Only *S. dysenteriae* type 1 expresses a cytotoxic Shiga toxin (Stx) similar to Shiga toxin-producing pathogenic *E. coli* (STEC), including enterohaemorrhagic *E. coli* (EHEC) 0157:H7. Disease caused by *S. flexneri* and *S. boydii* can be either mild or severe. Most cases of shigellosis are self-limited and usually not fatal except in the elderly, immunocompromised and undernourished children, particularly under the age of 4. In healthy individuals, if left untreated, clinical illness usually persists for one to two weeks (although it may be as long as a month) and the patient recovers. In some instances, there can be protracted asymptomatic shedding of the pathogen.

Other complications of shigellosis in addition to HUS include dehydration, toxic megacolon (rare), and reactive arthritis with joint pain and inflammation. In addition, seizures have been reported in 5.4% of shigellosis cases involving children. Another chronic sequellae occurring in 2% of the population infected with *S. flexneri* is the subsequent development of reactive arthritis, particularly in persons who carry the histocompatability marker HLA-B27. Symptoms include urethritis, conjunctivitis, and arthritis with a recently ascribed fourth characteristic as ulcerations of the skin and mouth. Patients with reactive arthritis can have symptoms lasting from months to years (http://www.cdc.gov/nczved/divisions/dfbmd/diseases/shigellosis/).

The incubation time ranges from eight to 92 hours and illness usually appears in less than three days. An important facet of *Shigella* pathogenesis is the extremely low infective dose (ID_{50}): 10 cells of *S. dysenteriae*, 140 cells for *S. flexneri* to less than 500 cells for *S. sonnei*. Based on studies in which volunteers became ill when oral doses as low as 200 cells were given, this number is considered the average ID_{50} for *Shigella* spp.

Matrix complexity can be one factor that may influence the data for ID_{50}. Gastric juices at a pH of 2 can be an effective part of the host's innate defense and has been shown to effectively kill microbial pathogens in 15 minutes. However, the influence on the matrix from water to very complex foods which harbor *Shigella* may have a significant effect on the survival of this pathogen in the stomach and indirectly impact the infectious dose. As an example, when 180 wild-type *S. flexneri* 2a were administered with bicarbonate buffer instead of milk, an increase in the shigellosis rate of 43% was noted indicating that resistance to gastric juice partially accounts for the high bacterial infectivity.

Certain at-risk populations, including the very young (below the age of 5 years), the elderly, or persons with decreased immune function, are more likely to be more susceptible to infection. Due to the low infective dose of *Shigella*, the high communicability of bacillary dysentery underscores the pathogen's great explosive potential for person-to-person spread as indicated in foodborne and waterborne outbreaks. The person-to-person transmission is common in several situations, particularly in day-care settings where toddlers commonly

practice poor personal hygiene, in institutions such as nursing homes and prisons, or in crowded, confined populations.

At the host cellular level, shigellosis is caused when *Shigella* species attach to and penetrate colonic epithelial cells of the intestinal mucosa. After invasion, they multiply intracellularly and spread intercellularly to contiguous epitheleal cells, but penetrate only as far as the lamina propria resulting in tissue destruction. As the infection progresses, dead cells of the mucosal surface slough off, resulting in the presence of blood, pus, and mucus in the stools. The dysentery stage of disease correlates with extensive bacterial colonization of the colonic mucosa. Watery diarrhea may or may not precede dysentery. At this stage of the disease, transient multiplication of bacteria occurs as the pathogen passes through the small bowel, probably leading to less effective reabsorption of jejunal secretions in the colon due to the destruction of the colonic mucosa. Foci of individually infected cells produce microabscesses that coalesce, forming large abscesses and mucosal ulcerations.

8.3 Characteristics of agent

Shigella species are members of the family *Enterobacteriaceae* and, as indicated above, may be considered a clone to the genus *Escherichia*. Shigellae are Gram-negative, nonmotile, non-sporulating, facultative anaerobic, rod-shaped bacteria. Serological grouping is based solely on the O-antigen present in the lipopolysaccharide since the members of this genus lack flagella (H antigens) and capsules (K antigens). They are also genetically closely related to the members of the genus *Salmonella*. Biochemical characteristics that distinguish shigellae from other enterics include an inability to ferment acetate, mucate and lactose (although some strains of *S. sonnei* may ferment mucate or lactose upon prolonged incubation), the lack of citric acid, inositol, salicin or adonitol utilization as a sole carbon source, and the inability to synthesize lysine decarboxylase. In addition, *Shigella* species require nicotinic acid for growth in a minimal synthetic medium, are oxidase negative, and none produce H_2S nor gas from glucose except for *S. flexneri* 6 and *S. boydii* 14. *Shigella dysenteriae* strains have the additional property of not being able to ferment mannitol. *Shigella dysenteriae* type 1 expresses an active β-galactosidase but does not produce catalase, an extremely rare feature among *Enterobacteriaceae*. Although *S. sonnei* strains have only one serotype, they can be subdivided into biovars on the basis of their ability to hydrolyze O-nitrophenyl β-D-galactyl pyranoside [ONPG], xylose and rhamnose. *S. sonnei* isolates, on solid agar medium, commonly undergo a transition from a smooth colony to a rough colony form. This phenotype is due to the relative instability of a plasmid that harbors the LPS encoding genes in *S. sonnei* during bacterial growth. Plasmid-encoded genes for synthesis of the LPS O-side chain are also present in *S. dysenteriae* type 1.

Shigella and EIEC are considered the only obligate pathogenic strains of *E. coli*; their propagation and survival are solely dependent upon their extremely narrow host range. Humans may be considered the only natural host of these pathogens. Although higher primates can develop shigellosis, this is most likely due to their close proximity to infected humans, and there is no evidence that the disease occurs naturally in the wild in nonhuman primates. *Shigella* are usually excreted for a few weeks after the illness, with more than 10% of infected individuals, notably children, excreting the pathogen for longer than 10 weeks. The role of asymptomatic carriers of *Shigella* in the maintenance of this pathogen in the world's population has been an intriguing thought. In highly endemic areas, up to 50% of all people with *Shigella* infections may have no apparent symptoms or signs and, as such, these

asymptomatic carriers of *Shigella* may exacerbate the maintenance and spread of this pathogen in developing countries. Two studies, one in Bangladesh and the other in Mexico, showed that *Shigella* were isolated from stool samples collected from asymptomatic children under the age of five years.

8.4 Epidemiology

According to the WHO website, http://whqlibdoc.who.int/publications/2005/9241592330.pdf, *Shigella* species cause approximately 80 million episodes of bloody diarrhea and 700,000 deaths each year throughout the world, with 99% of those occurring in developing countries (80% in Asia). These figures differ from the CDC report from 2010 but, overall, the number of people infected with shigellosis remains unquestionably high. Disproportionately, nearly 70% of the cases and approximately 60% of the deaths associated with this pathogen affect children younger than 5 years old. Although most cases of shigellosis are due to person-to-person transmission, outbreaks commonly result from food and/or water contamination, commonly in areas that are crowded, and with inadequate supply of potable water most likely due to poor sanitation.

Shigellae are commonly transmitted by the five Fs: the fecal-oral route, feces, direct person to-person contact (fingers), food, flies, and inanimate objects (fomites). More than two-thirds of all episodes of shigellosis are seen in children between one and five years old who tend to explore their environment with their mouths. Ingestion of contaminated water is also recognized as a mode of transmission for shigellosis. Nevertheless, *S. dysenteriae* sp. do not survive in water for more than two to three days. *Shigella flexneri* and *S. sonnei* can survive from 6–47 days and 35–39 days, respectively. Sustained endemic transmission and epidemics of bacillary dysentery are facilitated when crowding and poor sanitation conditions create an environment for direct fecal–oral contamination, such as day care centers, custodial institutions, mental hospitals, and nursing homes, or mass displacement and gathering such as refugee camps in time of war or political turmoil. Additionally, human immunodeficiency virus (HIV)-positive individuals may present more severe and persistent forms of shigellosis. Secondary attack rates following exposure to the primary case can be as high as 40% among household contacts.

Episodes of shigellosis appear to follow seasonal variations in certain countries. In arid countries, such as Egypt, transmission peaks in the hot dry season. This is primarily due to the consumption of contaminated water and decreased personal hygiene in times of water shortage. Conversely, the peak episodes in China and Thailand occur in the rainy season as a result of water-washed related transmission during heavy rains. These reflect the association of shigellosis with insanitary conditions that foster fecal transmission. In developed countries, the highest incidence of shigellosis generally occurs during the warmer months of the year when consumption of raw foods, fresh fruits and vegetables along with recreational facilities are the highest.

The distribution of *Shigella* spp. varies in different parts of the world. For example, *S. boydii* is not frequently encountered outside the Indian subcontinent where it was first identified. Ten years ago, the proportion of *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* was estimated to be respectively 60% (predominantly serotype 2a), 15%, 6% and 6% (30% of *S. dysenteriae* cases were type 1) in developing countries; and 16% (predominantly serotype 3a), 77%, 2% and 1% in industrialized countries, with nearly half of the cases reported among travelers. Travelers returning from India and neighboring countries, and East, West

or North Africa were at a higher risk for shigellosis. Importantly, the distribution of *Shigella* species also seems to evolve with time and with the economy of a region. This is somewhat borne out with the observation that *S. dysenteriae* dominated in the early parts of the 20th century but was replaced by *S. flexneri* in the 1930s and 1940s in the absence of epidemics, and more recently by *S. sonnei* in developed countries. Interestingly, when the economy of a country improves, *S. sonnei* becomes responsible for the majority of shigellosis outbreaks, as seen since 2000 in Thailand and Iran. Furthermore, in one study in six Asian countries that looked for a common link of diarrheal disease caused by *Shigella* with socio-economic factors, it was found that these diseases were associated with a particular *Shigella* spp., i.e., in less resource-rich countries the predominant specie was *S. flexneri*. In addition, it was observed that the variation of serotype (as also noted above in developing and industrialized countries) differed temporally and with regards to the geographic location, implying that the change in distribution may affect the effectiveness of any potential vaccine to use in areas of the world that have such shifts in serotypes.

It is not expected that *Shigella* would form biofilms to protect them from damaging environmental factors. They lack certain attributes necessary for biofilm formation including flagella, fimbriae and curli appendages and do not produce poly-β-1,6-N-acetyl-glucosamine or colonic acid polysaccharides (12). However, this may not preclude their incorporation into pre-established mixed-species biofilm communities.

8.5 Detection of organism

Analyses of food samples encompass different hurdles of consideration as compared to clinical specimens which reflect a much smaller number of matrices than foods. With a disparity of food matrices confronting an analytical laboratory, the primary means to detect *Shigella* species in foods is by isolating the pathogen. Currently, this is an arduous task since there are no enrichment medium or media that exist to selectively grow *Shigella* from food samples. Alternative means, such as molecular biology, immunological, or mass spectrophotometric-based methods, also face significant obstacles as a suitable isolation/detection protocol. Overall, the ability of laboratories to isolate *Shigella* from foods, particularly when this pathogen competes with the indigenous flora present in foods, is a formidable challenge.

Conventional microbiological methods entail the implementation of selective enrichment broths as well as selective and differential agar media. Although *Shigella* species do not appear to have any fastidious requirement for growth, they may be easily outcompeted by other indigenous bacterial populations found in foods. Most methods entail growth in broth medium followed by plating on selective agars. In *The Bacteriological Analytical Manual* (www.cfsan.fda.gov/~ebam/bam-6.html) one method is described to isolate shigellae from foods. Health Canada (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index_e.html) uses a very similar scheme with slight modifications. A protocol from the International Organization of Standards (ISO 21567:2004 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Shigella* spp.) provides an alternate method.

A range of selective agar media are recommended to plate cultures after growth overnight in broth. 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, is used to screen for lactose-negative colonies, as most *Shigella* are lactose negative. Eosin methylene blue (EMB) and Tergitol-7 agar are alternative low selectivity agars. Desoxycholate

and xylose-lysine-desoxycholate (XLD) agars are intermediate selective media and are preferred media to isolate *Shigella* spp. Although most *Shigella* spp. do not ferment xylose, some species, e.g., *S. boydii*, have variable reactions and may be missed. Highly selective media include *Salmonella-Shigella* and Hektoen agars. Some *Shigella* spp., such as *S. dysenteriae* type I, are unable to grow on the highly selective *Salmonella-Shigella* medium. Alternate agars have recently been introduced commercially. Chromogenic agars are newly developed plating media that can enhance the recovery of *Shigella* species from foods by selecting against indigenous microbial populations and concurrently provide colony color differentiation based on the chromogens used under different physiological parameters, e.g., metabolism, change in pH.

The polymerase chain reaction (PCR) techniques may be considered a more sensitive and specific technique than conventional culture techniques. Several single, nested, and multiplex PCR assays have been designed to routinely amplify marker(s) present in single or multiple copies (i.e., *ipaH*) in *Shigella* and EIEC bacterial genomes. However, they generally do not differentiate between the four *Shigella* spp., EIEC and/or serotypes. Targeting the O-serotype specific gene(s) using PCR or microarrays can bring more discriminatory power to the tests. Farfan et al. recently identified two markers that specifically differentiate between *S. flexneri*, *S. sonnei* and other diarrhoeagenic *E. coli*. More recent technological advances combine PCR with other commercially-available technology, such as the Luminex Assay (Bio-Rad), may have the ability to not only detect the presence of *Shigella* species in foods but also identify the serotype.

Immunological-based detection kits have been developed and used primarily in the clinical laboratory. These methods, particularly in automated instruments/systems, may have a use in food analytical laboratories as rapid and reproducible identification means, specifically if typical colonies are found on agar media used in the isolation of *Shigella*. If used directly on food samples, the number of targets, e.g., *Shigella*-specific lipopolysaccharides, could be well below the detection limit of the immunological assay. In some cases, certain serotypes of *Shigella* may not be able to be specifically identified since some O-antigens may cross-react with EIEC. Although immunomagnetic separation techniques have been reported in the literature, their application in food analytical laboratories has not been widely documented.

Newer technology includes the development of biosensors, capturing fluorescence generated via a sandwich immunoassay, and different forms of mass spectrometry are more than likely in their infancy at this time. For example, MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, can be used as a rapid identification scheme targeting specific pathogens. This technology is based on the comparison of protein profiles of the bacterium at the time of analysis. However, different growth conditions may produce different protein profiles via mass spectrometry, and therefore stringent adherent to any growth protocol must be followed. Other factors include the presence of exogenous substances that may hinder specific identification of the bacterial population present.

8.6 Physical methods for destruction

Since *Shigella* may not survive well outside its host, humans, its introduction into foods can only occur by a infected food handler with poor personal hygiene or during processing, which includes irrigation, harvesting and hand packaging. In recent food-related outbreaks that occurred around the world, a common source is a raw vegetable. Although *Shigella*

species are readily eliminated by pasteurization and other means, e.g., irradiation, implementing these technologies on raw produce is either impractical or not readily available in a large-scale operation.

As with most microbial pathogens found in foods, factors that affect growth and survival of *Shigella* spp. in foods include not only pH, a_w, salt concentration and temperature, but also the chemical components of the food matrix and the indigenous microbial flora. In general, *Shigella* species are inactivated at 63 °C for 5 min, are killed by pasteurization and its survival and growth dependent upon several intrinsic and extrinsic factors. Its growth range is from 7–46 °C with an optimum temperature at 37 °C. In regard to pH, the range is from 5–8, yet the pathogen does not survive well in acidic foods. *Shigella* species survive in frozen and chilled foods and dies slowly in foods with reduced a_w.

In some foods, the addition of nitrites, salts or organic acids can have an effect on the growth or survival of shigellae. However, these additional compounds may not be applicable to raw foods, such as produce, or processed products, which may affect taste or other food qualities. Overall, *Shigella* are introduced into foods usually after handling and any treatment prior to processing would be rendered ineffective at this point. For the consumer at home, the message that vegetables and fruits should be properly washed should be heeded.

8.7 Prevention and control

The importance of developing an efficacious vaccine against *Shigella* has been highlighted by the WHO as this world-wide organization has devoted much attention to this issue. Natural infections offer about 72% protection against a second episode of shigellosis due to the homologous serotype, but only offer less than 30% protection against a heterogenous serotype, suggesting *Shigella* O antigen is the key antigen for protection. A vaccine active against *S. sonnei*, *S. dysenteriae* type 1, *S. flexneri 2a*, *S. flexneri* 3, and *S. flexneri* 6 would cover more than 80% of the strains currently causing morbidity and mortality in both developed and developing countries. Recent work has focused on the development of vaccines against *S. sonnei*, *S. dysenteriae* type 1 and *S. flexneri* 2a. For *S. sonnei*, improved attenuated strains of *S. sonnei* WRSS1 were used as live oral vaccines; a live-attenuated strain of S. *dysenteriae* type 1 has been developed but not tested yet for its efficacy in humans. For *S. flexneri* 2a, two approaches have been tested: a hybrid complex of *S. flexneri* 2a lipopolysaccharide and the virulence factors IpaB, IpaC, and IpaD has been constructed and tested as an intranasal spray; the other vaccine is a live, attenuated strain that was tested in adults and children (ages 8–10 years) in Bangladesh.

Other means to control the spread of *Shigella* would be to ensure potable water supplies as well as to ensure that water used for irrigation and processing is pathogen-free. As noted above, in many instances the poor personal hygiene of infected individuals can lead to contamination of foods. And since the infectious dose of *Shigella* species is quite low, eliminating this source may have a pronounced effect on a safe, global food supply.

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9 *Vibrio vulnificus, Vibrio parahaemolyticus* and *Vibrio cholerae*

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9.1 Introduction

The genus *Vibrio* contains bacteria that are indigenous to estuarine and marine environments. Worldwide, species that are pathogenic to humans include *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. In recent years, fundamental discoveries have been made about how environmental signals modulate gene expression and virulence of *Vibrio* spp., raising the question 'What are the roles of virulence genes in aquatic environments where they most likely evolved?'

In this chapter we provide the reader with an overview of the ecology, pathogenicity, and virulence of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae*.

9.2 *Vibrio vulnificus*

9.2.1 Introduction

In the USA, *Vibrio vulnificus* is the leading bacterial cause of reported death attributed to the consumption of seafoods, and a species that produces one of the highest mortality rates (~60%) among known foodborne pathogens. As such, it has had a profound effect on federal and state public health policy, demonstrated in many states by mandatory consumer health advisories posted at retail stores that sell raw molluscan shellfish. At the federal level, some have recommended regulations that would prohibit the sale of summertime raw oysters harvested from the Gulf of Mexico.

It is relatively easy to link *V. vulnificus* infections to oysters, in that other foods are rarely implicated. There are two primary reasons why molluscan shellfish present increased risk to human health. First, molluscan shellfish filter and concentrate particles, including bacteria, from surrounding seawater. Second, shellfish are commonly eaten raw or undercooked, thus allowing live microbes easier access to human tissues.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

Once inside a susceptible host, *V. vulnificus* causes fulminant infections and extensive invasion of tissues. Susceptibility is believed to result from defects in host defense systems, most of which do not involve the immune system. One predisposing condition, hemochromatosis, is characterized by high levels of free iron in human tissues that accelerates the growth of *V. vulnificus*. In the case of primary hemochromatosis, mutations in the *hfe* gene cause increased uptake of iron in the intestines. In secondary hemochromatosis, disease can result from alcoholic cirrhosis of the liver or hepatitis. Other compromising conditions include diabetes mellitus and immunodeficiency, such as that caused by HIV. In the majority of fulminant infections, disease and death often occur within 24 hours of consuming oysters or seawater contacting broken skin.

The highly opportunistic nature of the disease is illustrated by large numbers of *V. vulnificus* that are consumed in a meal of raw unprocessed Eastern oysters (*Crassostrea virginica*) harvested from the Gulf of Mexico. A dozen such raw oysters typically contain more than 10 million *V. vulnificus*. Furthermore, evidence supports the idea that infection results from specific *V. vulnificus* strains that infect specific human subpopulations (possibly certain tissue types), within high-risk categories. This notion is supported by the fact that hundreds of thousands of people with immunocompromising disease eat raw oysters every year that contain millions of *V. vulnificus*, yet less than 100 individuals present with clinical disease.

9.2.2 Ecology

Vibrio vulnificus is an autochthonous estuarine organism found in temperate and tropical waters throughout the world and is easily isolated from seawater, sediment and marine life. Like other *Vibrio* spp., it attaches to aquatic surfaces, including plankton, sediment and fish, as well as to tissues of molluscan shellfish. Due to filter-feeding mechanisms, levels of *V. vulnificus* in molluscan shellfish may be >100-times higher in shellfish than in seawater.

Individual oysters contain multiple strains of *V. vulnificus*, sometimes more than 100 per oyster, as evidenced using pulsed-field gel electrophoresis (PFGE). This finding demonstrates the challenge for retrospective studies of infections, since the pathogenic strain needs to be enumerated within the total *V. vulnificus* flora. This approach is supported by reports showing that only one PFGE profile is found in the blood of an infected individual, even though multiple strains were ingested.

The ecology of *V. vulnificus* in seawater is markedly affected by temperature and salinity. Higher numbers occur in seawater with a salinity range of 5 to 20 parts per thousand (ppt), such as those in Gulf of Mexico estuaries. Within this salinity range, *V. vulnificus* grows above ~20 °C. Below 15–20 °C, viability declines but cells can persist in sediment, oysters and other marine life.

The effect of salinity on *V. vulnificus* viability is well illustrated in environments where temperature is relatively constant year round, such as Hawaiian coastal waters. *Vibrio vulnificus* is not isolated from high salinity seawater or from freshwater upwellings; however, it is easily isolated at high salinity–fresh water interfaces. These observations have been confirmed in laboratory microcosms, showing that viability is enhanced at 5–10 ppt salinity.

Currently there are no microbiological indicators for *V. vulnificus* analogous to fecal coliforms. However, predictive models with parameters for temperature and salinity have shown good utility and have been integrated with remote sensing instruments (e.g., satellites) to identify low- and high-risk harvest areas. These models are based on large databases that include parameters for temperature, salinity, and *V. vulnificus* levels in seawater, shellfish, and seawater. Other environmental studies have found associations between *V. vulnificus*

levels and environmental parameters such as chlorophyll and seawater turbidity. The predictive models are used to form the basis of risk assessments produced in different countries and by World Health Organization/Food and Agriculture Organization panels. Due to susceptibility to temperature and salinity, the ecology of *Vibrio* spp. is markedly affected by climate change. As such, predictive models will be useful for projecting effects of changing environments on human health risk.

The first ecological studies were published in the early 1980s, shortly after *V. vulnificus* was identified as a new species. In the 1990s, large environmental surveys were conducted to determine *V. vulnificus* levels associated with health risk, and strategies that could be used to manage risk. One study measured temperature and salinity in US coastal seawater and oysters. At sites along the Gulf of Mexico, *V. vulnificus* numbers increased with water temperature up to 26°C and were constant at higher temperatures. High (>10,000/g) *V. vulnificus* levels were found in oysters from salinities of 5 to 25 ppt. Lower numbers (<100/g) were found at salinities greater than 28 ppt, typical of Atlantic Coast sites. A second study measured *V. vulnificus* in seawater, sediment and oysters collected at monthly intervals from commercial shellfish harvesting sites in 15 coastal states (Maine, Connecticut, Rhode Island, Massachusetts, New Jersey, Virginia, South Carolina, Florida, Mississippi, Louisiana, Texas, California, Oregon, Washington, Hawaii). Results showed that of two predictors (temperature and salinity), temperature had the highest correlation with *V. vulnificus* levels in oysters. A linear regression formula was derived that predicted levels of *V. vulnificus* in Gulf of Mexico shellfish based on seawater temperature and salinity.

More recently (2007), market oysters were collected from retail establishments from nine states in the USA and analyzed by the Most Probable Number (MPN) method for *V. vulnificus* levels. The bacterium was distributed seasonally and geographically by harvest region and the highest ($>10^4$ MPN/g of oysters) levels of *V. vulnificus* were observed during summer months in Gulf Coast oysters.

The impact and control of *V. vulnificus* have been addressed in other countries. For example, from March 2002 to June 2003 molecular methods were used to detect and enumerate *V. vulnificus* in oysters from two estuaries along the southwest coast of India. Fifty-seven percent of samples were positive and levels ranged from <10 CFU/g to 10^3 CFU/g at both estuaries. The results indicated that *V. vulnificus* densities in a tropical country are mainly controlled by salinity rather than temperature.

Vibrio vulnificus was also recovered from Tilapia aquaculture systems in Bangladesh. Phylogenetic analyses showed that Bangladeshi isolates differed remarkably from fish or environmental isolates collected elsewhere and were more related to clinical isolates.

In a US study conducted from 2005 to 2010, drought was shown to affect levels of *V. vulnificus* in water and oysters, via elevated seawater salinity. When salinity returned to normal levels, *V. vulnificus* was detected in seawater but not oysters, indicating that during the drought, oysters were colonized with salt-tolerant species that displaced *V. vulnificus* and prevented recolonization.

Vibrio vulnificus exists in biofilms on sediments and on various other physical forms in the marine environment. As for other aquatic bacteria, *V. vulnificus* may utilize quorum sensing to modulate attached and free-swimming forms, and the expression of virulence genes. A gene homologous to the transcriptional regulator *lux*R of the *lux* operon found in *Vibrio harveyi* has been reported for *V. vulnificus*, showing >75% nucleotide identity. A similar finding was reported for *V. cholerae* and *V. parahaemolyticus*, indicating that the *lux* operon may have been inherited from a common ancestor, and is widespread in marine *Vibrio* spp.

There has been significant controversy concerning the relevance of the so-called 'viable but non-culturable' (VBNC) state to the ecology of *V. vulnificus*. Researchers report that following prolonged exposure to physiological stressors, such as low temperature and low nutrients, *V. vulnificus* cannot be cultured using conventional microbiological techniques. Studies show that when temperature is favorable, the VBNC form becomes culturable ('resuscitated') on conventional media. Another explanation for resuscitation of VBNC cells is that culturable cells are present in test flasks in low undetectable numbers, and/or are attached to and released from vessel walls.

VBNC cells have been shown to possess some enzyme activity. However, this is not unexpected, in that enzymes possess activity even after cellular DNA has degraded and replication is no longer possible. It is possible that the VBNC state more likely reflects the current state of technology to culture stressed cells, and that the term 'VBNC' will be redefined as nonculture-based methods better define the terms 'life' and 'viability.'

Bacteriophage also influence the ecology of *V. vulnificus*. In one study, of 60 *V. vulnificus* strains tested, 87% were susceptible to one or more phage types. The role of phage in the ecology of *V. vulnificus* remains poorly defined but has been proposed as a potential remedial method to reduce *V. vulnificus* and target pathogenic strains in post-harvest shellfish.

9.2.3 Pathogenicity and virulence

Vibrio vulnificus infections are reported throughout the world, indicating the ubiquitous nature of the species and wide distribution of virulent strains. The vast majority of *V. vulnificus* foodborne infections is restricted to seafoods harvested from temperate and tropical waters where high levels of the organism occur in warm months. Wound infections occur in the same geographical regions as foodborne infection; however, these have also been reported in areas where seawater salinity and temperature are suboptimal for *V. vulnificus* viability.

The U.S. Centers for Disease Control and Prevention (CDC) estimates that approximately 96 foodborne cases occur in the USA annually, but only half of the cases are believed to be reported. The incubation period for *V. vulnificus* disease is approximately 12 to 72 hours after exposure. Foodborne infections often progress rapidly into primary septicemia characterized by formation of edematous skin lesions on limbs. Fatalities may occur within 24 hours, especially in individuals with hepatic disease and immunocompromising conditions. In healthy individuals, *V. vulnificus* is believed to cause illness within 16 hours, which manifests in vomiting, diarrhea and abdominal pain. More extreme infections cause fever, chills, decreased blood pressure (septic shock) and blistering skin lesions. In addition to septicemia, gastroenteritis and wound infections, *V. vulnificus* can also cause serious ocular infections. Ocular trauma by shellfish from contaminated water has been shown to be the most common risk factor for *Vibrio* conjunctivitis and keratitis.

In an overview of *V. vulnificus* disease in the US, 422 infections were reported between 1988 and 1996. Of these, 45% were wound infections, 43% primary septicemia, 5% gastroenteritis and 7% from unknown exposures. Of those who developed primary septicemia, 61% died. All successful trackbacks of implicated oyster product showed that oysters were harvested from the Gulf of Mexico when the water temperature was >22 °C.

A very recent 2012 epidemiological study shows that the incidence of vibriosis increased between 1996 and 2010 in the USA. During this period, 7700 cases of vibriosis were reported by the Cholera and Other *Vibrio* Illness Surveillance (COVIS) system. Over this 14-year period, 1446 cases of *V. vulnificus* infection were reported, with an associated 31.9% mortality rate.

Prior to 1996, epidemiological reports showed that cases of human *V. vulnificus* disease were sporadic. However, in the summer and autumn of 1996 and 1997, an outbreak of invasive *V. vulnificus* infection occurred in Israel in people who had recently handled fresh, whole fish purchased from inland artificial fishponds. Overall, 62 cases of wound infection and bacteremia were reported. The isolates were indistinguishable by restriction fragment length polymorphism (RFLP)-PCR, and could not be typed by PFGE. Based on phenotype and RFLP tests, the authors concluded that the outbreak was caused by a new strain of *V. vulnificus* classified as biogroup 3.

Vibrio vulnificus virulence has been estimated by various methods including LD_{50} in mice, comparing opaque (virulent) versus translucent (avirulent) colony morphotypes, the presence of specific membrane proteins and extracellular enzymes, resistance to animal host defense systems, the presence of specific genes and DNA profiles using various RFLP techniques.

A multi-agency extensive study was conducted to learn about environmental levels associated with human disease. Total *V. vulnificus* levels in oysters were measured following harvest from a commercial site in Apalachicola Bay, Florida over a 3-year period. During this time, the incidence of human *V. vulnificus* infections reported in Florida and associated with oysters harvested from the Apalachicola Bay sampling location were recorded. Environmental *V. vulnificus* levels were considered if case-associated oysters were harvested within 72 hours of the Apalachicola Bay sampling. Eight *V. vulnificus* infections met these criteria, showing that *V. vulnificus* disease occurred when *V. vulnificus* levels exceeded 1000 CFU per gram of oyster meat (range 10^3 per gram to 10^5 per gram). This concentration translated to approximately 3×10^5 *V. vulnificus* in one dozen raw oysters.

In a separate but related study, *V. vulnificus* was enumerated in oysters directly linked to human infections. Logistical constraints included identifying cases, obtaining implicated oysters from homes and retail outlets, and collecting clinical samples from patient specimens. In a total of four cases where clinical specimens were obtained, each patient displayed a single strain defined by PFGE. This finding has been substantiated in other reports. In one human infection, oysters obtained from the implicated restaurant were analyzed to determine both the concentration and genetic diversity of *V. vulnificus* isolates. The oysters contained 9.6×10^3 *V. vulnificus* per gram of oyster, indicating that the patient ingested approximately 6×10^5 CFU *V. vulnificus*. PFGE tests of oyster isolates revealed eight unique DNA profiles among the ingested strains. The pathogenic strain was present in the implicated oysters at 2×10^3 CFU per gram, indicating approximately 1×10^5 CFU were consumed.

9.2.3.1 *Iron*

Vibrio vulnificus can cause septicemia and serious wound infections in patients with iron-overload and liver disease. Indeed, saturation of transferrin appears to be an important prerequisite for infection. The ability of *V. vulnificus* to acquire iron from the host has been shown to correlate with virulence. Catechol siderophores produced by *V. vulnificus* help it acquire iron from transferrin, and catechol siderophore mutants showed reduced virulence in an infant mouse model.

The ability of *V. vulnificus* to acquire host iron may directly result in growth of the pathogen to high levels. This has been confirmed in studies of the survival of *V. vulnificus* in whole blood from healthy volunteers and patients with chronic hepatitis, liver cirrhosis and hepatoma. Researchers demonstrate that bacterial numbers in human blood differ significantly between hepatoma patients and healthy volunteers, and increase with the severity of

patient liver dysfunction. Survival of *V. vulnificus* in whole blood also shows a positive correlation with serum ferritin concentration and the percentage of transferrin iron saturation. Survival of *V. vulnificus* in blood correlates with reduced phagocytosis by neutrophils, likely mediated by capsular polysaccharide.

9.2.3.2 *Extracellular toxins*

Reports of the *V. vulnificus* disease process describe intense acute cellulitis that spreads along subcutaneous tissue, resulting in severe tissue destruction. These pathogenic effects are primarily mediated by exoenzymes that damage the host cell membrane. Mouse skin damage can be caused by a single intradermal injection of *V. vulnificus* or by injecting an extracellular metalloprotease.

Nearly all environmental and clinical isolates of *V. vulnificus* produce some form of extracellular enzyme with cytolytic and/or cytotoxic activity. A long list of exoenzymes has been described including collagenase, elastase, hemolysin, DNase, lipase, mucinase and phospholipase.

Among metalloproteases from various bacterial species, the *V. vulnificus* metalloprotease has high hemorrhagic activity. Of two major basement membrane components (laminin and type IV collagen), type IV collagen was easily digested by *V. vulnificus* metalloprotease. Researchers hypothesize that degradation of type IV collagen may cause destruction of the basement membrane, breakdown of capillary vessels and leakage of blood components including erythrocytes. Researchers cloned the gene for the metalloprotease and demonstrated that it causes edematous changes of the skin that extend throughout the dermis and subcutaneous tissues, and into adjacent muscles.

Although various reports substantiate pathogenic effects of isolated *V. vulnificus* metalloprotease, its obligate role in virulence is questioned. A protease-deficient mutant was isolated by in vivo allelic exchange and found to be as virulent as parent strains. In addition, the mutant could not be distinguished from the parent strain in enhancement of vascular permeability, invasion from peritoneal cavity into blood stream, growth in blood and utilization of hemoglobin and transferrin.

9.2.3.3 *Capsule*

Significant attention has been given to the role of capsular polysaccharide (CPS) in virulence. Research has focused on defining cell surface markers that correlate with virulence. These studies examined lipopolysaccharide and capsule antigens, and expression of specific types of capsular polysaccharide (i.e., carbotypes). At least 15 capsular types have been reported for *V. vulnificus*. Some polysaccharide antigens appear to be associated with human clinical strains.

The quantity of CPS positively associates with *V. vulnificus* virulence in mice. Investigators report that large capsules are associated with low LD_{50}, resistance to the bactericidal action of human serum, anti-phagocytic activity and tissue invasion.

Although multiple CPS types have been described in the literature, virulence does not appear to correlate with a specific CPS type. Numerous reports confirm that the quantity of CPS expressed by *V. vulnificus* can result in reversible opaque and translucent colony morphologies. In contrast to translucent colony morphotypes, opaque morphotypes are associated with virulence and resistance to phagocytosis by human and oyster phagocytes.

Translucent variants express intermediate quantities of CPS and expression of CPS varies with growth phase (i.e., increases during logarithmic phase and declines in stationary

culture). Temperature also has an effect on CPS expression, whereby more CPS is produced at 30 °C than at 37 °C.

Following *V. vulnificus* infection, IgG antibodies can be demonstrated in patient serum that react with CPS. Anti-CPS antibodies have also been demonstrated in persons without a history of *V. vulnificus* infection, thus indicating occurrence of cross-reacting antibodies. Investigators report no significant difference between antibody levels to one of three capsular types tested, when comparing shellfish industry workers and persons with low exposure to *V. vulnificus*.

An epimerase is necessary for *V. vulnificus* to express extracellular CPS. It is common to many strains of *V. vulnificus* that express serologically-distinct extracellular CPS.

There has been substantial research to elucidate detailed CPS structures of *V. vulnificus*, in the hope of defining common structures of pathogenic strains. The complete structure of the polysaccharide from pathogenic *V. vulnificus* strain ATCC 27562 has been published.

9.2.3.4 *Other virulence factors*

Increased mortality observed in cirrhotic mice resulting from *V. vulnificus* infection depends on an in vivo tumor necrosis factor-alpha (TNFα) response. Specifically, when cirrhotic mice pretreated with TNFα receptor immunoadhesin were challenged with a low dose of *V. vulnificus*, the increased mortality compared to that of controls was prevented. Capsular polysaccharide is also thought to have a role in tissue production of TNFα. TNFα can be detected in the serum of animals challenged with an encapsulated parent strain; however, the unencapsulated strain is quickly eliminated, thus preventing detection of serum TNFα. Capsular polysaccharide has also been found to be a better inducer of TNFα than lipopolysaccharide (LPS).

9.2.3.5 *Strain diversity*

The primary environmental niche for *V. vulnificus* is the marine environment. Though this bacterium shows high strain diversity in oysters, single strains are isolated from patients, indicating that strains are not equally virulent. Strains have been classified on the basis of phenotypic properties and virulence in eels. Biotype 2 is virulent for eels, negative for indole reaction and serologically homogeneous (serogroup E). Whereas strains of biotype 1 are avirulent in eels, indole positive and serologically heterogeneous. The laboratory of Amaro has conducted extensive research of the serological reactions of both biotypes 1 and 2, and suggests that biotype 2 be designated as serovar E. A third biotype with the potential to cause outbreaks of human wound infections has also been proposed.

Molecular typing methods are superior for differentiating *V. vulnificus* at the intraspecies level, as phenotypic typing using API 20 E, API 20 NE and BIOLOG microplate discriminate poorly between biotypes, and can misidentify species. Following years of adaptation and evolution in the marine environment, *V. vulnificus* isolates display high genetic divergence when DNA is measured by RFLP techniques, including PFGE, ribotyping (RT) and randomly amplified polymorphic DNA (RAPD).

Until the mid-1990s, very little was known about the variety of *V. vulnificus* strains that exist in the environment and those that cause human disease. An early report describes RT

profiles of different clinical *V. vulnificus* isolates. Among the 10 restriction enzymes tested, *Hind*III was the most discriminatory. The superior resolution of *Hind*III for RT has been reported by others.

Various researchers have shown that multiple isolates from the same patient have the same RT, PFGE or RAPD profile. In contrast, clinical isolates from different patients have unique RFLP profiles. RT patterns of *V. vulnificus* remain unchanged after successive in vitro and in vivo passage. In a broad survey of *V. vulnificus* strains, researchers found high variation among clinical and environmental isolates, as determined by PFGE. In contrast, RT profiles of isolates following *Hind*III digestion showed more conservative patterns of genomic diversity. Various RT clusters were determined but none were identified that positively correlated with environmental source or pathogenic potential. Interestingly, some RT clusters show a lower percentage of clinical isolates than others.

Wong and co-workers reported that PFGE using *Not*I was less discriminatory than *Sfi*I. *Not*I was able to separate clinical isolates but not environmental isolates by geographic region. Investigators report that certain *V. vulnificus* strains were not typeable by either enzyme, suggesting this may be due to degradation of DNA. Bisharat and colleagues used multilocus sequence analysis (MLST) of seven housekeeping genes from each chromosome to determine the genetic diversity of 159 *V. vulnificus* isolates (biotypes 1, 2 and 3) collected from clinical and environmental sources worldwide. They found 70 unique sequence types (STs) and 82 isolates were classified in one of four MLSTs. Moreover, isolates of biotype 1 were more genetically diverse (66 MLSTs) than that of the isolates of biotype 3 (a single MLST). They also divided *V. vulnificus* into two clades based on MLST. Clade 1 strains were often isolated with environmental samples, while clade 2 strains were mainly associated with human disease. Clade 2 strains appear to possess inherently greater virulence.

Recently, several groups developed PCR-based assays to detect high-virulence strains. Nilsson and co-workers observed two major groups of *V. vulnificus*, designated type A (environment-associated) and B (clinical-associated), based on a 17-nucleotide difference throughout the sequence of the small subunit 16S rRNA gene. Vickery and co-workers developed a real-time PCR assay targeting the heterogenous regions of the 16S rRNA gene to type 85 isolates including the 67 isolates used in the previous study. Type B strains constituted 76% of clinical isolates, whereas types A and AB accounted for only 9 and 15%, respectively. Gordon and colleagues used this scheme in a quantitative PCR format to assess A/B ratios in clinical isolates and those harvested from permitted and prohibited shellfishing beds regulated by fecal coliforms levels. Fifty-three percent of the clinical isolates were type A and 47% type B, indicating an imperfect association with strain virulence. A PCR assay was developed targeting the virulence-associated gene (*vcg*), corresponding the VV0401 the open reading frame to type 35 clinically-associated (type C) and 35 environmentally-associated (type E) isolates. Ninety-three percent of type E isolates were recovered from environmental sources, whilst 90% of the C isolates were of clinical origin.

SOLiD™ sequencing of four *V. vulnificus* strains representing different clades (1 and 2) and biotypes (1 and 2) was used for comparative genomic analysis. This method was able to identify numerous genes that are common to the most virulent strains but lacked by attenuated strains. These virulence genes encode Flp pili, GGDEF proteins and genomic island XII. Such results will lead to a more thorough understanding of the molecular pathogenesis of *V. vulnificus*.

Future research should also elucidate the relationship of the double chromosome recently described for various *Vibrio* spp. A recent report shows that *V. vulnificus*, like

V. fluvialis and various serovars and biovars of *V. cholerae*, possesses two circular replicons of 3.2 and 1.9 Mb.

9.2.4 Detection

A well-established method for the isolation, detection and enumeration of *V. vulnificus* is found in the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual*. Many of the methods for preparing and enriching samples for *V. vulnificus* were derived from earlier procedures developed for *V. parahaemolyticus* and *V. cholerae*.

Isolation begins by diluting the sample 10- and 100-fold (and further dilutions for enumeration) in alkaline peptone water (APW) and blending for two minutes at high speed. The APW broth is then incubated at 35–37 °C for 18–24 hours. After incubation and without shaking the flask, a loopful of surface growth is transferred to a plate of mCPC agar that is incubated for 18–24 hours at 39–40 °C. *Vibrio vulnificus* produces flattened translucent yellow colonies with opaque centers. For further tests, colonies are streaked for isolation on a nonselective media such as tryptic soy agar containing 1–2% NaCl.

PCR and gene probe methods are available as confirmatory tests for *V. vulnificus*. Therefore, there is less demand to perform a battery of biochemical tests to speciate suspect colonies. When these phenotypic methods are used, *V. vulnificus* can be differentiated from non-*Vibrio* using TSI, KIA, and arginine glucose slants, oxidation- fermentation and oxidase tests, and various other methods.

The characteristics below are presumptive for *V. vulnificus*:

- Gram-negative
- Growth at 42 °C, positive
- Arginine dihydrolase test, negative
- Lysine decarboxylase test, positive
- Ornithine decarboxylase test, positive
- Voges–Proskauer test, negative
- NaCl test: 0% NaCl, negative; 3, 6% NaCl, positive; 8% NaCl, negative
- Sucrose fermentation, negative
- ONPG test, positive
- Arabinose fermentation, negative
- Sensitivity to O/129, sensitive to 10 and 150 µg
- TSI reaction: alkaline slant (rarely acidic)/acid butt; gas production, negative;
- H_2S, negative
- Hugh–Leifson test: glucose oxidation and fermentation, positive
- Cytochrome oxidase, positive

A direct-plating colony hybridization method detects *V. vulnificus* in environment samples, using an alkaline phosphatase-labeled oligonucleotide probe for the cytolysin (*vvh*A) gene. The main steps in the method are direct-plating samples on nonselective media followed by colony lift, hybridization and detection. This method allows rapid isolation and enumeration without enrichment.

Another method is a SYBR green I-based real-time PCR assay for detection of *V. vulnificus* in oyster homogenates. In this procedure, a pair of primers (L-vvh and R-vvh) is used to amplify a 205-bp sequence of the hemolysin (*vvh*) gene. This assay permits detection of 10 CFU/g of homogenate within eight hours.

9.2.5 Prevention and control

Persons with immunocompromising conditions need to take precautions to reduce the risk of *V. vulnificus* infections. These safeguards include not consuming raw shellfish from high-risk harvesting areas, cooking shellfish thoroughly, avoiding cross-contamination of food with raw seafood, avoiding exposure of open wounds or broken skin in high-risk environments, and wearing protective gloves when handling raw shellfish.

The Food & Agriculture Organization of the United Nations and the World Health Organization have produced risk assessments that can be used to develop and implement risk management policies that minimize the risk of *V. vulnificus* disease. The US FDA produced a similar risk assessment that is used to control growth of *V. vulnificus* through post-harvest time–temperature control.

Shellfish companies and researchers have also developed innovative post-harvest treatment processes. These include heat-treatment of live shellstock at 50 °C for 10 minutes, high pressure processing of shellstock, as well as freezing. Depuration is not an effective treatment to reduce levels of *Vibrio* spp. in shellfish.

9.3 Vibrio parahaemolyticus

9.3.1 Introduction

Vibrio parahaemolyticus infections are associated with the consumption of raw shellfish, cross-contaminated food and exposure of wounds to seawater. It is the cause of numerous outbreaks of foodborne illnesses where raw or undercooked seafoods are consumed. In Japan and the US, *V. parahaemolyticus* is one of the more frequent bacterial causes of foodborne illness. Recent and large outbreaks in Chile and the US, as well as potential links to climate change, have renewed interest in this pathogen.

The ecology of *V. parahaemolyticus* is more diverse compared to *V. vulnificus* and *V. cholerae*, mostly due to a greater tolerance for high salinity. It is isolated from a variety of entities in the marine environment, including shellfish, finfish, plankton, sediment and seawater. It is recovered from biotic and abiotic entities in marine environments when temperature and salinity are compatible with viability.

This pathogen can be typed by capsular (K) and lipopolysaccharide (O) antigens based on a well-established serological typing scheme. Of particular note is the O3 serotype that has been increasingly linked to outbreaks throughout the world, indicating that this serotype may be better adapted for survival in aquatic environments.

The thermostable direct hemolysin (*tdh*) and the TDH-related hemolysin (*trh*) genes and their gene products (TDH and TRH) serve as useful tools to identify virulent *V. parahaemolyticus* strains. Research continues to elucidate other virulence mechanisms that more fully explain pathogenicity in humans.

9.3.2 Ecology

In recent years, large surveys have been conducted to measure *V. parahaemolyticus* levels in seafoods and to determine specific environmental factors that influence viability, potentially leading to implementation of effective risk management practices. For example, densities of *V. parahaemolyticus* possessing *tlh*, *tdh*, and *trh* virulence marker genes were measured in

coastal waters of Mississippi and Alabama over a 19-month sampling period (February 2006 to August 2007). Densities in water, sediment and oysters were significantly associated with surface water temperature. Turbidity was strongly associated with densities of *V. parahaemolyticus* in all sample types. Therefore, the role of turbidity in predicting the risk of this bacterium may be more important than previously thought.

The seasonal distribution of total and pathogenic *V. parahaemolyticus* was studied from 2004 to 2005 in Chesapeake Bay oysters and seawater. Total and pathogenic *V. parahaemolyticus* were detected in 79 and 3% of the oyster samples, respectively, by a direct-plating method. Levels of *V. parahaemolyticus* in oysters varied seasonally and correlated positively with water temperature, turbidity and dissolved oxygen.

Levels of total and pathogenic *V. parahaemolyticus* in Pacific oysters collected from commercial growing areas in New Zealand's North Island (December 2008 to April 2009) were determined. *Vibrio parahaemolyticus* was detected in 95% of oyster samples, with levels ranging from <3 MPN/g to 1500 MPN/g. Only 3% of samples were positive for pathogenic *V. parahaemolyticus* and no significant correlation was observed between levels of this bacterium in oysters and the environmental parameters tested.

In 2007 to 2008, a seasonal study was conducted to quantify *V. parahaemolyticus* in tidal water, fiddler crab burrow water and intestinal pore water at the North Inlet Estuary in Georgetown, South Carolina. Levels of *V. parahaemolyticus* were significantly higher within burrow water than in creek and intestinal pore water. The results indicated that the highest level of this bacterium occurs during warmer months and that infaunal burrows are sites of *V. parahaemolyticus* enrichment.

In a large survey (1996–1997) of seafood products imported into Europe, *V. parahaemolyticus* was isolated from 45.9% of 686 samples of seafood from Southeast Asian countries. The incidence rates in shrimp, crab, snail, lobster, sand crab, fish and crawfish were 75.8, 73.3, 44.3, 44.1, 32.5, 29.3 and 21.1%, respectively. However, none of the isolates possessed *tdh* or *trh*. The occurrence, diversity and pathogenicity of *Vibrio* spp. was also examined in two estuaries along the Italian Adriatic coast, showing that *V. parahaemolyticus* was the second most-commonly isolated *Vibrio* spp species.

It is well known that all *Vibrio* spp. produce chitinase, an enzyme that hydrolyzes chitin. Chitin is the most abundant carbohydrate-based polymer in the marine environment and is found in the exoskeletons of various forms of marine life, including plankton. *Vibrio* spp. colonize chitinous surfaces of plankton and are involved in remineralization of molted exoskeletons. Interestingly, investigators have reported that, unlike *V. cholerae* non-O1 and *Aeromonas hydrophila*, *V. parahaemolyticus* failed to colonize either live or dead copepods. Unlike copepods, the alimentary tract of estuarine gastropods (*Clithon retropictus*) supported the growth of TDH-producing strains in summer months, even in the presence of high levels of TDH-negative *V. parahaemolyticus*.

Another factor that may contribute to the persistence of *V. parahaemolyticus* in the environment is resistance to phagocytes, such as molluscan hemocytes. Using a tetrazolium dye reduction assay to study factors governing the killing of bacteria by oyster hemocytes, opaque *V. parahaemolyticus* strains resisted oyster cellular host defenses better than translucent strains.

Vibrio parahaemolyticus has been shown to produce molecules capable of inducing luminescence in *Vibrio harveyi*, indicating that quorum signaling may help it adapt to communal life on surfaces in the marine environment. For example, when grown on a surface or in a viscous layer, *V. parahaemolyticus* differentiated to swarmer cells capable of movement over and colonization of surfaces. The bacterium can also switch between translucent and opaque colony types.

With the recent global spread of the O3:K6 strain, there is speculation that it may possess survival characteristics that distinguish it from other serotypes. Investigators found that all O3:K6 strains possess a common plasmid, pO3K6, having 8782 base pairs and 10 open reading frames (ORF). Interestingly, a single-stranded phage was isolated from one O3:K6 strain and when the double strand was produced with DNA polymerase, it was identical to pO3K6 by restriction fragment polymorphism. ORF8 may be useful for tracking the spread of O3:K6 strains since it is unique to O3:K6 strains isolated since 1996.

Filamentous phage have been isolated from various strains of *V. parahaemolyticus* and are a suspect vector for transferring genetic material among *V. parahaemolyticus* and other *Vibrio* spp. The overall gene structures of phage Vf12 and Vf33 have both conserved and distinctive regions and there are similarities in conserved genes with that of phage of *V. cholerae* and coliphage of *Escherichia coli*.

9.3.3 Pathogenicity and virulence

Vibrio parahaemolyticus causes three major clinical syndromes, the most common being gastroenteritis with symptoms of diarrhea, abdominal cramps, nausea, vomiting, headache and low-grade fever. Occasional bloody diarrhea with stools has been reported. In general, the illness is self-limiting with an incubation period of 15 hours and symptoms ranging from 4 to 96 hours.

Primary septicemia is reported for individuals with underlying chronic illness. Its ability to invade tissues is supported by research showing that *V. parahaemolyticus* isolates invade Caco-2 cells, a human colon carcinoma-derived cell line.

In the US, *V. parahaemolyticus* is the most common cause of bacterial seafood-borne disease. Illness is typically associated with cooked seafood cross-contaminated with seawater or uncooked seafood. Forty outbreaks, constituting over 1000 cases of *V. parahaemolyticus* infection, were reported to the CDC by four Gulf Coast states between 1973 and 1998.

Between May and September of 1997, over 250 human *V. parahaemolyticus* infections occurred in the Pacific Northwest of the USA and were associated with raw oyster consumption. All illnesses were caused by a strain with the O4 serotype that possessed the *tdh* gene. At the time of the outbreak, seawater temperatures were 1 to 5 °C higher than normal in the implicated estuaries; the authors suggested that El Niño-induced weather changes may have produced higher levels (i.e., >11,000 CFU/g) in shellfish.

In the following year, more than 300 *V. parahaemolyticus* infections were linked to consumption of oysters harvested from Galveston Bay, Texas. All clinical specimens displayed one PFGE type and were the O3 serotype. Interestingly, this serotype was also associated with other recent outbreaks in various geographical regions of the world. In the same year, oysters and clams harvested from the Long Island Sound in New York were implicated in 23 culture-confirmed cases of *V. parahaemolyticus* serotype O3 gastroenteritis. This was the first reported outbreak of *V. parahaemolyticus* linked to consumption of shellfish harvested from New York waters. Water temperature was reportedly 8°F higher than in the previous two-year period.

According to the State of Alaska Epidemiology Bulletin, an outbreak of *V. parahaemolyticus* causing diarrhea was reported in July 2004. Of the 189 victims interviewed, 132 (70%) stated that they had consumed raw oysters or seafood-related food.

Daniels and co-workers studied epidemiological data along with environmental factors that may have contributed to recent outbreaks of *V. parahaemolyticus*. These outbreaks involved four states in the US – Florida, Alabama, Louisiana and Texas – that participated

in the Gulf Coast Surveillance System. In their study, 345 cases were reported to the CDC between 1988 and 1997. This represented a large increase compared to the period 1973–1988 when only 40 cases were reported. Of the 345 cases, 59% suffered from gastroenteritis, 34% wound infections, 5% septicemia and 2% from other exposure. Among individuals with acute gastroenteritis, 88% had eaten raw oysters during the week before the illness. Khan and co-workers carried out a study on isolates of an outbreak in Texas, New York and Pacific Northwest from 1997 to 1998 that involved more than 400 cases. Since 1998, the number of outbreaks reported has been on the rise.

The COVIS study (2012) reported 3460 cases of *V. parahaemolyticus* disease between 1996 and 2010, with 714 hospitalizations, and an associated mortality rate of 0.7%. In contrast, the CDC FoodNet surveillance system reported 820 infections, 131 hospitalizations and four deaths.

A number of outbreaks of *V. parahaemolyticus* were reported in Southern Chile from 2004 to 2007. The bulletin revealed that there were 1500 cases in 2004, 3600 cases in 2005, 900 cases in 2006 and 475 cases in 2007. According to this report, there was a decrease in the number of cases as years progressed. A more recent outbreak in the USA occurred during May 20–July 31, 2006, in which health departments in New York City, New York state, Oregon and Washington reported a total of 177 cases of *V. parahaemolyticus* infection.

9.3.3.1 *Spread of the O3 serotype*

Prior to 1995, there was little evidence that specific serotypes of *V. parahaemolyticus* were spreading over geographical regions. In analyses of hundreds of strains collected over time, a variety of K serotypes were reported. Compelling evidence is presented that the O3:K6 pandemic of *V. parahaemolyticus* resulted from the spread of a single clone. *Vibrio parahaemolyticus* O3:K6 strains isolated from cases of diarrhea in Calcutta, India beginning in February 1996 and strains isolated from Southeast Asian travelers since 1995 belonged to a unique clone possessing *tdh* but not *trh*. These data agree with a previous report of clonality using arbitrarily primed PCR. Human illness resulting from this clone has increased in Bangladesh since 1997 and also in Taiwan, Laos, Japan, Thailand, Korea, and the USA between 1997 and 1998.

Researchers provide further evidence for this clonality by analysis of the *tox*RS sequence. They found that *tox*RS sequences of representative strains of the O3:K6 clone differed from those of O3:K6 strains isolated before 1995 by at least seven nucleotide base positions within a 1346-bp region. Furthermore, they developed a PCR method that targeted two of the base positions unique to the new O3:K6 clone. This method differentiated 172 strains belonging to the new O3:K6 clone from O3:K6 strains isolated from earlier time periods and only showed cross-reactions with *tdh*-positive and *trh*-negative strains belonging to O4:K68 and O1:K untypeable serovars that were isolated from travelers beginning in 1997. The authors proposed that these strains diverged from the new O3:K6 clone by alteration of O:K antigens.

9.3.3.2 *Hemolysin*

The 46-kDa homodimer secreted TDH is a major virulence factor of *V. parahaemolyticus*. TDH is a pore-forming toxin and has been extensively studied for its ability to hemolyze mammalian erythrocytes. Moreover, TDH induces a variety of cytotoxic effects that lead to cell degeneration and loss of viability. TDH also causes intestinal fluid secretion and induces a rapid transient increase in intracellular calcium that inhibits the cell cycle. The resulting morphological alterations appear to depend on the structure of the microtubular network.

Before molecular methods were available, TDH activity (hemolysis; termed the 'Kanagawa Phenomenon') was detected on blood agar supplemented with NaCl. Kanagawa Phenomenon (KP)-positive strains possessed both *tdh*1 and *tdh*2 genes. The *tdh*2 gene is primarily responsible for hemolytic activity of KP-positive strains, although both genes may be relevant to pathogenicity. In this regard, KP-negative strains have been isolated from clinical cases. Investigators demonstrated that two bases (positions 24 and 34) within the *tdh*2 promoter sequence are largely responsible for the difference in the promoter strength between the *tdh*2 and *tdh*1 genes. They showed that a single base substitution of the *tdh* promoters of KP-negative strains at position 34 was sufficient to increase the expression of *tdh* genes to the KP-positive level. For this reason, *tdh* genes of KP-negative strains may be significant since they can produce a KP-positive subclone by a single point mutation.

The binding of *tdh* to cell membranes is temperature-independent. For human erythrocytes, bound hemolysin produces cell disruption in a temperature-dependent manner. Some researchers propose that ganglioside asialo-GM_2 is the most potent receptor, but binding may also involve asialo-GM_1 and lactocerebroside.

TDH induces phosphorylation of two proteins (25 and 22.5 kDa) on human erythrocyte membranes. Phosphorylation of a 25 kDa protein is essential for hemolysis by TDH after binding. This protein differs from the 37 kDa protein (glyceraldehyde 3-phosphate dehydrogenase) that disappears from erythrocyte membranes treated with TDH.

The N-terminal region of TDH is likely involved in the cell binding process, while the region near the C-terminal may be involved in post-binding events. These interpretations were derived from studies using TDH/TRH chimeric proteins and two monoclonal antibodies that recognized different epitopes on TDH.

Interestingly, TDH causes a dose-dependent increase in intracellular free calcium in both human (Caco-2) and rat (IEC-6)) cell monolayers. The TDH-activated ion influx pathway could cause not only calcium influx but also sodium and manganese ions. The effect was reversible only at low toxin concentrations. Conversely, at high TDH concentrations, calcium-independent cell death was induced.

TDH is cytotoxic to the Intestinal 407 cell line, which is accompanied by damage to the plasma membrane and lysosomes, as well as cellular degeneration. Although an increase in cytosolic free Ca^{2+} has been observed in cells treated with TDH, the morphological effects on Intestinal 407 cells are not mediated by Ca^{2+}-dependent pathways. Others have hypothesized that the enterotoxicity caused by TDH in the nontumoral rat crypt-derived cell line IEC-6 results from induced intestinal chloride secretion using GT1b as a putative receptor and Ca^{2+} as a second messenger.

One research group proposes that a fourth hemolysin (TDH/II) is produced by *V. parahaemolyticus*, in addition to TDH, TRH and TDH/I. This hemolysin was characterized from a KP-negative, O13:K untypeable clinical isolate and found to stimulate vascular permeability in rabbit skin and be lethal to mice. Purified TDH/II and viable cells of the Vp-TDH/II-producing strain both induced fluid accumulation in ligated rabbit intestine.

Interestingly, a correlation was reported between urease-positive *V. parahaemolyticus* strains and the *trh* gene. These genes lie in close proximity with the *tdh* gene on the chromosome. Pulsed-field gel electrophoresis of *Not*1-digested DNA from 115 clinical isolates, followed by Southern hybridization with probes to *tdh*, or *trh*, or *ure* genes, display a single fragment. The majority of strains (81%) had two copies of *tdh* on the chromosome and no copies of *trh* or *ure*. Seven percent of the strains possessed the *tdh*, *trh* and *ure* genes on chromosomal DNA. Of these latter strains, all three genes were detected on a single *Not*I

fragment. Using long and accurate polymerase chain reactions, the distance between *trh* and *ure* was determined to be less than 8.5 kb.

9.3.3.3 *Iron*

Regulation of iron and its role in pathogenesis of *V. parahaemolyticus* is not well understood compared to other virulence factors. Under iron-restricted conditions, *V. parahaemolyticus* produces a siderophore, vibrioferrin, along with two outer membrane proteins of 78 and 83 kDa. The ferric vibrioferrin-binding protein of 78 kDa may be a receptor for ferric vibrioferrin involved in the early events of vibrioferrin-mediated iron uptake.

It has also been shown that *V. parahaemolyticus* utilizes heme and hemoglobin as iron sources, and contains chromosomal DNA similar to several other *Vibrio* spp. with heme iron utilization genes. Some suggest that the heme iron utilization systems of certain pathogenic *Vibrio* spp., particularly *V. parahaemolyticus* and *V. alginolyticus*, are similar at the DNA level, the functional level and in the case of *V. parahaemolyticus*, the amino acid sequence or protein level, to that of *V. cholerae*.

The growth of pathogenic and nonpathogenic strains of *V. parahaemolyticus* on iron-limited agar plates is enhanced by ferritin, lactoferrin and transferrin at 30 μM, and also by hemin, hemoglobin and ferric ammonium citrate at 100 μM. This property is also believed to enhance virulence, as demonstrated in suckling mice and adherence to mouse intestine.

9.3.3.4 *Adherence*

The OMP of KP-positive strains appears to be involved in attachment of *V. parahaemolyticus* to epithelial cells. Using antisera raised in rabbits, subsequently absorbed with the LPS fraction, the anti-outer membrane protein serum and its Fab (IgG) fragment inhibited adherence of KP-positive strains to rabbit intestinal epithelial cells in vitro. Preincubation of rabbit intestinal epithelial cells with outer membrane preparations also inhibited adherence of KP-positive strains. A similar effect was not observed with anti-LPS serum or pretreatment of rabbit intestinal epithelial cells with LPS.

In more detailed studies of structures involved in adherence, a positive correlation was observed between cell-associated mannose-sensitive hemagglutination and adherence of *V. parahaemolyticus* to rabbit enterocytes. The hemagglutinin was a heat-labile protein consisting of four identical subunits of approximately 26 kDa. Adherence of *V. parahaemolyticus* to rabbit enterocytes was inhibited by pretreatment of the bacterial cells with D-mannose and with the Fab fraction of immunoglobulin G against the purified hemagglutinin. Immunoelectron microscopy showed that the hemagglutinin was on the bacterial cell surface and not pili.

9.3.3.5 *Bile*

Several investigators propose that bile in the human intestine stimulates production of TDH. Either glycocholic acid or taurocholic acid stimulated 4- to 16-fold greater production of TDH into the cell medium compared to medium without the bile acids. Some investigators suggest that a bile acid-containing environment, similar to that found in the human intestine, favors growth of virulent strains and that bile acids enhance the expression of some virulence factors. They found that the addition of bile or bile acid deoxycholic acid to estuarine water-cultured bacteria led to an increase in the direct viable count and colony counts among virulent strains. The effect was not observed for avirulent strains and could be reversed by

extracting bile acids from the test matrix. In addition, they found that adding bile to laboratory cultures enhanced Congo red binding, bacterial capsule size and adherence to epithelial cells. Others propose that ToxR is a conserved protein involved in the modulation of outer membrane proteins and bile resistance of pathogenic *Vibrio* species, and that ToxR-mediated bile resistance is an early step in the evolution of *Vibrio* spp. as intestinal pathogens.

9.3.3.6 *Strain diversity*

A fundamental discovery about the structure of the *V. parahaemolyticus* genome was reported. Researchers found that *V. parahaemolyticus* strains, similar to most *Vibrio* spp., possess two chromosomes. Specifically, a physical map of the genomic DNA (5.1 Mb) for *V. parahaemolyticus* strain AQ4673 showed two circular replicons of 3.2 and 1.9 Mb. The presence of chromosomes rather than large plasmids was indicated by the observation of 16S rRNA genes on both the replicons.

Various laboratories have evaluated different methods to characterize or type *V. parahaemolyticus* strains of clinical and environmental origin. Researchers analyzed isolates by enterobacterial repetitive intergenic consensus sequence (ERIC) PCR, RFLP in rRNA genes (ribotyping), PFGE and RFLP analysis of the genetic locus (*fla*) encoding polar flagellum. They reported that ERIC and RT were the most useful typing methods when used in combination, while *fla* RFLP was less discriminatory. A high level of DNA degradation was observed when applying PFGE tests with various restriction endonucleases.

In a separate examination of *V. parahaemolyticus* strains that emerged in Calcutta, India from February 1996 to June 1998, investigators could not demonstrate RFLP in the gene encoding TDH. Clonal diversity was shown by RFLP of *rrn* operons and by PFGE.

Recently (2006 to 2007), PFGE, ERIC and repetitive extragenic palindromic PCR (REP-PCR) were used to identify sources of virulent strains and to measure persistence in time and space, of particular clones. In this study, environmental *V. parahaemolyticus* strains were isolated from the Italian Adriatic Sea. A number of genetic clusters or clones appeared to persist over time and reappeared in marine environments for subsequent months.

Automated RT (*Eco*RI) was compared to PFGE (*Not*I) to determine the genetic diversity of 22 O3:K3 isolates collected before and after 1996. RT yielded 13 patterns and was not able to separate post-1996 O3:K3 isolates from pre-O3:K3 isolates. PFGE produced 22 patterns and was able to discriminate pre- and post-1996 O3:K3 isolates. These results indicate that PFGE is a more discriminatory and reliable tool to identify isolates belonging to the pandemic clonal complex.

Matsumoto and co-workers used a novel group-specific PCR (GS-PCR) assay targeting sequence variability in the *tox*RS gene to identify pandemic O3:K3 isolates. This method was able to discriminate 100% of the pandemic isolates from 166 isolates representing 28 serovars.

MLST has also been used to identify pandemic strains and to study the phylogenetic relationships. Seven housekeeping genes located on chromosome II and/or chromosome I were used to type 100 *V. parahaemolyticus* isolates recovered from different geographical regions from 1951–2005. These isolates represented 35 serotypes from clinical (n=37) and environmental (n=63) sources. Three distance clusters and 62 unique allelic profiles were observed. The first cluster contained all pandemic isolates, the second cluster contained clinical and environmental isolates from the Gulf of Mexico and the third comprised varying sources along the Pacific coast. The pandemic cluster contained isolates collected from four continents. The results of this study indicate that MLST is useful to discriminate pandemic from nonpandemic isolates, as well as identify the geographic origin or relatedness of nonpandemic strains.

Genome analysis of two prepandemic and three nonpandemic *V. parahaemolyticus* isolates revealed that the *trh*- and *tdh*-positive strains had different pathogenicity island and mobile elements. In addition, single nucleotide polymorphism (SNP) analysis showed that 94% of SNPs among O3:K6 and O4K68 pandemic isolates were within a 141 kb region that surrounds the O- and K-antigen-encoding gene clusters. Moreover, comparison of the core genes of *V. parahaemolyticus* with those of *V. vulnificus* and *V. cholerae* indicates differences between these three pathogenic species. Of the core genes, 40–59% were conserved and 14–24% of the core genes were species-specific.

9.3.4 Detection

A standard method for the isolation, detection, and enumeration of *V. parahaemolyticus* is found in the FDA *Bacteriological Analytical Manual*. Sample preparation and enrichment are similar to that described above for *V. vulnificus*. On TCBS agar, *V. parahaemolyticus* colonies are green or blue-green, round, and 2–3 mm in diameter. *Vibrio parahaemolyticus* can be differentiated from non-*Vibrio* using TSI, KIA, and arginine glucose slants, oxidation fermentation and oxidase tests and other methods. *Vibrio parahaemolyticus* will not grow in 0% NaCl but does in 3–8% NaCl.

The characteristics below are presumptive for *V. parahaemolyticus*:

- Gram-negative rod
- Growth at 42 °C, positive
- NaCl test: 0% NaCl, negative; 3, 6, and 8% NaCl, positive; 10% NaCl, negative
- Sucrose fermentation, negative
- ONPG test, negative
- Arabinose fermentation, positive
- TSI appearance: alkaline slant/acid butt; gas production, negative; H_2S, negative
- Hugh–Leifson test: glucose oxidation and fermentation, positive
- Cytochrome oxidase, positive
- Arginine dihydrolase test, negative
- Lysine decarboxylase test, positive
- Ornithine decarboxylase test, positive
- Voges–Proskauer test, negative
- Sensitivity to O/129: sensitive to 150 μg, resistant to 10 μg

Researchers have conducted extensive serological analyses of *V. parahaemolyticus* isolates that form the basis of a common serological typing scheme. Currently, there are 12 O serogroups and at least 70 K antigens. Advances are being made to replace typing antisera with gene probes.

The presence of the thermostable direct hemolysin (*tdh*) and the TDH-related hemolysin (*trh*) genes and their gene products (TDH and TRH) serve as useful tools to identify potentially virulent *V. parahaemolyticus* strains. Historically, the Kanagawa reaction has been used to demonstrate the presence of TDH using Wagatsuma agar. While it measures the functionality of hemolysins, it is a tedious process and more subjective than molecular-based methods that detect *tdh* and *trh* genes.

Recently, several investigators developed real-time PCR assays, or quantitative PCR (qPCR), for the detection of total and pathogenic *V. parahaemolyticus* in water and shellfish using primers amplifying sequences of the *tlh* (species-specific gene), *tdh*, and *trh* genes in

conjunction with a dual-labeled flurogenic probe. This procedure involves preparation of samples, amplification of target DNA followed by detection and interpretation of results. The assay is sensitive (1 CFU/reaction), specific and rapid (~24 hours).

9.3.5 Prevention and control

Most *V. parahaemolyticus* infections can be prevented by properly cooking seafood and avoiding contamination of cooked seafood with seawater and raw seafood. As stated for *V. vulnificus*, persons with immunocompromised health should avoid consuming raw shellfish. Wound infections can be prevented by avoiding exposure of open wounds to warm, brackish seawater. Industry and government risk management strategies are similar to those described in the above section for *V. vulnificus*.

9.4 *Vibrio cholerae*

9.4.1 Introduction

Vibrio cholerae is the etiological agent of pandemic cholera, a human gastrointestinal disease manifested by severe diarrhea, dehydration and electrolyte imbalance. The endemic nature of cholera in various areas of the world illustrates that transmission occurs when untreated human waste and domestic water mix. Of the various human gastrointestinal pathogens, *V. cholerae* has been the subject of numerous studies to elucidate the disease process and how it survives in the environment. Today, there is a wealth of new information that illustrates the complex bacterial and host processes that produce cholera, as well as factors that promote its survival in aquatic environments.

Historically, a small subset of *V. cholerae* strains was considered to be capable of causing epidemic cholera. These strains produced cholera toxin (CT) and expressed the O1 antigen. Other *V. cholerae* strains were not pathogenic or caused mild diarrhea, and were referred to as 'non-O1' strains. However, this dogma was dispelled in the early 1990s when another serovar, *V. cholerae* O139, caused an epidemic in India. It is believed that the *V. cholerae* O139 epidemic strain likely emerged from the seventh pandemic O1 El Tor strain through a genetic rearrangement involving the horizontal transfer of exogenous O antigen and capsule genes. It is suggested that environmental *V. cholerae* O22 may have been the source of the exogenous DNA resulting in the emergence of epidemic *V. cholerae* O139.

In recent decades, very profound discoveries have been made about the virulence of *V. cholerae*, including discovery of two replicons, the virulence cassette and how environmental signals control and orchestrate the expression of specific genes. These findings show that processes leading to cholera are dynamic. In addition, they offer new insights into potential virulence properties of other bacterial pathogens.

For a more in-depth discussion of the pathogenicity, virulence and ecology of *V. cholerae*, the reader is directed to other publications, including those by Barua and Greenough, Wachsmuth and co-workers, and Kaper and co-workers.

9.4.2 Ecology

Vibrio cholerae replicates in environmental niches, including fresh and estuarine waters, in association with plants and animals, and in foods of plant and animal origin. The primary environmental factors that affect growth are temperature, nutrient level, acidity and water activity (a_w).

In the aquatic environment, *V. cholerae* is part of the endogenous (autochthonous) microflora, where growth is enhanced by moderate-to-high temperature and low-to-moderate salinity. The ecology of *V. cholerae* has been extensively studied in cholera-endemic regions of the world where researchers have investigated environmental factors associated with seasonal cholera.

In this regard, plankton support the growth and survival of *V. cholerae* that colonizes exoskeletons of certain zooplankton and phytoplankton species. In recent studies, satellite imagery has been used as an indirect means to assess the risk of *Vibrio* spp. infections by monitoring global surface water temperature and associated plankton levels.

The relationship among local incidence of cholera, prevalence of *V. cholerae* in the aquatic environment and bacteriophage that target O1 and O139 serogroups, was investigated in Dhaka, Bangladesh over a three-year period. The number of cholera patients varied seasonally and coincided with the presence of pathogenic *V. cholera*e strains in water samples that lacked detectable cholera phage. During interepidemic periods, water samples contained cholera phage but no viable *V. cholerae*. These results indicate that seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phage. Moreover, cholera phage may play a role in the emergence of new *V. cholerae* pandemic serogroups or clones.

Environmental and epidemiological surveillance of *V. cholerae* in another cholera endemic region, Chandigarh, India, was studied from 2007 to 2008. *Vibrio cholerae* was isolated from 59.5% of water and plankton samples, and two-thirds of the isolates were resistant to various antibiotics. The prevalence of this organism correlated with water and air temperature, but not rainfall.

More recently (2010), water and seafood samples were analyzed for *V. cholerae* during the cholera outbreak in Haiti. Eleven and 33% of water and seafood samples, respectively, were positive for toxigenic *V. cholera*e serotype Ogawa.

The ecology of *V. cholerae* has also been studied in developed countries where cholera is not endemic. Water and sediment samples were collected for one year (2002 to 2003) from six sites on the east coast of North Carolina and analyzed by molecular methods. *Vibrio cholerae* was detected infrequently and at very low levels; its presence correlated with water temperature and total *Vibrio* levels. These results indicate that environmental conditions at these sites were not suitable for its growth and survival.

An ecological and molecular epidemiological study was carried out in aquatic environments of Okayama, Japan, from 2007 to 2009. Though *V. cholerae* O1 strains were rarely observed, non-O1/non-O139 strains were isolated frequently. These strains possessed *hlyA* (a gene encoding hemolysin) and *tox*R genes (a gene controlling the pathogenic island of the *V. cholerae* genome), but not *ctx*A gene (the gene of cholera toxin), indicating potential horizontal transfer of virulence factor genes among strains.

In 2010, 138 fish and shellfish samples were collected from Swiss markets and analyzed for the occurrence of *V. cholerae*. This bacterium was isolated from three samples and none of the isolates contained *ctxA* gene.

9.4.3 Epidemiology, pathogenicity and virulence

Common vehicles for cholera are contaminated water and food. Transmission can occur through direct ingestion of contaminated water or via foods that support the growth of *V. cholerae*. Infection with *V. cholerae* is often mild or subclinical, but symptoms may sometimes be severe. The incubation period of cholera ranges from several hours to five days and depends on inoculum size. Common symptoms are the sudden onset of 'rice-water'

stools, vomiting, muscle cramps and anorexia. Approximately one in 20 infected people show severe symptoms such as dehydration, increased heart rate and shock.

Human volunteer experiments show that high inoculum levels (~10^{11} CFU) are required to survive passage through the stomach acid barrier, colonize the small intestine and cause diarrhea. In contrast, only 0.001% of this inoculum level is needed to produce acute diarrhea when stomach acid is neutralized with sodium bicarbonate or with non-acidic food.

Once in the small intestine, flagella transport *V. cholerae* to mucosal enterocytes where it attaches and proliferates to concentrations as high as 10^7 to 10^8 CFU per gram of small intestine. While actively colonizing the mucosa and closely approximating themselves to enterocytes, *V. cholerae* produces cholera toxin (CT) that is responsible for changes in fluid transport across epithelial surfaces of the small bowel.

The expression of several *V. cholerae* virulence factors is regulated by *Tox*T and two membrane proteins, TcpP/H and ToxR/S. These are required for *tox*T transcription. Toxigenic *V. cholerae* strains are lysogens of CTXPhi, a filamentous phage that encodes cholera toxin. The receptor for CTXPhi is the toxin-coregulated pilus (TCP), the genes for which reside in a large genetic element, the TCP pathogenicity island.

Adherence to intestinal mucosa involves a variety of factors. TCP consists of long filaments attached to the lateral surface of *V. cholerae* O1. When the *tcp*A gene is mutated, human volunteers do not experience diarrheal illness. Other factors that have a potential role in *V. cholerae* colonization of the small intestine include accessory colonization factor (ACF), core-encoded pilus, LPS, mannose-sensitive hemagglutinin, mannose-fucose-resistant hemagglutinin, outer membrane protein U and the polysaccharide capsule of O139 strains.

Following attachment and colonization of the small intestine, CT is produced and affects normal ion transport across epithelial cell surfaces. This leads to chloride secretion by the cell, water loss and severe diarrhea. CT is comprised of two subunits, A and B. Subunit A (CT-A) is present as a single subunit and possesses toxic activity that results in activation of membrane-bound adenylate cyclases. There are five B (CT-B) subunits that are receptors for epithelial GM_1 ganglioside, which bring the holotoxin in proximity to the epithelial cell surface.

Using monoclonal antibodies, at least three immunological forms of CT-B have been described. The classical biotype of *V. cholerae* produces CT-1, the El Tor biotype produces CT-1 and CT-2, and non-O1 serotypes of *V. cholerae* produce CT-1, CT-2, and CT-3. Clinical strains of *Vibrio mimicus* produce CT-1. Epitopes that are common to CT and the heat-labile enterotoxin (LT) of *E. coli* are located in the GM_1 ganglioside-binding site. Three of the four genetic forms of CT-B match the three immunological forms.

CT catalyzes the transfer of ADP-ribose of NAD to the G_s protein, a regulator of adenylate cyclase. This action leads to increased Cl^- secretion by intestinal crypt cells and decreased NaCl-coupled absorption by villus cells. The net movement of water from the cell into the intestinal lumen results in the watery diarrhea manifested in cholera.

Lysis of sheep erythrocytes is one test used to differentiate the El Tor biotype of *V. cholerae* from the classical biotype, although more recent El Tor strains have less hemolytic activity. The role of El Tor cytolysin/hemolysin in human infections has been assessed and the recombinant strain still caused diarrhea in 33% of the volunteers. Therefore, its importance as a virulence factor is not completely resolved. A single base-pair difference in classical and El Tor promoters is responsible for differential regulation of virulence gene expression in these two biotypes.

Vibrio cholerae also produces a toxin that changes the permeability of the small intestine by affecting the cytoskeletal structure of the intercellular tight junction (*zonula occludens*). Normally, the tight junction prevents the movement of compounds through the intercellular space. This toxin (Zot), in conjunction with other virulence factors, may contribute to the

diarrheal symptoms of cholera. The *zot* gene has been demonstrated in various O1 and non-O1 strains, and is co-regulated with the *ctx* gene. Another *ctx-zot* co-regulated factor is the accessory cholera enterotoxin (ace).

There is other evidence showing that prostaglandins (PGE) may have a significant role in the symptoms of cholera. Cholera patients have elevated concentrations of PGE_2 in their jejunum and PGE_2 causes fluid accumulation in ligated rabbit ileal loops. Furthermore, the enteric nervous system may be affected by *V. cholerae* infection, causing changes in intestinal secretion and absorption.

9.4.3.1 *Strain diversity*

Epidemiologists and evolutionary microbiologists are interested in the origin of bacterial strains, and rely heavily on the use of phenotypic and genotypic characteristics as discriminatory tools. Although useful information can be derived from phenotypic properties of bacteria, the genome offers more detailed and stable characteristics.

Serology was one of the earliest methods used to type *V. cholerae* and showed that the O1 serogroup could be subdivided into Ogawa and Inaba serotypes. Also, O1 strains could be divided into two biotypes, classical and El Tor, based on phenotypic tests including erythrocyte hemolysis and agglutination, sensitivity to polymyxin B and biochemical reactions. With the discovery of isoenzymes, the multilocus enzyme electrophoretic technique was used to type *V. cholerae* strains. The genetic diversity of *V. cholerae* strains has also been studied using various RFLP techniques, including PFGE, RT and specific gene RFLP patterns, as well as sequencing CT genes. Both RT and PFGE have demonstrated geographic and temporal stability, although PFGE is more discriminatory for typing *V. cholerae* isolates than RT.

A variation on this technique is PCR-based methods such as amplified fragment length polymorphism (AFLP), RAPD-PCR, and ERIC-PCR, and REP-PCR. Recently, 43 *V. cholerae* of different serogroups from various sources were characterized using RAPD-PCR, ERIC-PCR and REP-PCR. These approaches were evaluated on typeability, reproducibility, stability and discriminatory power. ERIC-PCR was more discriminatory and VCR-PCR proved useful in separating non-O1/non-O139 strains. RAPD-PCR and REP-PCR were not suitable due to lack of reproducibility and stability.

Whole genome sequencing typing (WGST) of 23 *V. cholerae* strains isolated from a variety of sources over the past 98 years revealed 12 distinct lineages. All seventh pandemic clones share nearly identical gene content. The results of this study also recommend that genome assortments, not serogroup, should be used to define pathogenic *V. cholerae* clones as this bacterium undergoes extensive genetic recombination via lateral gene transfer.

WGST, PFGE and antimicrobial susceptibility were used to characterize 24 recent *V. cholera*e recovered from five different districts in Nepal. In addition, the 24 genomes were compared to 10 previously sequenced *V. cholerae* isolates, including three from the Haitian outbreak. The results of this study, indicating that the Haitian isolates were brought from Nepal, show that WGST is a powerful tool for epidemiological investigations.

9.4.4 Detection

Methods for the isolation, detection, and enumeration of *V. cholerae* are found in the FDA *Bacteriological Analytical Manual*. The methods for preparing and enriching environmental and food samples for *V. cholerae* are similar to those described for *V. vulnificus*, above, with

some modification. On TCBS agar, *V. cholerae* (E1 Tor and classical biotypes) appears as large, smooth, yellow (sucrose-positive) and slightly flattened colonies with opaque centers and translucent peripheries. Tolerance to salt is a simple method to differentiate *V. cholerae* from other *Vibrio* spp. Specifically, by using agar or broth containing 0 and 3% NaCl, *V. cholerae* and *Vibrio mimicus* grow in the presence of added salt, whereas most other *Vibrio* spp. only grow in 3% NaCl.

Diagnostic antisera and monoclonal antibodies can be used to identify the Ol group of *V. cholerae* as well as the subgroup Inaba (factors AC) and Ogawa (factors AB). Other antisera are available to identify serotype O139. For serology, colonies should originate from nonselective agar to avoid false-positive and false-negative reactions. Cultures confirmed as Ol can be further identified as classical and El Tor biotypes using polymyxin B sensitivity, hemolysin test, Voges–Proskauer test, and bacteriophage susceptibility.

These characteristics are considered to be presumptive of *V. cholerae*:

- Gram-negative rods
- TSI or KIA: acid slant/acid butt; gas production, negative; H_2S, negative
- Hugh–Leifson test: glucose fermentation and oxidation, positive
- Cytochrome oxidase, positive
- Arginine dihydrolase test, negative
- Lysine decarboxylase test, positive
- Ornithine decarboxylase test, positive
- Growth at 42 °C, positive
- NaCl test: 0% NaCl, positive; 3% NaCl, positive; 6% NaCl, negative
- Sucrose fermentation, positive
- ONPG test, positive
- Arabinose fermentation, negative
- O/129 sensitivity, sensitive to 10 and 150 g O/129

For detection of *Vibrio cholerae*, a multitarget molecular beacon-based real-time nucleic acid sequence-based amplification (NASBA) assay has been developed. Target genes include *ctx*A, *tcp*A, *tox*R, hemolysin (*hyl*A), and the 60-kDa chaperonin product (*gro*EL). This assay depends on the simultaneous activity of three different enzymes such as RNaseH, avian myeloblastosis virus reverse transcriptase and T7 RNA polymerase. Molecular beacon probes for the five different targets are labeled with 6-carboxy-fluorescein (FAM) at the 5'- and Dabsyl (a universal quencher) at the 3'-ends. The NASBA assay is a sensitive, isothermal (41 °C) transcription-based amplification system that specifically detects messenger RNA. The main steps in this assay are production of single-stranded RNA amplicons and detection by hybridization with a sequence-specific probe, such as a molecular beacon. The entire assay can be completed within three hours.

A quadruplex real-time PCR assay is available to detect and identify *Vibrio cholerae* O1 and O139 strains, and determine virulence potential. In this method, *ctx*A, *hyl*A, O1-specific *rfb*, and O139-specific *rfb* genes are targets. The four probes are labeled with four different fluorophores to differentiate O1 and O139 strains from non-01, non-O139 and to determine if bacteria contain toxin genes.

Another procedure is a validated real-time assay based on the TaqMan technology for simultaneous detection of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* with an Internal Amplification Control. The target genes are *toxR* gene for *V. parahaemolyticus*, the s*odB* gene *V. cholerae* and *vvha* gene for *V. vulnificus*. In addition, the assay differentiates pathogenic

and nonpathogenic isolates of *V. parahaemolyticus* and *V. cholerae*. The reported sensitivity of the assay is 1 CFU/25 g of samples after one-step enrichment.

9.4.5 Prevention and control

Cholera epidemics typically occur in geographical regions where there is an increased risk of human feces contaminating drinking water and food. In these situations, it is important to ensure a safe drinking water supply, that food is handled safely and that human feces are safely disposed. Industry and government food safety risk management strategies are similar to those described in the above sections for *V. vulnificus* and *V. parahaemolyticus*.

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10 *Yersinia enterocolitica*

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10.1 Introduction

While not a major foodborne pathogen, *Yersinia enterocolitica* is responsible for approximately 100,000 cases per year of foodborne illness in the United States. The pathogen was discovered 70 years ago in the United States by Schleifstein and Coleman, who named it *Bacterium enterocoliticum*. Little was known about this bacterium until the early 1960s when clinical and veterinary microbiologists reported the isolation of bacteria similar to *B. enterocoliticum* from patients and animals. Consequently, these isolates were classified and named *Y. enterocolitica* (i.e., pertaining to the intestine and colon). The reclassification of this organism makes possible a more meaningful evaluation of the distribution of this species and its pathogenicity. Subsequent studies showed its ubiquitous distribution in the environment and its presence in food and clinical samples.

10.2 Nature of illness

Yersinia enterocolitica is recognized as a foodborne pathogen, and the disease caused by this bacterium is called yersiniosis. Symptoms of yersiniosis include severe abdominal pain that suggests an appendicitis-like attack, as well as fever, diarrhea, headache, and vomiting. The very young and very old are most susceptible to *Y. enterocolitica* infection. The incubation period for yersiniosis is 24–36 h or longer. The infection is self-limiting and generally resolves in 1–3 days. A large number of food-associated outbreaks of yersiniosis have been

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reported in developed countries and *Y. enterocolitica* can be isolated from 1–2% of all human cases of acute enteritis. The estimate for the number of cases of foodborne yersiniosis is 97,656/year (range 30, 388–172,734/year) in the United States resulting in an estimated 29 deaths/year (range 0–173).

10.3 Characteristics of agent

Yersinia enterocolitica are members of the *Enterobacteriaceae* family. It is a Gram-negative, oxidase-negative, catalase-positive, nitrate-reductase-positive, cold-tolerant facultative anaerobic rod, 0.5–0.8 × 1–3 μm in size, and exhibits significant pleomorphism. The organism is not motile when grown at 37 °C but is motile at 22–25 °C, with relatively few peritrichous flagella when grown at less than 30 °C. In addition, the bacterium is urease positive, ferments mannitol, and produces gas from glucose. *Yersinia enterocolitica* differs from most members of the family Enterobacteriaceae in that it grows slowly at 37 °C.

Yersinia enterocolitica is a cold-tolerant pathogen. It can grow at temperatures as low as 0 °C. However, the bacterium can grow at temperatures as high as 44°C, with the optimum temperature being 32–34 °C. The bacterium is inactivated at 50 °C. The pH range for growth is pH 4.5–8.5, with an optimum of pH 7–8. The organism can grow in the presence of 0.5–5% of NaCl. *Yersinia enterocolitica* can survive in frozen conditions for at least 12 weeks. The organism survives various stresses that would be imposed by the food environment and food processing conditions. Surviving cells of *Y. enterocolitica* exposed to stresses such as heat (45 ° to 55 °C), freezing (–20 °C), anaerobic atmospheres (N_2/H_2; CO_2/H_2; vacuum), osmotic stress (NaCl) retained the virulence plasmid (pYV). The retention of pYV during exposure to these stressful events indicates that the organisms could lead to disease if present in food.

Human pathogenic strains of *Y. enterocolitica* have a number of properties that confer virulence on the organism. Several of these strains exhibit a marked temperature-dependent expression of genes that are correlated with the presence of a 70- to 75-kbp pYV that is directly involved with virulence. A number of pYV-mediated phenotypic characteristics including colony morphology, low-calcium response (Lcr), Congo red (CR) uptake, crystal violet (CV) binding, autoagglutination (AA), serum resistance, tissue culture detachment, and hydrophobicity (HP) are correlated with virulence of *Y. enterocolitica*. At a low level of calcium ions, pYV also encodes for 'Yops' (*Yersinia* outer membrane proteins: i.e., a set of proteins secreted by pYV-bearing virulent strains), which are important virulence factors. The delivery of Yops into the host cell subverts or modulates normal host cell signal transduction and cytoskeletal functions. Another pYV-encoded, calcium-independent, outer membrane protein, YadA, mediates cellular attachment and entry. These physiological traits associated with pYV are expressed only at 37 °C. However, growth at 37 °C in laboratory culture also fosters the loss of pYV and the concomitant disappearance of the associated virulence characteristics. Elements encoded by the chromosome are also necessary for virulence. Pathogenic *Y. enterocolitica* share two chromosomal loci, *inv* (i.e., the invasion loci that mediates the penetration of host cells) and *ail* (i.e., the attachment-invasion loci that mediates bacterial attachment to host cells), which are involved in the first step of pathogenesis. The heat-stable enterotoxins (Ysts) are encoded by *yst* genes and may be involved in the induction of diarrhea. However, the role of Ysts in the pathogenesis *Y. enterocolitica* is not fully understood.

10.4 Epidemiology

Infection by *Y. enterocolitica* is a zoonotic diease. The organism is commonly found in the tongue, tonsils, and feces of pigs and this fact indicates that the meat from colonized pigs can be contaminated during slaughter. It is possible that contaminated pork can lead to cross-contamination of other foods during the preparation of meals, particularly those food items that are not cooked. The presence *Y. enterocolitica* in pig fecal matter may account for a large percentage of environmental contamination of soil and water. Ruminants are rarely reservoirs for *Y. enterocolitica*. However, it is interesting that milk and other dairy products have been associated with several outbreaks of *Y. enterocolitica* infections but it is not clear to what extent dairy products initiate sporadic cases of disease. Poultry do not appear to be carriers of pathogenic *Y. enterocolitica*. Pets (dogs and cats), rodents (rats), and wild animals may be fecal carriers of pathogenic *Y. enterocolitica*. The intimate contact between humans (particularly children) and pets suggests that pets may be a potential source of transmission of *Y. enterocolitica*. It is probable that domestic pigs constitute the largest reservoir of pathogenic *Y. enterocolitica*.

The primary transmission route of *Y. enterocolitica* into the human body is fecal–oral through ingestion of raw or inadequately cooked pork (due to fecal contamination during slaughter) or foods contaminated by an infected food handler. Untreated water has also been implicated in the disease. Common food vehicles in outbreaks of yersiniosis are meat (particularly pork), milk, dairy products, powdered milk, cheese, tofu, and raw vegetables. Since *Y. enterocolitica* can grow at low temperatures, even refrigerated foods are potential vehicles for the growth and dissemination of these organisms. The majority of food isolates differ in biochemical (based on biochemical reactions, termed biovars) and serological (based on lipopolysaccharide surface O antigens, termed O serovars) characteristics from 'typical' clinical strains and are usually called 'nonpathogenic' or 'environmental' *Yersinia* strains.

Person-to-person transmission may occur if a person with *Y. enterocolitica*-induced diarrhea handles objects used by another person (for example, children's toys) or if an infected person with poor personal hygiene prepares food, especially raw or uncooked foods. Animal-to-human transmission may occur if an individual comes into contact with the feces of animals (rodents, pets, domestic and wild animals) carrying *Y. enterocolitica* or with water or food contaminated by those animals.

Blood transfusion from an infected donor has been associated with *Y. enterocolitica*-associated sepsis. Rarely, an individual may be infected if a wound is inoculated from a *Y. enterocolitica*-contaminated environmental source. Thus, while contaminated food and water are the major routes of transmission of *Y. enterocolitica* to humans, other routes have also been implicated.

Outbreaks of yersiniosis are uncommon considering the widespread occurrence of *Y. enterocolitica* in the environment, its ability to colonize and persist within animals, and its ability to grow at refrigerated temperatures. Most of the human infections caused by *Y. enterocolitica* are sporadic. The data presented in Table 10.1 suggest that outbreaks are rare and contribute little to the total incidence of *Y. enterocolitica* disease. It is believed that 90% of *Y. enterocolitica* infections are foodborne. In the United States, *Y. enterocolitica* is a minor foodborne bacterial pathogen since only 0.027% (97,656/3,645,773) of the foodborne illness caused by bacteria is due to *Y. enterocolitica*.

Table 10.1 Recent outbreaks of *Yersinia enterocolitica*

Country, date	Serotype, biotype	Vehicle	Number of cases
Pennsylvania, March 24 to August 5, 2011	?	Glass-bottled water, milk, ice cream	16
Norway, March 2011	O:9	Bagged salad mixtures	17
Pennsylvania, 2008 to 2010	?	?	8
Australia, July 2009	?	Asian roast pork, BBQ pork	3 (family outbreak)
Japan, July 2006	O:9, 2	Pork (?)	3 (family outbreak)
Norway, February 2006	O:9, 2	Ready-to-eat pork	11
Japan, August 2004	O:8	Salads	42
Croatia, January 2002 (oil tanker)	O:3	?	22
United States, November 2001–February 2002	O:3, 4	Pork chitterlings	12

10.5 Bacteria–human host interaction

The most common symptom of *Y. enterocolitica* infection is gastroenteritis with a clinical picture of self-limited diarrhea associated with mild fever and abdominal pain. Mesenteric lymphadenitis (inflammation of the mesenteric lymph nodes) mimicking appendicitis may occur. Transient carriage, as well as excretion of *Y. enterocolitica*, can result from infection and can lead to person-to-person transmisson. In a small number of *Y. enterocolitica* infections, post-infectious sequelae are seen. An important sequela is reactive arthritis (a sterile arthritis in which bacteria are not present in the affected joint fluid) which often occurs in individuals carrying the HLA-B27 tissue serotype but is not limited to that serotype. Another complication that may result as a consequence of infection is a skin eruption termed erythema nodosum (inflammation of the fatty layer of skin). *Yersinia enterocolitica* septicemia may be seen in individuals with underlying diseases, in particular those patients with iron-overloading conditions such as thalassemia, sickle cell disease, hemochromatosis, or chronic hemodialysis. Age can influence the clinical picture of *Y. enterocolitica* infection. Gastroenteritis predominates in infants, children, and young people. Reactive arthritis is seen in young adults, and skin complications are common in adult females.

Yersinia enterocolitica infection is initiated through the ingestion of contaminated water or food. The organisms colonize the intestinal lumen and transmigrate through the M cells (antigen-sampling cells) across the epithelial lining of the small intestine and colonize the underlying Peyer's patches (lymphoid tissue). Subsequently, *Y. enterocolitica* may spread via lymph or blood into the mesenteric lymph nodes or to extraintestinal sites (liver, spleen). Adhesion, invasion, and survival of the organism depend on virulence factors encoded by chromosomal or pYV genes.

Specific antibodies are produced in humans infected by *Y. enterocolitica*. Immunity against *Y. enterocolitica* is based on both innate and acquired host defense mechanisms. Expression of Yops (*Yersinia* outer proteins) and YadA (*Yersinia* adhesion A) give the organism the ability to evade the host's innate defenses such as phagocytosis by polymorphonuclear leukocytes and macrophages, and attack by the complement complex. Protective acquired immunity against *Y. enterocolitica* is cell-mediated rather than antibody-mediated. Cell-mediated immunity and phagocyte-dependent protective responses depend on type 1 T helper (T_h1) cells. T_h1 cells are responsible for the production of

cytokines (interferon-gamma, interleukin-2, and tumor necrosis factor beta) which activate macrophages. *Yersinia enterocolitica* can evade the host's innate immune system; however, in most cases, infections induce a self-limited disease suggesting that the evasion strategies of *Y. enterocolitica* against the innate system are only partially successful. The organism is not efficient in evading acquired immunity and the disease is limited by phagocytosis and killing by T cell-activated macrophages.

10.6 Detection of organisms

10.6.1 Virulence determinants

The plasmid-associated virulence determinants have been used to differentiate between virulent and avirulent strains of *Y. enterocolitica*. Thus, these virulence determinants provide a rapid, reliable, and simple method for isolation and detection of plasmid-bearing virulent *Y. enterocolitica* (YEP^+) strains from foods. The main disadvantage of the use of these pYV-borne virulence determinants is the instability of the virulence plasmid. Incubation of strains at 37 °C for isolation fosters the loss of pYV resulting in plasmidless avirulent (YEP^-) strains; however, pYV-associated phenotypes are only expressed at 37 °C. Because of the instability of pYV at 37 °C, it is difficult to isolate YEP^+ strains after initial detection. As a consequence, detection has been hampered in clinical, regulatory, and quality control laboratories that employ an incubation temperature of 37 °C for isolation/detection of the organism.

10.6.2 Congo red binding

Congo red binding has been used to screen pYV-bearing *Y. enterocolitica* strains for virulence. When YEP^+ and YEP^- strains were cultivated at 37 °C for 24 h on a CR-containing, low-calcium brain heart infusion agarose (CR-BHO) medium, two types of readily discernible colonies were observed. The YEP^+ cells absorbed CR and formed red pinpoint colonies (CR^+) (Fig. 10.1A). The YEP^- cells failed to bind the dye and formed much larger white or light orange colonies (CR^-) (Fig. 10.1B). The size and colony morphologies of YEP^+ strains on CR-BHO also showed Lcr. The CR binding test was correlated with the presence of pYV with a number of virulence-associated properties and with mouse virulence, for a wide variety of pathogenic serotypes of *Y. enterocolitica* (Table 10.2). Thus, the binding of CR by YEP^+ strains consistently and efficiently allows differentiation of virulent (YEP^+) and avirulent (YEP^-) strains of *Y. enterocolitica*. In an investigation of a *Yersinia* outbreak in Los Angeles Country, California, in 1992, the CR binding technique detected 1 YEP^+ isolate per 300 colonies recovered on CR-BHO from each of seven patients. These data highlight the sensitivity of the CR binding technique for detection of YEP^+ cells in clinical samples that contained predominantly YEP^- cells due to incubation of samples at 37 °C during the initial isolation of the organism.

Since incubation at 37 °C causes the loss of pYV, an additional advantage of the CR binding technique is that it can be used to isolate *Y. enterocolitica* cells carrying pYV. The ability of the CR binding technique for recovery of YEP^+ cells varied from 5 to 95%, indicating strain variation in the stability of pYV. The YEP^+ strains showed all of the expected plasmid-associated properties, including virulence in the mouse (Table 10.2). By using the CR binding recovery technique mentioned above, the Food and Drug Administration (FDA) investigators recovered and enhanced the level of plasmid carriage

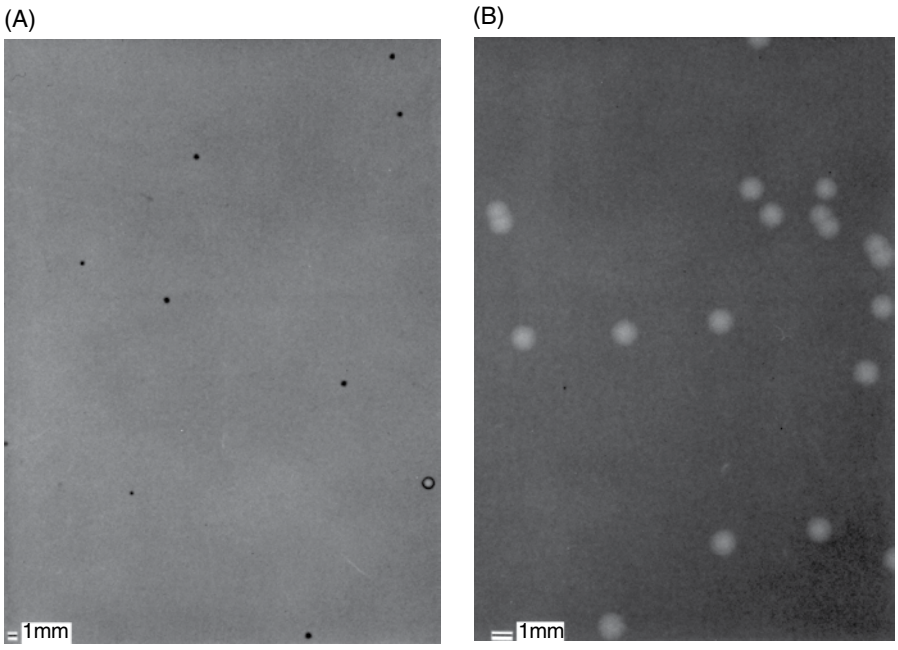

Figure 10.1 Congo red binding of colonies of *Yersinia enterocolitica* cells grown on CR-BHO for 24 h at 37 °C. (A) YEP$^+$ cells showing pinpoint red colonies. (B) YEP$^-$ cells showing large white or light-orange colonies. The concentration of CR used in the binding assay was 75 µg per ml. © American Society for Microbiology, Journal of Clinical Microbiology, Bhaduri et al., 29, 1991, 2341–2344.

of YEP$^+$ strains from 0.3% to over 92% from clinical samples obtained during a 1992 outbreak of yersiniosis in Los Angeles County, California. Thus, the recovery technique is useful to screen and to isolate viable YEP$^+$ cells, even if they are present at very low levels among a mixture of cells.

10.6.3 Isolation of pathogenic YEP$^+$ strains from foods

The increasing incidence of *Y. enterocolitica* infections and the role of foods in some outbreaks of yersiniosis have led to the development of a wide variety of methods to isolate this bacterium from foods. The unstable nature of the virulence plasmid during incubation at 37 °C complicates the isolation of YEP$^+$ strains by causing the overgrowth of virulent cells by plasmidless derivatives and can eventually lead to a completely avirulent culture. Since the population of *Y. enterocolitica* in foods is usually low, and since the natural microflora suppresses the growth of this organism, isolation methods usually involve selective enrichment followed by plating onto selective media.

Several approaches have been taken to enrich and isolate *Y. enterocolitica* from food. One method employs prolonged enrichment for 2–4 weeks at refrigeration temperatures to take advantage of the psychrotrophic nature of *Y. enterocolitica* and to suppress the growth of any background flora. Due to the extended time period needed for this method, efforts have been made to devise selective enrichment techniques employing shorter incubation times and higher temperature, thus making them more practical for routine use. However, high levels of indigenous microorganisms can overgrow and mask the presence of YEP$^+$ and nonpathogenic

Table 10.2 Correlation among CR Binding Technique, Virulence, and Virulence-Associated Properties of Original and Recovered Plasmid-Bearing Strains of *Y. enterocolitica*

Strains[a]	Serotype	CM[b]	CV[c] Binding	LCR[d]	CR[e] Binding	AA[f]	HP[g]	Plasmid (70–75 kbp)[h]	Diarrhea in Mice[i]
GER	O:3	+	+	+	+	+	+	+	+
GER-RE	O:3	+	+	+	+	+	+	+	+
GER-C	O:3	–	–	–	–	–	–	–	–
EWMS	O:13	+	+	+	+	+	+	+	+
WEMS-RE	O:13	+	+	+	+	+	+	+	+
EWMS-C	O:13	–	–	–	–	–	–	–	–
PT18-1	O:5:0:27	+	+	+	+	+	+	+	+
PT18-1-RE	O:5:0:27	+	+	+	+	+	+	+	+
PT18-1-C	O:5:0:27	–	–	–	–	–	–	–	–
O:TAC	O:TACOMA	+	+	+	+	+	+	+	+
O:TAC-RE	O:TACOMA	+	+	+	+	+	+	+	+
O:TAC-C	O:TACOMA	–	–	–	–	–	–	–	–
WA	O:8	+	+	+	+	+	+	+	+
WA-RE	O:8	+	+	+	+	+	+	+	+
WA-C	O:8	–	–	–	–	–	–	–	–

[a] Strains are from the FDA. Recovered strains are designated as RE. Plasmidless avirulent YEP$^-$ strains are designated as C (cured).
[b] CM: Colony morphology: In a calcium-adequate brain heart infusion agar (BHA) medium, YEP$^+$ cells appeared as small colonies (diameter 1.13 mm) as compared to larger YEP$^-$ colonies (diameter 2.4 mm).
[c] CV binding: Crystal violet binding. YEP$^+$ cells appeared as small dark violet colonies (diameter 1.13 mm) on BHA. YEP$^-$cells appeared as larger white colonies (diameter 2.4 mm) on BHA.
[d] Lcr: Low-calcium response. Calcium-dependent growth at 37 °C. YEP$^+$ cells appeared as pinpoint colonies of diameter 0.36 mm as compared to the larger YEP$^-$ colonies of diameter 1.37 mm on CR-BHO.
[e] CR binding: Congo red binding. YEP$^+$ cells appeared as red pinpoint colonies (diameter 0.36 mm) on CR-BHO. YEP$^-$ cells appeared as large white colonies (diameter 1.37 mm) on CR-BHO.
[f] AA: Autoagglutination.
[g] HP: Hydrophobicity.
[h] Presence and absence of pYV as determined by PCR amplification of *virF* gene.
[i] Fecal material consistency was liquid; diarrhea was observed starting on days 3 and 4 post-infection.

Y. enterocolitica strains. Enrichment media containing selective agents such as Irgasan, ticarcillin, and potassium chlorate are effective for enhancing recovery of a wide spectrum of *Y. enterocolitica* strains from meat samples. However, no single enrichment procedure is adequate for recovery of a broad spectrum of pathogenic *Y. enterocolitica* from foods. Since there is no specific plating medium for the isolation of YEP$^+$ strains, cefsulodin–irgasan–novobiocin (CIN) and MacConkey (MAC) agars are commonly used to isolate presumptive *Y. enterocolitica* from foods. The initial isolation of presumptive *Y. enterocolitica* from enriched samples on CIN and MAC agars adds an extra plating step, and the picking of presumptive *Y. enterocolitica* requires skilled recognition and handling of the colonies. The unstable nature of the virulence plasmid further complicates the detection of YEP$^+$ strains since isolation steps may lead to plasmid loss and the loss of associated phenotypic characteristics for colony differentiation. Moreover, the presumptive *Y. enterocolitica* colonies isolated should be confirmed as YEP$^+$ strains. Biochemical reactions, serotyping, biotyping, and virulence testing are essential for differentiation among YEP$^+$, YEP$^-$, environmental *Yersinia* strains, and other presumptive *Yersinia*-like organisms. Biochemical tests using commercially-available systems such as

analytical profile index (API) 20E test strips give similar reactions among these organisms and are not conclusive. Serotyping of major O and H antigens differentiates between pathogenic and environmental *Y. enterocolitica* but fails to discriminate between YEP^+ and YEP^- strains. Likewise, biotyping does not confirm the presence of the virulence plasmid in YEP^+ strains. Several plasmid-associated phenotypic virulence determinants, including colony morphology, AA, serum resistance, tissue culture detachment, HP, Lcr, and CV binding, have been used to indicate the potential virulence of *Yersinia* isolates. These methods require specific reagents and conditions and do not give definite results. In addition, most of these procedures are costly, time consuming, complex, and impractical for routine diagnostic use in field laboratories. Although virulence can be demonstrated effectively using laboratory animals, this test is not suitable for routine diagnostic use. Molecular techniques such as deoxyribonucleic acid (DNA) colony hybridization, DNA restriction fragment length polymorphisims, and the polymerase chain reaction (PCR) have also been successfully applied to the detection of virulent strains. However, these techniques are complex and time consuming. These methods detect only the presence of a specific gene, not the actual presence of the live organism. Although virulence is plasmid mediated in all strains examined, the plasmids involved differ in molecular weight. Thus, in epidemiological studies, it is not sufficient to search for plasmids of a particular molecular weight as an indicator of *Y. enterocolitica* virulence. Unfortunately, methods described in the literature for the isolation of pathogenic *Y. enterocolitica* from foods do not treat confirmation of virulence in presumptive or known *Y. enterocolitica* isolates recovered from selective agars as an integral part of the detection method. The most rapid enrichment procedure available for the isolation of a wide spectrum of *Y. enterocolitica* serotypes does not include the identification of isolates as YEP^+ strains.

An improved homogenization-based procedure for selective enrichment, identification and maintenance of various pathogenic YEP^+ serotypes from pork samples has been developed. This procedure is suitable for ground and liquid food samples wherein bacterial contamination is distributed throughout. In some cases, food slurries are used for enrichment, and the presence of food in the enrichment medium increases the background microflora and requires increased time for enrichment of YEP^+ strains. The CR binding technique is also not applicable for both detection and isolation, because the appearance of a red pinpoint colony is masked by the background microflora. Hence, it is necessary to first isolate presumptive *Y. enterocolitica* on selective agar plates and then subsequently identify any YEP^+ strains by the CR binding and Lcr techniques. This approach takes six days to complete from sample enrichment through confirmation of YEP^+ strains, and as few as nine colony-forming units (CFU) of YEP^+ strains per gram of spiked ground pork can be recovered from spiked samples.

A method for simultaneous detection and isolation of pathogenic YEP^+ serotypes from enriched swab samples of various foods was reported. This procedure is applicable to foods that have a physical surface for swabbing, and such surfaces are often the primary site of contamination. Since the actual food sample was not used and since there was a low level of competing microflora, the time for enrichment of YEP^+ strains and their subsequent confirmation by the CR binding and Lcr techniques was appreciably reduced (Fig. 10.2). This technique allowed for recovery of YEP^+ serotypes from various foods spiked with as low as 0.5 CFU/cm^2 within 4 days.

The above-mentioned homogenized slurry and swabbing techniques were effective for recovery of YEP^+ strains from naturally contaminated porcine tongues. The PCR assays validated these methods for detection of YEP^+ strains (Fig. 10.3). The YEP^+ serotypes isolated by these two procedures expressed plasmid-associated virulence characteristics and were positive in the mouse virulence test.

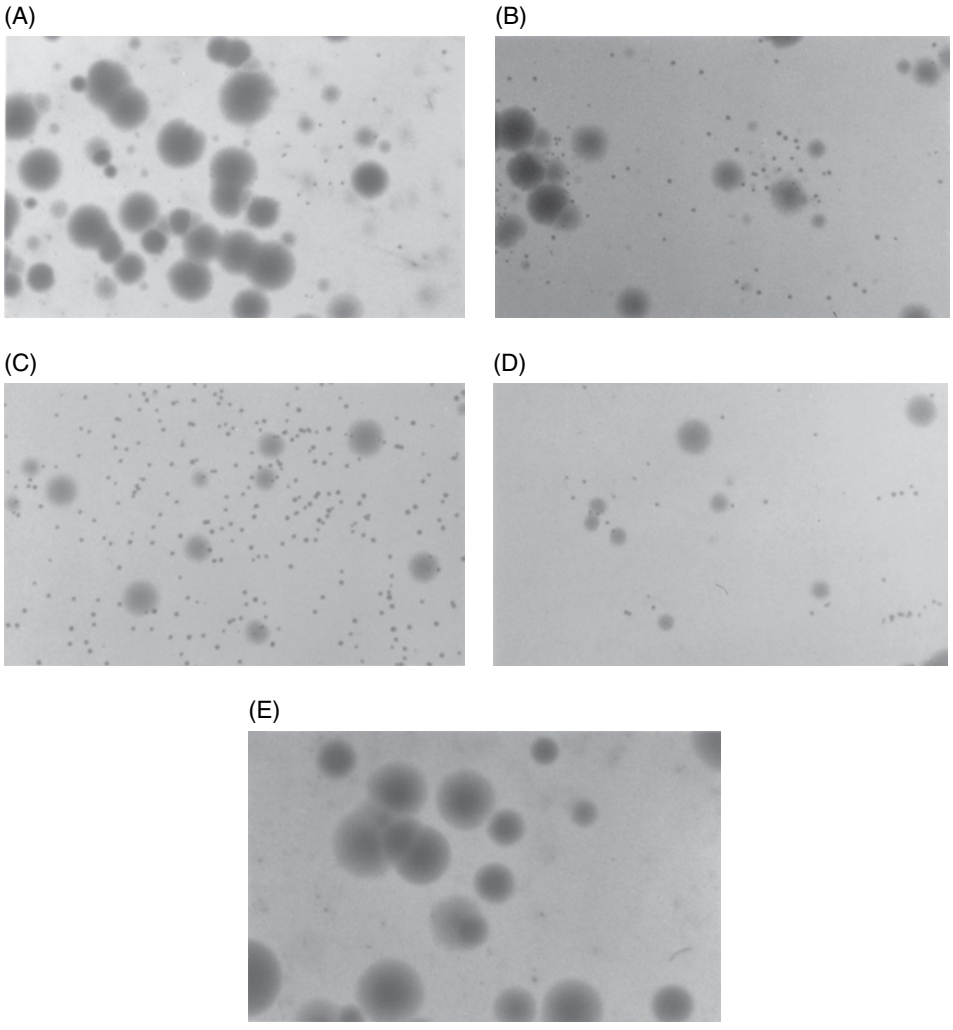

Figure 10.2 Recovery of YEP+ strains as red pinpoint colonies on CR-BHO from (A) artificially contaminated pork chops, (B) ground pork, (C) cheese, (D) zucchini, and (E) naturally contaminated porcine tongue. © American Society for Microbiology, Appl. Environ Microbiol, Bhaduri and Cottrell, 63, 1997, 4952–4955.

10.7 Prevention and control

The patterns observed in foodborne *Y. enterocolitica* outbreaks indicate that post-processing steps are the main causes of food contamination and subsequent human illness. Specific prevention and control measures of foodborne yersiniosis include the following:

1. Special care should be taken during incision and removal of the intestines, tongue, pharynx, and tonsils of pigs to avoid cross-contamination.
2. Meat products, particularly pork, should be handled with care; food utensils, equipment, and countertops should be thoroughly cleaned to prevent cross-contamination.

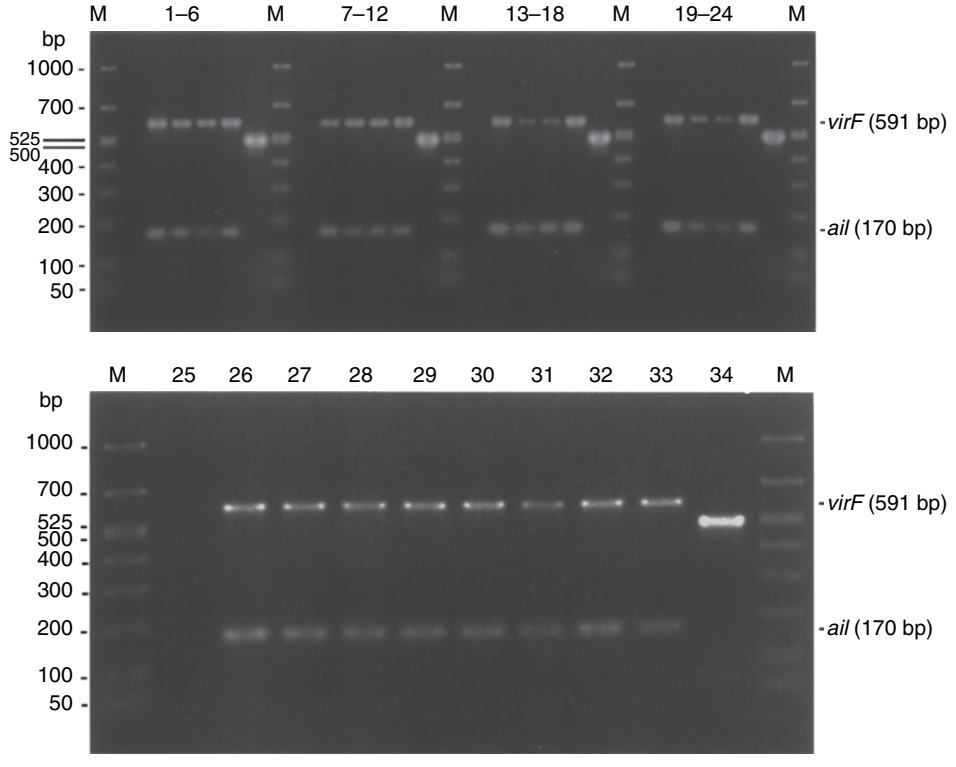

Figure 10.3 Confirmation of CR^+ clones isolated from various artificially contaminated foods and from naturally contaminated porcine tongue as YEP^+ strains by multiplex polymerase chain reaction (PCR) using chromosomal *ail* gene and *vir*F gene from pYV. Lane M=50–1000 bp ladder marker. Negative control with no template (lanes 1, 7, 13, 19 and 25). CR^+ colony showing the presence of 170 and 591 bp products with mixture of both *ail* and *vir*F primers from chromosome and pYV respectively: pork chops (lanes 2–4), ground pork (lanes 8–10), cheese (lanes 14–16), zucchini (lanes 20–22) and porcine tongues (lanes 26–32). Positive control with purified DNA from YEP^+ strain showing the presence of 170 and 591 bp products with mixture of both *ail* and *virF* primers from chromosome and pYV respectively (lanes 5, 11, 17, 23 and 33). Positive control for PCR assay with λ as DNA template (lanes 6, 12, 18, 24 and 34). © American Society for Microbiology, Appl. Environ Microbiol, Bhaduri and Cottrell, 63, 1997, 4952–4955.

3. Refrigerated foods should be thoroughly cooked or heated at temperatures sufficient to kill the pathogen. Likewise, cooked and refrigerated foods should be heated to a steaming temperature to kill *Yersinia* before consumption.
4. Precautions for prevention of fecal–oral spread of the pathogen should be practiced. Water supplies should be free from animal and human fecal waste.
5. Hands should be washed with soap and hot water after handling raw foods, including pork, as well as before serving food and eating food

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11 *Campylobacter*

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11.1 Introduction

Species within the genus *Campylobacter* have emerged over the last decades as significant clinical pathogens of human public health concern. This microorganism is responsible for 400–500 million cases of infection each year worldwide, in which most cases (approximately 95%) are caused by *C. jejuni* or *C. coli*, although *C. upsaliensis*, *C. hyointestinalis*, *C. lari*, *C. fetus*, and *C. sputorum* biovar sputorum have also been demonstrated to be implicated as gastrointestinal pathogens. These data, however, are likely to underestimate the actual disease burden because it is estimated that only 1 in 7 cases is reported in the United Kingdom and only 1 in 38 cases in the United States.

Campylobacters may have been discovered in 1886 by Theodor Esherich from the colons of infants who had died of what he called 'cholera infantum.' However, it was not until 1972 that Dekyser and Butzler isolated *Campylobacter* from the blood and feces of a previously healthy young woman with acute febrile hemorrhagic enteritis. *Campylobacter*, which is ubiquitous in nature and is found in the intestinal microbiota of a large number of wild and domestic animals, is currently recognized as the most frequent foodborne pathogen and remains one of the most prevalent bacterial foodborne pathogens in the industrial world to date.

11.2 Nature of the illness caused by *Campylobacter*

Campylobacteriosis, the acute gastrointestinal illness caused by several *Campylobacter* species, has been described as an emerging foodborne disease. The illness can be presented with a wide range of symptoms, and the infection is usually self-limiting. Therefore, the majority of patients require no more than supportive treatment, e.g., maintenance of hydration and electrolyte balance. However, occasionally infection leads to death in infants and young adults (5% of estimated food-related deaths). Infections occur at all ages, although peaks are described for children below the age of four and persons between 15 and 39 years.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

C. jejuni has an infective dose of between 500 and 10,000 organisms and following an incubation of 2–5 days, symptoms develop. Several reports have indicated that incubation could be as short as ~24–72 h and could extend up to 10 days. In approximately half of patients, diarrhea is preceded by a febrile period with malaise, myalgia, abdominal pain, and fever. Fresh blood may appear in the stools by the third day. In addition, severe abdominal pain may mimic acute peritonitis. Occasionally, some patients, especially teenagers or young adults, develop peritonitis from acute appendicitis; but in most patients, inflammation of some part of the ileum and jejunum with mesenteric adenitis occurs.

This bacterium has been identified as an important risk factor for the development of inflammatory bowel disease. Local complications such as cholecystitis, pancreatitis, and peritonitis rarely occur, and immunoproliferative small intestinal disease also has been associated with this bacterium. Bacteremia is detected in less than 1% of patients, and it occurs most often in patients whose immune system is severely compromised. Some patients develop erythema nodosum or polyarthralgia (i.e., reactive arthritis). Extra-intestinal infections, including meningitis, osteomyelitis, and neonatal sepsis, are rare. *Campylobacter* infections are also associated with post-infectious complications, including Reiter syndrome and Guillain–Barré syndrome (which is an acute polyneuropathy that affects the peripheral nervous system, where the most typical symptom is an ascending paralysis beginning in the feet and hands and migrating towards the trunk, and in some cases a change in sensation or pain as well as dysfunction of the autonomic nervous system is observed).

Variations in clinical manifestations could be due to the wide genetic diversity that exists between isolates in both animals and humans. During the past decade, especially since the publication of the first *C. jejuni* genome sequence, major advances have been made in understanding the pathobiology and physiology of this organism. The genome of *C. jejuni* is composed of one circular chromosome with 1,641,481 base pairs thought to encode 1654 proteins and 54 stable RNA species. It reveals several genes with homopolymeric G tracts prone to phase variation via a slipped-strand mispairing mechanism. In addition, it has become clear that *Campylobacter*, in contrast to other diarrhea-causing bacteria, does not express a large number of classical virulence factors. It also has been demonstrated that *C. jejuni* contains about 22 variable regions on the chromosome, principally in regions that encode for surface-accessible carbohydrate structures, such as the capsule, lipooligosaccharide, and flagellum.

Due to the absence of a suitable animal model, the virulence properties of these bacteria have mostly been investigated using in vitro models. This, in part, has limited our knowledge of the pathogenicity of this organism. The main route of *C. jejuni* and *C. coli* human infection is through improperly handled or undercooked poultry, whereas illness caused by *C. lari* and *C. upsaliensis* may be due to proximity to water and shellfish and handling of pets, livestock, or livestock carcasses. Following their consumption, *C. jejuni* passes through the duodenum and is exposed to bile secretion. Bile resistance is primarily mediated by the CmeABC multidrug efflux pump. After this passage, the bacteria colonize the lower intestinal tract (ileum, jejunum, and colon), which is a crucial step for pathogenesis. Adherence may be required for this bacterium to resist intestinal peristalsis and expulsion. Several factors have been identified that mediate adherence to cultured cells including flagella, *Campylobacter* adhesion to fibronectin (CadF), PEB1a, PEB4, and jejuni lipoprotein A (JlpA). The presence of the *fla*A (flagella) and the *cad*F genes has been detected among all *C. jejuni* isolates. To date, flagella and motility are the most well-defined colonization factors. Flagella allow bacteria to penetrate the mucous layer covering intestinal cells using their polar flagella and 'corkscrew' motion. It has been shown that a correlation exists between

the severity of clinical symptoms in infected individuals and the degree to which *C. jejuni* isolates adhere to cultured cells. Administration of a mixture of motile and nonmotile *C. jejuni* to human volunteers resulted in the isolation of only motile *C. jejuni*, implying a significant role of motility in colonization. Also, flagella are involved in autoagglutination and biofilm formation. Other bacterial factors are currently under scrutiny to determine their precise roles in the invasion process, such as flagellin production, sialylation of lipooligosaccharides (LOS), chemotaxis, capsular polysaccharides (CPS), production of heat shock proteins (which are associated with thermal stress), and *Campylobacter* invasive antigens (Cia).

The specific role of the cytolethal distending toxin (CDT) in pathogenesis remains unclear. This toxin causes morphological changes (cytoplasmic distention) and seems to be important for cell cycle control and induction of host cell apoptosis. In addition, CDT has been recognized as a major pathogenicity-associated factor. It causes eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and leading to cell death. CDT is the product of three genes designated as *cdtA, cdtB*, and *cdtC*, which encode proteins with molecular masses of 30, 32, and 20 kDa, respectively. The *cdt* genes are very conserved among the different *Campylobacter* strains. Genetic and biochemical evidence indicates that all three polypeptides are required for cellular intoxication. Detection rates for the *cdtA*, *cdtB*, *cdtC*, and *cdtABC* cluster genes are reported to be 98%, 96%, 92%, and 88%, respectively; however, there are differences in CDT production. Isolates that were PCR negative for one or more individual *cdt* toxin genes also produced low or no CDT toxin. Despite the high prevalence of CDT genes, only 27.6% of *C. jejuni* and 2.8% of *C. coli* strains showed evidence for cytotoxin production in HEp-2 cells.

Treatment with antibiotics such as macrolides and fluoroquinolones is usually administered only in severe infections in infants, the elderly, immunocompromised individuals, and patients with extra-intestinal manifestations.

11.3 Characteristics of *Campylobacter*

Campylobacter belongs to the epsilon class of proteobacteria, in the order *Campylobacteriales*; this order includes two other genera, *Helicobacter* and *Wolinella*. *Campylobacter* was initially classified as *Vibrio* spp. due to its spiral morphologies, and later Sebald and Véron (1963) postulated the new genus *Campylobacter* in 1963. Actually, the family *Campylobacteriaceae* consists of the species *Campylobacter, Arcobacter*, and *Bacteroides ureolyticus*, and it occurs primarily as commensals in humans and domestic animals. There are 17 species serotypes within the genus *Campylobacter*, which can be divided into more than 600 penner serotypes (heat-stable antigens) and more than 100 Lior serotypes (heat-labile antigens). All clinically relevant *Campylobacter* spp. are considered to be thermotolerant in nature. The thermophilic species include *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus*, while the nonthermophilic species include *C. concisus*, *C. curvus*, *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis*, *C. showae*, *C. sputorum*, and *C. rectus*. Within the *C. jejuni* species, two subspecies, ssp. doylei and ssp. jejuni, can be distinguished on the basis of nitrate reduction and cephalothin susceptibility.

Campylobacter is a fragile organism in the environment and requires special growth conditions. It grows in vitro with partial oxygen tension of 2–10%; however, it exhibits great flexibility in its adaptation mechanisms to survive environmental stresses, such as temperature

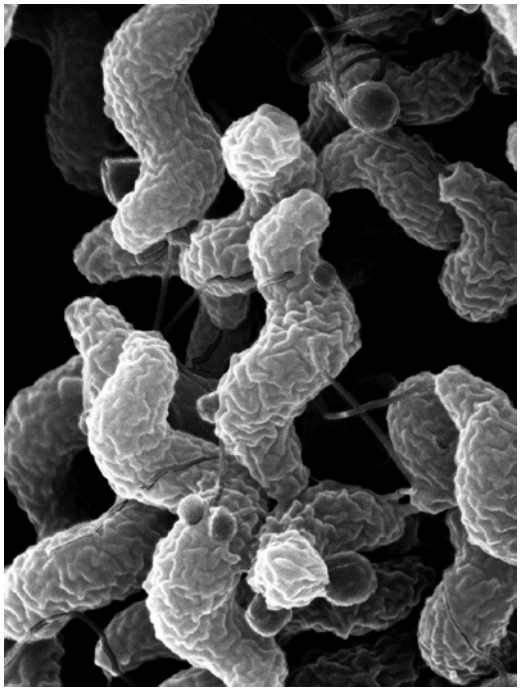

Figure 11.1 Electron microscope image showing the characteristic spiral, or corkscrew, shape of *C. jejuni* cells and related structures. Photo by De Wood; digital colorization by Chris Pooley. Agricultural Research Service, USDA.

shift, oxygen tension, and nutrient depletion, which usually occur during transmission between the environment and animal hosts and within the host's intestine. This adaptability is attributable to the genetically, metabolically, and phenotypically diverse population structure of *Campylobacter* and its capability to adapt in response to challenges. Even more, evidence indicates that *Campylobacter* strains could present phenotypic and physiological differences between strains grown under the same conditions.

Members of the family *Campylobacteraceae* are typically motile with a characteristic corkscrew-like motion via a single polar unsheathed flagellum at one or both ends of their cells (Fig. 11.1). This species requires complex growth media as it is not able to oxidize or ferment carbohydrates and has no lipase or lecithinase activity. *Campylobacteraceae* obtain energy from amino acids, or tricarboxylic acid cycle intermediates. Oxidase activity is present in all *Campylobacter* spp. except *C. gracilis*. Members of these genera have small genomes (1.6–2.0 megabases) and can establish long-term associations with their hosts, sometimes with pathogenic consequences.

The optimum temperature for growth of this organism is between 37 °C and 42 °C, it is sensitive to high temperatures and cannot survive pasteurization or most cooking procedures, and it is unable to grow below 30 °C, below pH 4.9, or in 2% sodium chloride. Furthermore, these bacteria are very sensitive to osmotic stress, aeration, and desiccation, and they do not survive well on dry surfaces. The bacteria have the ability to acquire adequate nutrients in vivo, which is an important characteristic for successful colonization. Amino

acids are the primary carbon source, especially aspartate, glutamate, serine, and proline. It has been shown that mutation of genes involved in the catabolism of aspartate (*aspA*) and serine (*sdaA*) reduced bacterial colonization in chickens.

A very important issue is that *C. jejuni* can enter a viable but nonculturable (VBNC) state in response to extremes in pH, moisture content, temperature, nutrient content, and salinity and cannot be detected via culturing methods. Once in a VBNC state, the organism must maintain an energy balance from substrate oxidation through respiration to grow, divide, and remain viable.

11.4 Epidemiology

Campylobacter is known to be commensal in cattle, swine, and birds; however, *C. jejuni* is often the predominant species in poultry, and *C. coli* is most prevalent in swine. *C. lari* is widespread in birds (seagulls in particular), but it has also been isolated from dogs and swine. In addition, *C. upsaliensis* frequently has been isolated from domestic dogs and cats. For more than 40 years, this organism has also been found as a frequent cause of different diseases in animals, causing abortions in bovines, enzootic sterility in cows, winter dysentery in calves, and swine dysentery. The natural reservoirs are wild birds, whose intestines offer a suitable biological niche for the survival and dissemination of *C. jejuni*.

Chickens are the most important source of human infection. Colonization of *Campylobacter* in chickens occurs around the third week of life, arising in the crop and intestine, in particular the caeca, and then multiplies. The body temperature of chickens happens to be close to the optimal growth temperature of these bacteria. During this colonization, chickens do not show signs of disease. *Campylobacter* is disseminated via feces, and pecking and coprophagy ensure a rapid spread between birds, increasing the probability of contamination if flocks are improperly handled.

The most recognized route of transmission of *C. jejuni* to humans is via consumption of contaminated poultry meat, accounting for 29% of reported cases. However, other routes have been reported such as contact with feces of cattle, sheep, poultry, and pigs. Companion animals, including cats and dogs, also carry *Campylobacter*. Contamination of the environment by domestic and wild animal feces presents an alternative exposure pathway for human infection, for example, soil, beach sand, sewage, groundwater, and drinking water. Humans may also be exposed to contaminated animal feces in the environment through outdoor activities such as camping, walking, and picnicking.

The thermophilic campylobacters *C. jejuni*, *C. coli*, *C. laris*, and *C. upsaliensis* are more associated with human gastrointestinal disease. However, the majority of cases (approximately 90%) are attributed to infections with *C. jejuni*, although *C. coli* is increasingly recognized as an important pathogen. These bacteria have been classified by the NIH as Category 'B' Bioterrorism Agents due to their ability to cause foodborne and waterborne outbreaks.

Over the last five years, campylobacteriosis was the most commonly reported zoonosis in the European Union, followed by salmonellosis and yersiniosis, accounting for an average of 25,000 cases of infectious intestinal disease annually in the UK; at a cost of four million pounds, it is estimated to be responsible for approximately 3.5 million cases of diarrheal illness per year in the United States. Also, it has been estimated that approximately 1% of the population in Western Europe is infected each year with this bacterium, and some reports have indicated that New Zealand has a higher reported rate of campylobacteriosis than other developed countries.

Young children remain most susceptible in both the developing and the developed world. However, it has been stated that *Campylobacter* is hyperendemic in developing countries, where poor sanitation and close human contact with animals are a common denominator. In industrialized countries, the incidence of *C. jejuni* and *C. coli* infections peaks during infancy and again in young adults aged 15–44 years.

Over 90% of human campylobacteriosis cases are sporadic, and most of them occur in the summer. Most infections are believed to result from the ingestion of contaminated food. *Campylobacter* species can survive on fresh and frozen retail poultry products and can easily enter into contact with humans, although the role of other nonfood exposures in the epidemiology of sporadic campylobacteriosis still remains unknown. It is estimated that handling, preparation, and consumption of broiler meat may directly account for 20 to 30% of human cases of campylobacteriosis in the EU.

Campylobacter spp. has also been found to form biofilms (assemblage of microbial cells that are associated with a surface and are enclosed in a matrix of primarily polysaccharide materials), which are found in watering supplies and plumbing systems of animal husbandry facilities and animal-processing plants. This form of growth could provide protection to the microorganisms from environmental stresses and antimicrobial agents that are used in sanitizing animal-husbandry facilities and animal-processing plants, allowing survival.

11.5 Detection of *Campylobacter*

Various methods have been developed to detect or enumerate *Campylobacter*. The Most Probable Number (MPN) method (using different enrichment media such as Bolton or buffered peptone water and incubating under microaerophilic conditions) is very useful for samples with few cells and allows for the estimation of the number of bacteria present in samples.

Methods for the detection of *Campylobacter spp*. were originally developed for clinical specimens. Some variations have been made according to the sample processed but, in general, enrichment media (such as Tran blood-free enrichment broth [BFEB], or Modified BEFB [M-BFEB], both incubated at normal atmospheres, or Bolton or Preston, incubated under microaerophilic conditions [anaerobe jars with a modified atmosphere using a Gas Pack or gas tanks with a mixture of 5% O_2, 10% CO_2, and 85% nitrogen]) is required for *Campylobacter* isolation in samples where the number of organisms is expected to be low, such as from foods and environmental samples, and this is not necessary for feces samples.

A great variety of selective media and procedures for the isolation of campylobacters have been developed; however, no single medium is sufficient for the isolation of all *Campylobacter* spp. The most often reported selective media for the isolation of *Campylobacter* from foods and feces are Abeyta-Hunt-Bark agar or modified Campy blood-free agar (mCCDA). Other selective agars for isolation of campylobacters from human and animal feces include Skirrow agar, Butzler agar, Campy-BAP, and *Campylobacter* Blood Free Medium (mCCDA); Preston agar is recommended for isolation and enumeration from feces and environment samples. The selective agar plates are incubated in a microphilic atmosphere. Addition of antioxidants such as superoxide dismutase, catalase, sodium dithionite, or histidine to the growth media significantly enhances the survival of *C. jejuni* at a pO_2 of 17 to 21%. Typical colonies are confirmed by phenotypical tests.

Recently, a two-step simple aerobic method for *Campylobacter* detection was proposed: M-BFEB coupled to PCR is a rapid and attractive alternative for isolation and identification of *C. coli* and *C. jejuni* from poultry. Also, a method using sample filtration followed by

Preston Selective *Campylobacter* agar (incubated under microaerophilic conditions) produced a 38.5% increase in the number of isolates.

When the diagnosis of infection is based exclusively on selective media for culturing, it appears that >95% of *Campylobacter* infections are caused by *C. jejuni* or *C. coli*. However, with refinements in isolation and identification methods, other related species, such as *C. upsaliensis*, *C. lari*, *C. fetus* subsp. *fetus*, *C. jejuni* subsp. *doylei*, *C. concisus*, *A. butzleri*, and *Arcobacter skirrowii*, have been isolated from human patients with diarrhea. The hippuricase gene is only found in *C. jejuni*, although some *C. jejuni* isolates are hippuricase-negative, thereby making it impossible to differentiate *C. coli* from hippuricase-negative *C. jejuni* using purely biochemical tests.

During assays for detection, two morphologies of *C. jejuni* have been described: culturable spiral forms and nonculturable coccoid forms. When faced with environmental stressors, bacteria can enter a VBNC state. The ability to culture bacteria in this state is lost even though the microorganism is alive and metabolically active, making their detection on culture media difficult during quality control tests. The risk and significance of detection failure could be high, since it has been demonstrated that stressed pathogens under the VBNC stage may be more virulent than those under nonstressing conditions.

Epifluorescence microscopy with viability staining distinguishes live and dead cells, and it allows for enumeration of VBNC cells. A better microscopic enumeration method is solid phase cytometry (SPC). In addition, the polymerase chain reaction (PCR) in different formats has been used for detection and enumeration of *Campylobacters* as it is an efficient, specific, and sensitive technique. However, inhibitors from media or food components are usually a limitation of PCR techniques. A further limitation of conventional PCR is the inability to distinguish DNA from live and dead cells and thus properly estimate viable cell numbers. Recently, real-time PCR quantification has been developed, and this technique may be sensitive enough for clinical applications. Vital colorants could be useful to distinguish between viable and nonviable cells. *Campylobacter* species can be further differentiated into definitive types by multilocus sequence typing (MLST, a method of identifying microbial isolates by their nucleotide sequence data), restriction fragment length polymorphism determination of the *flaA* gene (flaA-RFLP), pulsed-field gel electrophoresis (PFGE), and automated repetitive extragenic palindromic polymerase chain reaction (REP-PCR).

Other rapid methodologies have been developed to quantify the microorganism (SimPlate [Biocontrol], Campylobacter ID Agar [bioMerieux], Brillance CampCount Agar [Oxoid]), or to detect it based on immunological techniques (3 M Tecra Visual Immunoassay VIA [3 M], Assurance Gold Campylobacter EIA [Biocontrol], Transia Plate Campylobacter [Biocontrol], VIDAS System [bioMerieux]), biochemical techniques (Biolog Microbial Identification System [Biolog], Vitek 2 System [bioMerieux]), or molecular techniques (Phenotype Microarrays [Biolog], BAX System Real Time PCR Assay [Dupond], PCR kit for the qualitative detection of *Campylobacter* DNA [Biotecon Diagnostics], RiboPrinter® Microbial Characterization System [Dupond]). A simple serological method to confirm the bacterium is the Dryspot Campylobacter test [Oxoid].

11.6 Prevention and control measures

Advances in understanding *Campylobacter* biology have provided us with new opportunities to develop anti-*Campylobacter* strategies. Given the fact that contaminated poultry meat is a major source of human infections from *Campylobacter* species, reduction of this pathogen

in commercial chickens both at the pre-slaughter and post-slaughter stages has been a focus of investigation and has been targeted at four levels: (1) reducing the numbers of flocks colonized by *Campylobacter*; (2) reducing the levels of bacteria in the birds prior to slaughter; (3) strategies to avoid cross-contamination during slaughter from positive to negative birds; and (4) strategies to reserve contaminated meat for frozen products, which reduces the number of viable organisms.

Some interventions have been proposed to reduce *Campylobacter* colonization in chickens. One of the most important ways of introducing *Campylobacter* in a flock is human activity; thus, strategies to train personnel and methods for decontamination have been developed. Vaccination of birds has been used, but early attempts were mostly without success, since subunit vaccines based on flagellin fragments provided homologous protection in some instances. However, variation of this protein, in part caused by variation in its glycosylation states, limits cross-protection between strains. Live-attenuated *Salmonella* vaccines constructed to express immunogenic Campylobacter peptides (fragments of proteins CjaA [Cj0982c] or PeB1 ([Cj0921c], [Cj0113]) have also been applied; the last one especially has been very effective at protecting against different Campylobacter species.

Recent advances in the field of metagenomics have provided approaches to design competitive exclusion products to potentially reduce *Campylobacter* colonization. Recent work has revealed a possible association between the presence of a subspecies of *Megamonas hypermegale* and *Campylobacter* suppression.

If contamination cannot be avoided, it may be possible to eliminate the bacteria or decrease their load prior to slaughter. For this reason, bacteriophage therapy has recently received considerable attention. Administering lytic bacteriophages to artificially contaminated chicken carcasses or *Campylobacter*-colonized chickens reduced the level of *C. jejuni* contamination or colonization with varying success. However, it has been shown that bacteria develop resistance to their phages. Thus, administration of large numbers of phages shortly before slaughter could, in theory, reduce resistance and *Campylobacter* carriage. Also, administration of bacteriocin to chickens reduced the colonization level of *Campylobacter* by more than 10^6 colony-forming units per gram of cecal contents. These results suggest that bacteriocin treatment is a promising approach for the control of *Campylobacter*.

The use of different antimicrobial treatments based on chlorine, sodium chlorite, cetylpyridinium chloride, chlorine dioxide, ozone, peroxyacids, and trisodium phosphate (TSP) would help to control microbial populations during poultry processing. Furthermore, *Campylobacter* is relatively sensitive to low-dose radiation treatment and could be eliminated readily from poultry meat products by this method.

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12 *Arcobacter* and *Helicobacter*

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12.1 Introduction

Arcobacter, *Campylobacter*, and *Helicobacter* are closely-related microbes of the ribonucleic acid (RNA) superfamily VI of the Proteobacteria (epsilonproteobacteria). These microbes are Gram-negative, motile curved, or spiral-shaped rods that grow under microaerobic conditions. Their general features are summarized in Table 12.1. A tally of publications from 2000 through 2010 detailing various aspects of *Arcobacter* microbiology (n = 80) contrasts with those of *Campylobacter* (n = 3700) and *Helicobacter* (n = 13,000) and emphasizes the relative obscurity of this genus.

Herein we examine the evidence that *Arcobacter*, especially *Arcobacter butzleri*, and *Helicobacter*, in particular *Helicobacter pylori*, are potential human foodborne pathogens.

12.2 *Arcobacter*

The genus *Arcobacter* was proposed in 1992, following reanalysis of the aerotolerant species *Campylobacter* (Latin, 'curved rods') *cryaerophila* (Latin, 'loving cold and air'). The genus *Arcobacter* (Latin: 'arc-shaped bacterium') includes aerotolerant *Campylobacter*-like Gram-negative bacteria, which are motile by means of polar unsheathed flagella. *Arcobacter* was first isolated from aborted bovine fetuses and designated *C. cryaerophila*.

Unlike other *Campylobacter* species, *Arcobacter* grows in the presence of atmospheric oxygen (aerotolerant) and at temperatures (15–25 °C) that are lower than those used for incubation of *Campylobacter*. By 2011, 14 recognized species were documented in vertebrates, invertebrates (including shellfish), and diverse environmental niches, including marine habitats (Table 12.2). It has been suggested that members of the genus *Arcobacter* are primarily environmental microbes, based on its relatively large genome size (2.3 to 3.2 Mb) in contrast with pathogenic *Helicobacter* (1.65 Mb) and *Campylobacter* (1.64 Mb). The annotation of the complete *Arcobacter* genome led Miller and colleagues to conclude

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Table 12.1 Summary of Major Distinguishing Characteristics of Members of RNA Superfamily VI (epsilonproteobacteria)

Strain	Growth at 25 °C	Oxygen tolerance	Flagella	Genome size (Mb)
Helicobacter pylori J99	No	Microaerophilic	Multipolar, sheathed	1.65
Campylobacter jejuni NCTC 11168	No	Microaerophilic	Single polar, unsheathed	1.64
Arcobacter butzleri RM 4018	Yes	Aerotolerant	Single polar, unsheathed	2.3
Arcobacter nitrofigilis CI^T				3.2

Table 12.2 *Arcobacter* Species and Host Distribution

Species	Host
A. butzleri	Humans
	Livestock
	Water
A. cibarius	Broiler carcasses
A. cryaerophilus	Humans
	Livestock
A. defluvii	Sewage
A. ellisii	Mussels
A. halophilus	Hypersaline lagoon water in Laysan Atoll
A. marinus	Seawater, starfish, seaweed
A. molluscorum	Shellfish
A. mytili	Mollusks, brackish water
A. nitrofigilis	Roots of aquatic *Spartina* plant
	Mussels
A. skirrowii	Humans
	Preputial swabs of bulls
	Aborted fetuses
A. sulfidicus	Oceanic filamentous mats
A. thereius	Cloacal swabs of ducks
	Liver, kidney of aborted piglets
A. trophiarum	Pig

that members of the genus can be generalized as free-living organisms found predominantly in aqueous environments, and occasionally associated with livestock or isolated from food. This characteristic is important, especially as field surveys continue to report a rich diversity of *Arcobacter* in water supplies, poultry litter samples, other animal- or food-producing environments, as well as algal endosymbionts.

12.2.1 Nature of illness in animals and humans

Arcobacter species, like *Campylobacter*, may exist as commensals in livestock, including cattle, hogs, and – less frequently – in poultry. However, unlike *Campylobacter* and *Helicobacter*, *Arcobacter* spp. exists as free-living microbes inhabiting, for example, marine sediments. *Arcobacter* species mainly implicated in either human or veterinary clinical illness include *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*. With respect to public health

importance, to date *A. butzleri* is perhaps the most likely candidate to cause human illness, primarily enteritis. Despite its initial description and continued isolation from aborted livestock species, the role of *Arcobacter* as a veterinary pathogen is in question.

An evidence-based semiquantitative method for prioritization of foodborne zoonoses ranked *A. butzleri* as a microbe of 'Significant Importance'. In 2002, *Arcobacter* spp. were classified by the International Commission on Microbial Specifications for Foods (ICMSF) as emerging pathogens, although in contrast to *Campylobacter* it is of relatively minor importance in clinical medicine. To illustrate, in the United States alone *Campylobacter* causes ~2 million cases of gastroenteritis, 10,600 hospitalizations and 55 deaths annually. Yet for *Arcobacter* since its initial description in 1977 there are fewer than 300 reported sporadic cases, including a single outbreak of *Arcobacter* in Italian school children, worldwide. In humans, enteritis and occasionally septicemia occur in immunocompromised individuals, such as AIDS patients, the elderly or type 2 diabetics.

Culture methods may impact the prevalence estimates. In general, clinical surveys employ culture methods and high temperature incubation optimized for *Campylobacter*, which underestimate *Arcobacter* prevalence. To illustrate, a Danish survey of *Arcobacter* in human feces (n=1376) estimated a prevalence of *A. butzleri* of less than 0.1% using *Campylobacter* protocols. However, seven *A. cryaerophilus* isolates were recovered from 500 stool samples of clinically healthy human carriers (1.4%) in a Swiss study utilizing methods optimal for *Arcobacter*. Two European surveys independently ranked *Arcobacter* as the fourth most common cause of human campylobacteriosis, after *C. jejuni*, *C. coli* and *C. fetus*. In the first, an exhaustive eight-year study in Belgium of clinical stool samples (n=67,599) utilizing culture techniques suitable for *Arcobacter* estimated the prevalence of *A. butzleri* (3.5%) and of *A. cryaerophilus* (0.5%). Patients ranged in age from 30 days to 90 years, reported travel abroad, and underlying predisposing disease. In contrast to the bloody diarrhea of *C. jejuni*, patients with culturable *A. butlzeri* experienced acute (3 to 15 days, 50.8%) or chronic (more than two weeks to two months, 16.4%) watery diarrhea. Nearly 23% required hospitalization. Interestingly, 20% of culture-positive patients were asymptomatic. In a second study, utilizing *Campylobacter* selective media incubated at 37C, *A. butzleri* represented 1% of clinical isolates (n=2855) obtained during an 18-month study in France. Cases were associated with diarrhea (97%), blood in the stool (13.8%) with 58.6% requiring hospitalization. A smaller study in Texas of 353 patients reporting enteritis set the prevalence of *Arcobacter* of 0.6% (2/353) using culture protocols optimal for its isolation.

Consumption of contaminated water and poultry, as described below, as well as person-to-person contact and heightened susceptibility of the immunocompromised host coupled with the microbe's putative virulence factors identical to those of *Campylobacter* (*cadF*, *ciaB*, *cj1349*, *hecA*, *hecB*, *irgA*, *mviN*, *pldA*, and *tlyA*) augment transmission. Because it survives in the environment it is not surprising that *A. butzleri* has been reported in drinking water reservoirs in Germany, water treatment plants, livestock water supplies, rivers, canal waters of Bangkok, wet poultry litter, and water samples obtained from oil fields. In the United States, *A. butzleri* was recovered from a well serving an Idaho youth camp following an outbreak of waterborne enteritis. The presence of *A. butzleri* in unchlorinated water supplies suggested this as a possible source of contamination. Although clinical and environmental samples were not available for molecular comparison in this or any other water outbreak, consumption of contaminated water remains a recognized risk factor.

Infections with *Arcobacter* might be common in developing nations where contaminated water poses a risk factor. To illustrate, risk factor analysis of 19 patients with *A. butzleri*-associated illness indicated that 63% of the cases were linked to consumption or contact with

potentially contaminated water associated with travel. *A. butzleri* and *A. cryaerophilus* together comprised 3.9% of the 461 pathogens identified during the 14-month survey in South Africa. *A. butzleri* accounted for 16% of the *Campylobacter*-like isolates obtained from Thai children with diarrhea. In that study, contaminated water was suggested as a vehicle of transmission.

The clustering of the cases in an outbreak of *A. butzleri* in Italian schoolchildren with recurrent abdominal cramps suggested person-to-person transmission. *A. butzleri* has also been recovered from an acquired immunodeficiency syndrome (AIDS) patient with intermittent diarrhea in the absence of other enteric pathogens. Nonhuman primates are naturally infected with *Arcobacter* and may develop colitis, which may provide insight into its pathogenesis in humans.

12.2.2 Characteristics of agent

Arcobacter are morphologically similar to *Campylobacter* in dark-field microscopy. Rapid darting motility is observed; unusually long cells of >20 μm may be seen. Colonies are small (1 mm in diameter), generally nonpigmented, and convex with entire edges and may swarm on fresh agar.

Aerotolerance after initial isolation in a microaerobic environment and growth at 15–30 °C are the key features to distinguish *Arcobacter* from thermophilic *Campylobacter* species. However, *A. butzleri*, like *Campylobacter jejuni*, may grow at 42 °C. In one study, more than 75% of *A. butzleri* field strains examined grew at 42 °C.

Phenotypic traits may distinguish the three principle species of *Arcobacter* recovered from livestock and humans. The most reliable phenotypic tests to identify *A. butzleri* are negative or weak catalase production, growth in *Campylobacter* minimal medium, abundant growth on blood agar, growth on MacConkey agar, and resistance to cadmium chloride. In contrast, *A. cryaerophilus* is strongly catalase positive, does not grow on *Campylobacter* minimal medium or on MacConkey agar, and is sensitive to cadmium chloride. The heterogeneous species, *A. cryaerophilus*, consists of at least two subgroups whose identification may add insight into epidemiological associations. *A. skirrowii*, which is infrequently isolated, exhibits a strong catalase activity, reduces nitrate, but fails to grow on MacConkey.

12.2.3 Epidemiology

The epidemiology of *Arcobacter* may parallel that of *Campylobacter*. For example, *Arcobacter* species may be commensals of livestock. For cattle, *Arcobacter* (10%) and *A. butzleri* (1.5%) were detected in the feces of clinically healthy dairy cows, which may explain its presence in raw milk. Likewise, isolation of *Arcobacter* in live cattle feces predicts its presence in beef. That *A. butzleri* was cultured from 1.5% of minced beef samples examined (n=68) does not preclude environmental contamination during preparation.

Arcobacter is present in the feces of 40% of healthy pigs and is recovered from aborted porcine fetuses. However, there is no conclusive evidence establishing *Arcobacter* as a cause of abortion in livestock. *Arcobacter* was detected in 22% of ground pork samples (n=290) in the United States. In contrast, *Arcobacter* was cultured from only 0.5% (1 of 194) of pork samples in the Netherlands. The difference in handling between ground pork and minimally processed pork cuts as well as isolation methods may underlie the differences between the findings of the two studies. Despite its presence in livestock and red meats, no human cases of *Arcobacter* have been linked to consumption of either beef or pork.

Arcobacter, like *Campylobacter*, has been reported more frequently in poultry products than red meats. Thus, poultry meat may be a significant reservoir of *A. butzleri*. In France,

A. butzleri was recovered from 81% of poultry carcasses examined (n=201). Nearly half of the poultry isolates in that study were of serogroup 1. Because serogroups 1 and 5 are recovered from clinical cases, consumption of undercooked contaminated poultry may be a risk factor for human infection. In Canada, *A. butzleri* was recovered from 97% (121 of 125) of poultry carcasses obtained from five different processing plants. Again, serotype 1 was the predominant group isolated from Canadian poultry. In contrast, *Arcobacter* was detected in only 24% (53 of 224) of retail purchased poultry products in the Netherlands.

In the United States, a pilot study estimated the prevalence of *Campylobacter* and *Arcobacter* in whole turkey carcass rinses (n=300). Overall, *Arcobacter* (88%) was recovered more frequently than *Campylobacter* (54%). More specifically, *A. butzleri* (75%) was detected more often than either *C. jejuni* (27%) or *Campylobacter coli* (27%). In a tristate study, *Arcobacter* (77% of samples) and *A. butzleri* (57% of samples) were isolated from mechanically separated turkey meat (n=395 samples). This suggests that *Arcobacter*, in contrast to *C. jejuni*, survives the aeration associated with meat grinding. Adaptations to the chlorinated chiller tank environment used in poultry processing, such as replication at 5 °C, and enhanced survival in the presence of organic material, favor cross- contamination and yield the higher prevalence of *Arcobacter* on poultry carcasses when compared to its infrequent isolation from live birds. Finally, *A. butzleri* forms biofilms on stainless steel surfaces. especially when incubated in chicken meat juice (5 °C to 21 °C), an organic matrix, which enhances survival of both *C. jejuni* and *A. butzleri* at refrigeration temperatures (77 days at 5 °C).

The ease of recovering *Arcobacter* from poultry meat contrasts with its infrequent isolation from live birds, despite its presence in moist litter. The inability to colonize the intestinal tract of poultry contrasts with the high carriage rate of *C. jejuni* and *C. coli*. For example, *Arcobacter* (2.2%) and *Campylobacter* (60%) were detected in cloacal swabs of live turkeys (n=368). Its low recovery from live birds and its high prevalence on poultry carcasses suggest that extensive contamination occurs during processing and reflect the ability of *Arcobacter* to survive in cold processing waters.

Arcobacter spp. have been isolated or detected in seafood, shellfish, and raw milk. Given its recovery from dairy cattle (10%) and its tolerance to cold, air, and salt, it is probable that its prevalence in these foods may exceed that of *Campylobacter*.

12.2.4 Detection and differentiation of organism

Methods for collecting and transporting foods, water and fecal samples, poultry carcass rinses, and livestock carcass swabs are conducted generally as described for *Campylobacter*. Like *Campylobacter*, samples may be transported on ice in buffered peptone water or Cary-Blair transport media.

No single method is universally accepted for the recovery of *Arcobacter* from clinical specimens, foods, livestock, or water. This has stymied comparison of prevalence estimates. *Arcobacter* were first described from aborted bovine and porcine fetuses cultured in EMJH-P80 semisolid medium formulated to recover *Leptospira* from clinical samples. Use of media designed for *Campylobacter* when incubated microaerobically at 30 °C are adequate for *Arcobacter* isolation. To illustrate, *A. butzleri* was recovered from poultry meat using a modification of Rosef's enrichment broth described for the cultivation of thermophilic *Campylobacter*.

A rigorous comparison of five published methods for isolation from livestock feces revealed that a 24-hour enrichment in *Arcobacter* specific broth (supplemented with 5-fluorouracil, amphotericin B, cefoperazone, novobiocin, and trimethoprim), followed by plating to mCCDA (supplemented with amphotericin B, cefoperazone and teicoplanin), was the most sensitive

(71%) and specific (64%). Formulations have been developed specifically for *Arcobacter*, and at least one is commercially available.

PCR assays targeting species-specific 16S and 23S rRNA genes of the genus *Arcobacter* as well as protocols specific for *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* have been described. A multiplex PCR assay to simultaneously identify the major species of public health importance, namely *A. butzleri* and *A. cryaerophilus*, in livestock and foods are continuously improved. The multiplex PCR assay is conducted either on pure cultures or directly from enrichment, which foregoes the need for isolates, bypasses the ambiguities of biochemical identification, and is a suitable screen for field studies. As expected, prevalence estimates based solely on PCR protocols conducted from enrichments consistently exceed those based on microbiological culture, which suggests either a high percentage of false positives or fastidious microbes which are lost during culture. One platform for species identification of pure isolates amplifies a 527 bp fragment of 16S rRNA and then compares the sequences with the commercially-available MicroSeq 500 library.

Field strains of *A. butzleri* may be distinguished by serotyping. At least 72 serogroups are recognized. As with *Campylobacter*, however, the limited availability of serotyping reagents severely restricts the applicability of this method and has been replaced by molecular analysis.

The development of DNA-based typing methods has accelerated epidemiological studies. In addition to providing species identification, the genomic profiles can be used to show identity of isolates, inferring an epidemiological relationship, or their dissimilarity, indicating that the isolates are not related and thus may not be associated with a common source of contamination. Molecular fingerprints derived from restriction fragment length polymorphisms, have suggested person-to-person transmission in a nursery school outbreak in Italy and have indicated multiple sources of contamination of mechanically separated turkey meat in the United States. Advances in metagenomics, in which microbial communities are identified by 16S rRNA gene sequences, avoid the bias inherent in microbiological culture. Metagenomics identified the abundance and diversity of *Arcobacter* present in wet poultry litter, again emphasizing the environmental robustness of the genus. Molecular methods such as pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are powerful epidemiological tools for isolate profiling. The publication of the full *A. butzleri* genome sequence identified seven housekeeping genes identical to those of *Campylobacter* suitable for MLST (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt*).

12.2.5 Physical methods for destruction

Because of its adaptation to the environment, *Arcobacter* may be more resistant to inactivation (heating, freezing, etc,) than *Campylobacter*. By comparing D values (the irradiation dose that reduces by 10-fold the number of viable bacteria), *A. butzleri* (0.27 kGy) was found to be more resistant to irradiation than *C. jejuni* (0.18 kGy). Thus, irradiation doses (1.5 to 4.5 kGy) approved by the U.S. Food and Drug Administration for meats would kill *Campylobacter* as well as *Arcobacter*. *Arcobacter* grows over a pH range of 5.5–7.5 with optimal growth of *A. butzleri* at pH 6 and optimal growth of *A. cryaerophilus* at pH 7.0–7.5. Thermal tolerance studies indicate the thermal death time for a single strain of *A. butzleri* to be 2.5, 5, and 15 min at 60, 55, and 50 °C, respectively. Based on these data, *Arcobacter* will not survive minimum pasteurization treatment (63 °C for 30 min or 71.7 °C for 15 s) of milk. Thermal tolerances, as measured in D-values (time required to reduce by 10-fold the number of viable bacteria at a specific temperature), have been computed for survival of *Arcobacter* in both absence (phosphate buffered saline) or presence (ground

meat products) of biological matrices. When suspended in PBS, *Arcobacter* (0.03 to 5.81 min at 60 to 50 °C) is more thermotolerant than *Campylobacter* (0.88 to 1.63 min at 50 °C) with heat resistance (D-values) as predicted, enhanced in a food milieu.

An increase in NaCl is correlated with a decline in active water (aw) activity. With respect to salt tolerance, some strains of *Arcobacter* can survive in 5% NaCl, which corresponds to a water activity value of 0.968. In contrast, *Campylobacter* spp. are sensitive to drying and have minimal aw score of 0.990 (~0.85% NaCl). As Cerevenka concluded in reviewing inactivation strategies, multiple strains from multiple sources should be evaluated in order to compensate for the 'non-homogeneous behavior' of strains.

Arcobacter, in contrast to *Campylobacter*, grows at 15 °C, a trait reflected by the species name *cryaerophila*. This suggests that it can survive in the cold longer than typical *Campylobacter* species. For example, *A. butzleri* populations decreased by 0.5 $\log_{10}$ when held in ground water at 5 °C for 14 days, underscoring the environmental hardiness of the genus. In contrast, *C. jejuni* declined by six to seven $\log_{10}$ units when held for a similar interval.

12.2.6 Prevention and control

The limited epidemiological data available for *Arcobacter* suggest that transmission to humans results from consumption of either contaminated water or undercooked poultry. Chlorine inactivates *A. butzleri* as well as *C. jejuni* and *H. pylori*. Thus, disinfection practices normally used in drinking water are adequate to control these closely-related organisms. This is critical because of the epidemiological relationship noted between cases of human enteritis due to *A. butzleri* and consumption of contaminated drinking water. Thus, interventions to prevent human infection should focus on consumption of chlorinated drinking water and thorough cooking of poultry. These measures parallel those recommended for *Campylobacter*.

In summary, members of the genus *Arcobacter* encompass free-living organisms of aquatic niches, which are occasionally associated with food animals and infrequently isolated from humans. Its distribution in water, livestock, meats, shellfish, and fish reflects robust genetic adaptation to diverse environments.

12.3 *Helicobacter*

Members of the genus *Helicobacter* colonize humans and animals and cause enteritis, gastritis, and – rarely – abortions in livestock. By 2011, 36 species had been described from a variety of animal hosts, including primates, poultry, and domestic animals (Table 12.3). Each species maintains a restricted host range. Humans are the major hosts for *H. pylori*, which is the most common human bacterial infection in the world.

12.3.1 Nature of human illness

Helicobacter pylori is present in 95% of duodenal and in 70–80% of human gastric ulcer cases as well as in clinically healthy individuals, including family members of patients. It is unique among bacteria in being directly linked to human gastric carcinoma.

Antibodies to *H. pylori* are highest in individuals from rural settings and in populations of low socioeconomic status. Antibodies in >50% of the population occur in individuals living in developing countries. In contrast, antibodies are found in <50% of the population living in industrialized countries such as Australia, the United States, and France.

Table 12.3 *Helicobacter* Species, Host Distribution and Target Organ

***Helicobacter* Species**	**Host**	**Target organ**
H. pylori	Human, rhesus macaque	Stomach
H. acinonychis (H. acinomyx)	Cheetah, felines	Stomach
H. anseris	Geese	Intestine
H. aurati	Hamsters	Stomach
H. baculiformis	Cat	Stomach
H. bilis	Mouse	Bile, intestine, liver
H. bizzozeroni	Dog	Stomach
H. bovis	Cattle	Stomach
H. brantae	Geese	Intestine
H. canadensis	Geese	Enterohepatic
H. canis	Dog, humans	Enterohepatic
H. cetorum	Seals	Stomach
H. cholecystus	Hamster	Entrohepatic
H. cinaedi	Hamsters, human	Intestine
H. colifelis	Cat	Intestine
H. cynogastricus	Dog	Stomach
H. equorum	Horses	Intestine
H. felis	Dogs, cat	Stomach
H. fennelliae	Human	Enterohepatic
H. sp. flexispira taxon 8	Dogs, humans, mice	Intestine
H. sp. flexispira taxon 1	Sheep	Reproductive tract
H. ganmani	Rodents	Intestine
H. hepaticus	Mouse	Intestine
H. macacae	Monkeys	Intestine
H. mainz	Humans	Blood
H. marmotae	Woodchucks, rodents	Enterohepatic
H. mesocricetorum	Hamsters	Enterohepatic
H. muridarum	Rat	Stomach, caecum
H. mustelae	Ferret	Stomach
H. nemestrinae	Pig-tailed macaque	Stomach
H. pametensis	Pig, tern	Enterohepatic
H. pullorum	Poultry, human	Enterohepatic
H. rodentium	Laboratory mice	Enterohepatic
H. salomonis	Dog	Stomach
H. suis	Hog	Stomach
H. suncus	Shrew	Stomach
H. trogontum	Rat	Enterohepatic
H. typhlonius	Mouse	Intestine
H. westmeadii	Human	Blood culture
H. winghamensis	Human	Enterohepatic

12.3.2 Characteristics of agent

Helicobacter pylori is a Gram-negative microbe that is motile by means of four to six unipolar sheathed flagella, microaerophilic, and spiral shaped. The sheathed flagella may be adaptations to survival in gastric juices. Growth occurs between 30 and 37 °C, but not at 42 or 25 °C. Humans are the only significant reservoir of *H. pylori*, which colonizes the mucosa of the antrum of the stomach where few acid secretory parietal cells are located.

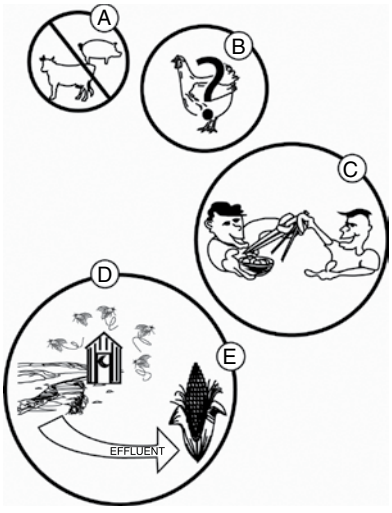

Figure 12.1 Possible food-related routes of transmission of *Helicobacter pylori*. (A) *H. pylori* has not been detected in cattle or swine. Thus, there is no evidence in support of transmission of *Helicobacter* species to humans via consumption of beef and pork. (B) However, *H. pullorum* may be transmitted to humans by consumption of contaminated poultry products. (C) Since *H. pylori* is present in saliva, transmission by chopsticks has been suggested. (D) *H. pylori* has been cultured from human feces. Thus, flies may serve as reservoirs or mechanical vectors of transmission of *H. pylori* and disseminate it from feces by routes similar to other enteric pathogens. (E) Contaminated feces or sewage effluent may pollute the water supply which is consumed or used to irrigate vegetables. *H. pylori* may survive in flies.

12.3.3 Epidemiology

H. pylori may be transmitted to humans by (a) fecal–oral spread; (b) oral–oral spread via salivary secretions; and, less likely, (c) ingestion of contaminated foods and water (Fig. 12.1). Close contact, poor sanitary conditions, familial crowding, confinement in submarines, and clustering in institutions have also been identified as risk factors for *H. pylori* infection.

Fecally-contaminated foods and water are potential vehicles of transmission. *H. pylori* has been detected in human feces either by culture, which is the 'gold standard', or by PCR. As expected, the PCR format has been shown to detect more positive samples (more 'sensitive') than culture, electron microscopy, or histological examination. Because of the potential for 'false positive results' as well as high interspecies similarity (98 to 100%) among some helicobacters species especially when amplifying 16S rDNA targets, identification should incorporate other 'informative macromolecules' such as 23S rRNA, *glmM*, *hsp60*, *ure B* and the *gyrB* genes. For example, study of milk products detected the phosphoglucosamine mutase gene (*glmM*) of *H. pylori* by nested PCR in 34.7% of raw milk samples, but failed to culture *H. pylori*.

Fecally-contaminated fruits, vegetables, and shellfish may transmit *H. pylori*. In a study of 1815 Chileans under the age of 35, *H. pylori* antibodies were detected in >60% of lower socioeconomic groups. Seropositivity correlated with age, low socioeconomic status, and consumption of uncooked vegetables. Another risk factor that reached marginal significance was consumption of uncooked shellfish. In contrast, no significant difference in *H. pylori* seroprevalence was found between vegetarians and meat eaters in the United States. No isolations of *H. pylori* have been reported from vegetables, fruits, shellfish, or seafoods.

Water has been proposed as a vehicle of transmission based on PCR detection of *H. pylori* in water, including rivers and sewage-contaminated water. In communities of Lima, Peru, the water source may be a more important risk factor than socioeconomic status in acquiring *H. pylori* infection. One study evaluated 407 Peruvian children (aged two months to 12 years) from families of low and high socioeconomic status. Children from high-income families whose homes received municipal drinking water were 12 times more likely to be infected than those from high-income families whose water supply came from community wells. Thus, the municipal water supply may present a greater risk of infection than the socioeconomic status.

Results of serosurveys of veterinarians and slaughterhouse workers have suggested that *H. pylori* is a zoonosis and is therefore transmitted from livestock to humans. Antibodies to *H. pylori* were detected more frequently in slaughterhouse workers exposed to animal carcasses than in clerks employed at the same abattoir. However, no pre-employment serological titers were included in that study to evaluate pre-existing infection status. Also, comparisons between the clerical and nonclerical staff with respect to age, socioeconomic status, and country of origin were not provided. As previously noted, infection status increases with age and reflects social economic status as well as country of birth. That antibodies to *H. pylori* are higher in persons in contact with sheep is provided as evidence of zoonotic transmission.

Evidence of infection by *Helicobacter*-like organisms in livestock, including catttle and hogs, is based in part on serosurveys. However, given the phylogenetic diversity of the genus (Table 12.3), use of nonspecific test antigens may predictably lead to false-positive reactions. Of all of the *Helicobacter* species, only *H. pullorum* may be transmitted from poultry to humans via consumption of undercooked chicken.

12.3.4 Detection of the organism

Methods to detect *Helicobacter* in foods are limited. Isolation protocols that have been used to culture the microbe from clinical specimens may be adapted to its recovery from foods. In general, a suspect food product, for example poultry samples contaminated with *H. pullorum*, are homogenized and plated onto nonselective and selective media. Nonselective media include chocolate agar or brain heart infusion agar supplemented with 5–10% defibrinated blood. Selective agars are commercially available. Freshly poured plates are inoculated, incubated (35 °C, 3–5 days) microaerobically (5–7% O_2, 5–10% CO_2), with the critical addition of hydrogen (8% H_2), in high humidity. Plates are examined for the presence of small pinpoint translucent nonhemolytic colonies. The presence of Gram-negative, curved, or spirally shaped bacilli that grow at 35 °C but not at 25 °C and are strongly urease positive is indicative of *Helicobacter*.

Polymerase chain reaction assays have accelerated the identification of the diverse species of *Helicobacter*, especially *H. pylori*, in fecal, gastric biopsy, and oral cavity samples. The PCR assays specifically designed for *H. pylori* target, for example, the *vac A* and *cag A* genes and the genes encoding the urease enzyme.

12.3.5 Physical methods for destruction

Thermal and cold tolerance data are limited for *H. pylori*. However, because of their phylogenetic relationship, it is probable that *H. pylori* is as sensitive to heat, cold, and irradiation as *C. jejuni* and *A. butzleri*. *Helicobacter pylori* survived longer in experimentally inoculated milk that was refrigerated (six days) than in milk held at room temperature (three days).

With respect to water treatment, chlorination studies completed in the United States by the Environmental Protection Agency indicated that *H. pylori* is as sensitive to standard chlorination regimens as *C. jejuni*, *A. butzleri*, and *Escherichia coli*. Isolation of *H. pylori* (n = 10 isolates) from drinking water in Basra, Iraq, was attributed to the low chlorine concentrations (<0.5 mg chlorine/liter) in the treated municipal water system. A Mexican study reporting 16S rRNA genes of *H. pylori* by PCR in seven water pretreatment samples (100%) failed to detect *H. pylori* in 20 samples after chlorination (0%). Undoubtedly, as laboratory protocols are refined the importance of water transmission may be clarified.

12.3.6 Prevention and control

Risk analyses indicate that *H. pylori* may be transmitted to humans via consumption of fecally-contaminated water. However, routine chlorination inactivates *Helicobacter* as well as *Campylobacter* and *Arcobacter*. Flies may transport *H. pylori* from feces to foods. Thus, intervention strategies that effectively halt fecal spread by insects of other foodborne agents should be effective.

Epidemiological evidence suggests that, in developing countries, consumption of sewage-contaminated vegetables may be a risk factor for *H. pylori* infection. Therefore, cleaning of raw vegetables with chlorinated water would most likely reduce the risk of infection.

To summarize, although other *Helicobacter* species have been recovered in livestock, *H. pylori* has not been confirmed in cattle, sheep, hogs or poultry, thus eliminating these meat animals as a source of infection. In developing countries, however, consumption of sewage-contaminated drinking water and vegetables may be risk factors for *H. pylori* infection. Isolation and/or specific detection of *H. pylori* in fruits, vegetables and meats will provide undisputable evidence for possible transmission via foods. In the absence of case control studies, there is minimal evidence for significant transmission of *H. pylori* or other *Helicobacter* species in foods.

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Arcobacter

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13 *Brucella*

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13.1 Introduction

Brucellae are Gram-negative, facultative, intracellular bacteria that can infect humans and many species of animals. Brucellosis is an ancient disease that can be traced back possibly to the 5th plague of Egypt around 1600 BC. Historically, a disease pattern compatible with brucellosis was also described by Hippocrates (450 BC). It has also been suspected as a disease occuring during the Roman Empire. In 1887 Sir David Bruce isolated the organism (*Micrococcus melitensis* at that time) responsible for brucellosis, also known as Malta fever, from a British soldier who died from the disease in Malta. This bacterium was renamed *Brucella melitensis* in his honor. In 1905, Zammit demonstrated, again in Malta, the zoonotic nature of *B. melitensis* by isolating it from goat's milk. Human brucellosis has always been associated with an animal (domestic or wild) reservoir of *Brucella* spp. However, the main sources of infection(s) as well as the routes of contamination may differ. Brucellosis is one of the most frequent bacterial zoonoses in low-income countries, where the control programs have not succeeded in eradicating this neglected zoonosis. The disease is a major cause of direct economic losses and an impediment to trade and exportation.

The genus *Brucella* has traditionally been classified into six species: *B. melitensis*, *B. suis*, *B. abortus*, *B. neotomae*, *B. ovis*, and *B. canis*, which are reflective of animal host preference. In 1985, it was proposed that the six *Brucella* species should be grouped as biovars of a single species based on DNA-DNA hybridization studies. The *Brucella* Taxonomic Subcommittee of the International Committee on Systematics of Prokaryotes adopted this proposition. However, the international community of *Brucella* researchers has never accepted this change and a return to the pre-1986 taxonomy was advocated and eventually adopted by the *Brucella* Taxonomic Subcommittee. The genus *Brucella* has been further expanded with a set of recently-discovered species. Such species include *B. ceti* and *B. pinnipedialis* that have been isolated from cetaceans and pinnipeds respectively. *B. microti* has been initially isolated from the common vole but later from the red fox and from soil. The latest described species is *B. inopinata* which was isolated from a breast implant infection in a woman with clinical signs of brucellosis.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

Interestingly, it has been suggested that the divergence of species in the genus *Brucella* could have been concomitant with the divergence of their mammalian hosts, 60 millions years (my) ago. However, recent comparative genome sequence analyses suggested that this is inconsistent with the fact that the hosts of *B. ceti* and *B. pinnipedialis* did not diverge at the same time. The ancestors of pinnipeds where carnivores and molecular data have been used to estimate the split between ursids and pinnipeds to 35.7 ± 2.63 (= mean ± SE) my, and fossil records report early pinnipeds 35 my ago. Cetaceans went back to the sea much earlier, the oldest known cetaceans date back to the Eocene, 55 my ago. According to genome sequence analyses most *Brucella* species probably diverged 86,000 to 296,000 years ago. The recent complete genome sequence analysis of *B. pinnipedialis* reference strain B2/94 revealed that the divergence time of *Brucella* spp. found today in marine mammals is totally incompatible with the divergence time of their hosts. A fortuitous contamination of cetaceans and pinnipeds, probably via the food chain, may explain better this transmission of *Brucella* to marine mammals. This also opens the remote possibility of *Brucella* from marine origin infecting terrestrial mammals.

13.2 Nature of illness in animals and humans

Brucellosis is an economically important disease in production animals worldwide. *B. melitensis*, *B. abortus*, and *B. suis* cause abortion and infertility in their natural hosts, goats and sheep, cattle and swine, respectively. The ability of *Brucella* spp. to successfully survive and replicate within different host cells explains their pathogenicity. Extensive replication of *Brucella* spp. in placental trophoblasts is associated with abortion in their animal preferential hosts, and persistence in macrophages leads to chronic infections that are a hallmark of brucellosis in both natural animal hosts and humans. Albeit their respective host preferences, *Brucella* spp. have also been isolated from a great variety of wildlife species. As a consequence, different wildlife species may act merely as spillover hosts (victims) or as reservoir hosts (vectors) of *Brucella* spp. for other animal species and humans. The source of naturally acquired brucellosis in humans is almost always to be found in the animal reservoirs, although a very few cases of human-to-human transmission have been reported.

More than 500,000 new cases of human brucellosis occur each year. Human brucellosis is an illness characterized by acute or insidious onset of fever, night sweats, undue fatigue, anorexia, weight loss, headache, and arthralgia. The overlap with a wide range of infectious and noninfectious diseases leads to a wide differential diagnosis, and misdiagnosis and confusion with other diseases has led to brucellosis being labelled as a 'major mimicker' and a 'disease of mistakes'. Among the diseases, it can be confused with tuberculosis, typhoid fever, infective endocarditis, leptospirosis, cryptococcosis, histoplasmosis, infectious mononucleosis, malaria, collagen vascular disease, chronic fatigue syndrome and malignancy. In chronic cases, psychiatric symptoms, mostly depression and mental disturbances, have been observed and ascribed to delayed convalescence.

13.3 Characteristics of *Brucella* species

The genus *Brucella* belongs to the family Brucellaceae within the order Rhizobiales of the class Alphaproteobacteria. The closest phylogenetic neighbor of the genus *Brucella* is the genus *Ochrobactrum*, a saprophyte that occasionally infects humans.

Six species were initially recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*. This classification is based mainly on differences in pathogenicity, host preference, and phenotypic characteristics. Four additional species have been included since 2007 in the genus *Brucella*. These comprise the species *B. ceti* and *B. pinnipedialis* isolated from marine mammals, with cetaceans (dolphin, porpoise, and whale species) and pinnipeds (various seal species) as the preferred hosts, respectively. According to several molecular typing analyses, one subgroup within *B. ceti*, which is composed exclusively of strains isolated from various dolphin species, has been proposed to constitute a separate species with the name *B. delphini*. The isolates from cetaceans from the Pacific have also been proposed to constitute a separate species. Three human cases with naturally-acquired infection by *Brucella* strains presumably of marine origin have been reported, one case of spinal osteomyelitis from a patient in New Zealand, and two neurobrucellosis cases from Peruvian patients. Interestingly, these human isolates presented the same genotype as cetacean strains from the Pacific. *B. microti*, first described in 2008, was isolated initially from the common vole but later also from the red fox and from soil. The latest species is *B. inopinata*, which was isolated from a human breast implant infection and represents the most distant *Brucella* species at the phenotypic and molecular levels relative to the others. The animal or environmental reservoir of the latter species is not known. New *Brucella* species will likely be described in the future, including isolates from baboons, from wild rodents in Australia, and for strain BO2 isolated from a patient with chronic destructive pneumonia. Strain BO2 and strains from wild Australian rodents have been proposed as novel lineages of the *B. inopinata* species.

The preferential hosts and the pathogenicity for humans of the 10 recognized *Brucella* species are depicted in Table 13.1.

Table 13.1 *Brucella* species, preferred host and pathogenicity for humans

***Brucella* species**	**Biovars**	**Preferential host(s)**	**Pathogenicity for humans**
B. melitensis	1–3	Sheep, goat	High
B. abortus	1–6, 9	Cattle	High
B. suis	1, 3	Swine	High
	2	Wild boar, hare	No[a]
	4	Reindeer, caribou	High
	5	Rodents	No
B. neotomae	–	Desert wood rat	No
B. ovis	–	Ram	No
B. canis	–	Dog	Moderate
B. ceti	–	Cetaceans	Unknown[b]
B. pinnipedialis	–	Pinnipeds	Unknown[b]
B. microti	–	Soil, common vole, red fox	Unknown
B. inopinata	–	Unkown	Unknown

[a] One case of *B. suis* biovar 2 infection in an immunocompromised hunter has been described in France.
[b] One human laboratory contamination has been described in the UK. Three naturally-acquired cases, presumably from marine origin, have been described. The isolates were, however, of MLSA genotype ST27, which has only been found to date in strains originating from the Pacific. The classification of marine mammal strains into species *B. ceti* and *B. pinnipedialis* is initially based on a number of isolates originating from the Atlantic. From: Godfroid et al., (2011). Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. Prev. Vet. Med. 102, 118–131.

13.4 Epidemiology

The epidemiology of animal and human brucellosis has recently been reviewed by Godfroid et al. (2011). Prevalence and epidemiology of brucellosis in livestock production has been described in many developing countries as seen by the number of reports generated in the past 10 years. Cattle seroprevalence estimates have been observed to range between 3 and 15%. Factors influencing prevalence include production systems, agro-ecological zones, husbandry practices, contact with wildlife, management factors. In cattle, brucellosis continues to contribute to economic losses associated with abortions, infertility and prolonged calving-to-conception intervals. Generally, in most developed countries, test-and-slaughter programs, together with compensation for farmers, accreditation and financial incentives for disease-free herds, allowed the achievement of a status close to eradication of brucellosis in livestock and, consequently, in humans. In cattle, the infection is predominantly caused by *B. abortus*, and is usually detected in pregnant females through abortions. The removal of sanitary barriers and the liberalization of exchanges in accordance with the World Trade Organization Agreement require the harmonization of the brucellosis health status among countries in order to eliminate the risk of contamination of a country with a favorable health status through importation of live animals and animal products from a country with a lower health status. In 2008, 12 EU member states were Officially Brucellosis Free (OBF) in cattle, as well as in sheep and goats. In 2008, 15 non-OBF member states reported bovine cases of brucellosis (herd prevalence equal to 0.12%). The situation seems to be less favorable in Southern European countries but is still below 1%. Because brucellosis has public health and international trade implications, all member states have an interest in obtaining and in maintaining this officially free status. Cases of cross-infections with *B. melitensis* were observed in herds mixed with sheep and goat flocks in southern Europe and are regularly reported in the middle east where it has become an emerging veterinary and public health problem.

B. suis biovars 1, 2 and 3 and *B. abortus* in pigs may have an impact on human health in the future. *B. suis* biovars 1 and 3 are found worldwide in most areas where pigs are kept. They affect both sexes of swine causing infertility, abortion, orchitis and lesions of bones and joints. The prevalence is generally low except in parts of South America and South East Asia. Within the EU, the epidemiological situation of brucellosis in pigs varies. Some countries are free from the disease, others report sporadic outbreaks and some report infections as an emerging problem. Available epidemiological evidence shows that *B. suis* biovar 2 is the most common agent, but biovars 1 and 3 can also occur. There is also evidence suggesting that wild boars currently remain the main source of infection for domestic pigs because several outbreaks of *B. suis* occurred in outdoor rearing farms. Enzootic *B. suis* biovar 2 has been described in wild boars and in hares (*Lepus europeaus*) in several member states of the European Union. In the south of the USA and in Australia, populations of feral swine appear to be heavily infected with *B. suis* biovar 1. Infections with *B. suis* in humans occur in people handling pigs on farms and during slaughtering and processing, including the hunting of feral swine. Recently, it has been shown in South Carolina, USA, that feral pigs were infected with *B. abortus* wildtype, S19 and RB51 vaccine strains besides *B. suis* biovar 1. The importance of this finding lies in that pigs were for the first time demonstrated to act as reservoir host of *B. abortus* in the absence of contact with cattle for more than 25 years. *B. suis* biovar 1 has also been isolated from pigs and humans in all central and South American countries. For example, in Argentina, *B. suis* biovar 1 is frequently isolated from

pigs but has also been isolated from people and cattle. As for *B. melitensis* infection in cattle, *B. suis* biovar 1 infection in cattle is an emerging veterinary and public health problem.

Currently, only three nomen species of the genus *Brucella* have an essential impact on public health, i.e., in order of their significance *B. melitensis*, *B. abortus*, and *B. suis*. Although human brucellosis is the most common bacterial zoonotic infection worldwide it is still a regionally neglected disease. Human brucellosis is known to be highly endemic in the Mediterranean basin, Middle East, Western Asia, Africa, and South America. Although animal brucellosis has been brought under control in several industrialized countries, human brucellosis occurs sporadically in individuals who acquire the infection abroad or by ingestion of unsafe, illegally imported animal products and in occupationally exposed groups. In 2008, a total of 619 confirmed human brucellosis cases were reported in the European Union (EU) (0.1 case per 100,000 inhabitants). The highest incidence was recorded in those member states not officially free from bovine and ovine/caprine brucellosis (Greece, Italy, Portugal and Spain). At EU level, a statistically significant decreasing trend was observed during the five-year period 2004–2008. The peak of reported cases was observed in spring and summer.

Studies conducted in North Africa and in the Middle East reported the occurrence of human brucellosis attributed to the presence of *B. melitenis* in livestock, while in sub-Saharan Africa *B. abortus* is mainly implicated. In areas where *B. abortus* is a major problem in cattle, seroprevalence rates in humans are estimated to be in the range of 1–5% but in areas where *B. melitensis* is endemic (mainly in the Middle East) higher prevalence rates have to be expected. The countries with the highest incidence of human brucellosis are Saudi Arabia, Iran, Palestinian Authority, Syria, Jordan and Oman. Consumption of raw milk continues to be the major mode of exposure in developing countries. Although *Brucella* can be transmitted directly and indirectly from its animal reservoir to humans, indirect transmission remains the highest overall risk and mainly occurs through the consumption of unpasteurized milk or dairy products. *B. melitensis* infection in cattle has emerged as a serious public health problem in some southern European countries and Israel as a result of the consumption of unpasteurized milk since *B. melitensis* is capable of colonizing the bovine udder. Moreover, in some South American countries, cattle are now believed to be more important than pigs as a source of *B. suis* biovar 1 infection for humans, because *B. suis* biovar 1 is capable of colonizing the bovine udder as *B. melitensis* does. Consequently, human brucellosis is mainly foodborne but can also be an occupational infection.

Few cases of brucellosis in humans caused by *B. canis* have been described. However, canine brucellosis in man might be underdiagnosed due to a low perception of the disease and a lack of valid serological tests. Human infections by strains of marine origin seem to have an atypical course but only three naturally acquired cases have been reported to date. The clinical importance of *B. inopinata* and the atypical *Brucella* strain BO2 closely related to *B. inopinata* is still unclear despite the fact that both agents have been isolated from diseased humans. Little is also known about the human pathogenicity of *B. microti* but in experimental cellular and murine models of infection *B. microti* exhibited a significantly higher virulence than other *Brucella* species.

13.5 Detection of organism

Classically, detection and identification of *Brucella* spp. has been based on cultural and phenotypic analysis (biotyping). Although undoubtedly providing valuable information, biotyping is a highly specialized and time-consuming approach requiring

experienced staff and well-optimized, noncommercial reagents ideally used under secured biological containment.

Initial PCR detection methods were based on the 16S rRNA and *bcsp31* genes. PCR methods based on the 16S rRNA amplify a DNA fragment common to all *Brucella* species but cross-react with members of the closely related genus *Ochrobactrum*. Other molecular markers such as *recA*, *omp2*, or the 16S–23S intergenic transcribed sequence have been described to identify members of the genus *Brucella*.

The presence of the mobile genetic insertion sequence element IS*711* (GenBank accession no. M94960), also known as IS*6501*, has been a useful target for molecular characterization of classical terrestrial mammal *Brucella* species and biovars based on the number and distribution of IS*711* copies within the bacterial genomes. Among classical *Brucella* species, IS*711*-based fingerprints proved to be stable, species specific (except *B. canis*), and to some extent biovar specific. Thus, the IS*711* element became the preferred target for general identification purposes due to its restricted occurrence in *Brucella* and the presence of multiple copies, allowing for unparalleled sensitivity and direct testing on clinical samples. One of the most popular PCR assays for the differentiation of *Brucella* species, designated AMOS PCR, is based on the polymorphism arising from species–specific localization of this insertion sequence IS*711* in the *Brucella* chromosome and can differentiate *B. abortus* (biovars 1, 2, and 4), *B. melitensis* (biovars 1, 2, and 3), and *B. ovis* and *B. suis* (biovar 1). Modifications of this assay have been introduced over the years to improve performance, and additional strain-specific primers were incorporated for identification of the *B. abortus* S19 and RB51 vaccine strains.

Regarding IS*711*, of particular interest are the marine mammal *Brucella* strains which have been shown to carry a higher number of the IS*711* element in their genome than terrestrial mammal *Brucella* species and biovars, with the exception of *B. ovis*. Several IS*711*-based molecular typing method studies have confirmed the classification into two marine mammal *Brucella* species with subgroups in each and correlating closely with classifications made using other molecular typing methods, such as multilocus sequence analysis (MLSA), multilocus VNTR (Variable Number of Tandem Repeats) analysis (MLVA), or *omp2a* and *omp2b* porin genes diversity analysis. It must be mentioned that this classification into *B. ceti* and *B. pinnipedialis* refers in particular to marine mammal strains isolated mostly from the Atlantic (around 300 strains isolated to date). Thus, for each of these marine mammal *Brucella* species and their subgroups, specific chromosomal IS*711* locations have been identified and PCR detection methods developed. Most recently, a novel IS*711* chromosomal location that is specific for the *Brucella* MLSA genotype ST27, previously associated with Pacific marine mammals and human zoonotic infection in New Zealand and Peru, has been identified. It supported the previous observation that this peculiar genotype is distinct from those commonly isolated from the Atlantic and currently classified within the species *B. ceti* and *B. pinnipedialis*. This novel IS*711* location, the distinct IS*711* locations in the group of strains consisting only of dolphin isolates (proposed as *B. delphini*), and other molecular data support the suggestion of additional *Brucella* species in marine mammal *Brucella* isolates besides *B. ceti* and *B. pinnipedialis*. The specific IS*711* location of MLSA genotype ST27 together with others may be of further use for the molecular identification of *Brucella* isolates of marine origin.

Recently, a new PCR assay called the Bruce-ladder PCR assay has proved to be useful to identify and differentiate for the first time all of the *Brucella* species and the vaccine strains in the same test. In contrast to AMOS PCR, this Bruce-ladder PCR is indeed able to specifically detect DNA from *B. canis*, *B. neotomae*, *Brucella* isolates from marine mammals,

B. abortus biovars 3, 5, 6, 7, and 9, and *B. suis* biovars 2, 3, 4, and 5. Other advantages are speed (the PCR can be performed in less than 24 h), minimal sample preparation (it works with whole-cell lysates), and reduced risks (PCR can be carried out with *Brucella* colonies, limiting the manipulation of live *Brucella*). The Bruce-ladder PCR thus seems to be a useful tool for the rapid identification of *Brucella* strains of animal or human origin, not only in reference centers but also in any basic microbiology laboratory worldwide. Real-time PCR assays based on some of the genetic markers described above were developed for *Brucella* species identification.

While the above molecular tools are undoubtedly valuable, none has significant resolution at the subspecies level. The use of MLSA has opened the way to detailed characterization of the global population structure of *Brucella*. These analyses confirmed the status of the classical species as distinct genetic entities, began to index intra-species diversity and relate this to historical biovar designations, and provided a framework for the placement of atypical or emerging *Brucella* isolates. Further, these analyses and the availability of more robust phylogenetic histories allowed the identification of canonical single nucleotide polymorphisms (SNPs) that could be exploited as the basis of rapid diagnostic tests. A number of SNP-based assays have recently been described that can rapidly identify *Brucella* isolates to the species and biovar level and identify vaccine strains.

A further major recent genome-driven advance has been the identification and exploitation of tandem DNA repeats as typing tools. These repeats have been exploited in many bacteria to develop a new generation of MLVA-based typing approaches but are likely to prove particularly valuable in *Brucella* which previously lacked any epidemiological tool with adequate resolution to facilitate reliable epidemiological trace-back. MLVA has proven highly efficient in characterizing outbreaks and in assessing the stability of vaccine preparations.

13.6 Prevention and control

In the developed world, control of animal brucellosis has been successfully achieved through the combination of vaccination and test-and-slaughter programs and human brucellosis through milk pasteurization, coupled with effective disease surveillance and animal movement control. In developing countries, however, control by test-and-slaughter is hardly achievable because of limited resources to indemnify farmers whose animals are slaughtered during such screening programs. While occupational exposure may be considered as a major mode of transmission, consumption of infected milk products from infected animals remains a major route of transmission even in nonendemic countries.

Successful eradication programs have always been costly, long, and hard to carry through. The difficulties in controlling and eradicating brucellosis reflect a variety of issues. The most important one is likely the animal management conditions (extensive breeding, transhumance, coexistence of several livestock species, etc.). Most often, endemic areas are in countries with marked infrastructural weaknesses, an aggravating circumstance since efficient use of current vaccines requires proficient veterinary services. This requirement relates in part to some of the limitations of currently-available brucellosis vaccines, and it seems likely that a perfect vaccine could greatly facilitate control and eradication. According to a recent review by Godfroid et al. (2011), the perfect brucellosis vaccine should: (1) trigger a solid and life-lasting immunity; (2) protect against infection by *Brucella* species other than those typical of a given host; (3) be innocuous regardless of the physiological state of

the animal; (4) be effective in a single dose; (5) not interfere with serological diagnostic tests; (6) not be virulent for humans or carry resistance to antibiotics; (7) not be shed in the environment; (8) be stable; and (9) be affordable. Indeed, some of these requirements have become apparent only after using the classical brucellosis vaccines, consisting of live attenuated strains, for more than half a century. Despite their limitations, such attenuated strains as *B. abortus* S19 and *B. melitensis* Rev.1 have been successfully used in some developed countries to eradicate brucellosis. However, their use in eradication programs poses the problem of distinguishing infected from vaccinated animals in serological tests. Although it is important to stress that this problem is of little or no significance in countries unable to implement testing and slaughtering programs, this has been considered the major drawback of these vaccines. Therefore, alternative vaccines devoid of diagnostically significant epitopes or antigens have been investigated in detail. Smooth lipopolysaccharide (S-LPS) is the most important antigen in infections by smooth brucellae (*B. abortus*, *B. melitensis*, *B. suis*) and the serologically immunodominant epitopes are carried in its O-polysaccharide section. The lipopolysaccharide of rough mutants (R-LPS) lacks the O-polysaccharide and carries only the core oligosaccharide-lipid A sections. Therefore, the use of rough mutants as vaccines should minimize the serological interference. One of them is now commonly used, and is the attenuated *B. abortus* vaccine strain RB51. This strain is a rough mutant obtained from virulent *B. abortus* strain 2308 following passage on media with rifampin and penicillin. It carries a mutation in the O-polysaccharide gene *wboA*, consisting of an IS*711* element insertion, but also other and unknown genetic defects. Concerning protection, controlled experiments show that RB51 is, however, inferior to S19. As expected, RB51 does not elicit significant amounts of antibodies to the O-polysaccharide so that its interference in brucellosis tests that use smooth *Brucella* suspensions is minimal. Nevertheless, RB51 has several drawbacks as it can induce abortions and can be excreted, and its use should be limited to nonpregnant animals. Since RB51 is more attenuated, it should be less dangerous than S19, and only very few human cases have been described. However, the number of unreported human cases might be higher because the absence of anti-O-polysaccharide antibodies makes RB51 infections undetectable in standard laboratory tests for human brucellosis. Therefore, adherence to biosafety practices should not be abandoned when using RB51. The resistance to rifampin is an additional drawback of RB51 because this antibiotic is often preferred over streptomycin or gentamicin (in all cases combined with doxycycline) for treatment of human brucellosis. Although introduced over 12 years ago, no country using RB51 has eradicated cattle brucellosis although success has recently been suggested in the Azores, Portugal. RB51 does not protect sheep against either *B. melitensis* or *B. ovis*.

Various other vaccine candidates (LPS defect mutants, protein deploid mutants, etc.) have been tested in animal experiments or have not gone beyond laboratory models. Thus further research is needed in vaccine development to try to obtain the best combination of efficient vaccine – efficient diagnostic test to eradicate brucellosis in countries where it remains a major public health issue and in any re-emerging situation.

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14 *Escherichia coli*

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14.1 Introduction

Escherichia coli is a normal inhabitant of the digestive tract of animals, including humans. It serves many beneficial functions in the body by synthesizing useful vitamins and by competing and suppressing the growth of pathogenic bacteria that may be present or ingested with food and water. With the exception of anaerobic bacteria, *E. coli* is one of the dominant enteric species in the human feces; therefore, it has been used as an indicator of fecal contamination for close to a century. The concept of indicators is based on the premise that if *E. coli* is detected in food and water, it is evidence that the product has been fecal contaminated and that pathogens may also be present. However, as *E. coli* can be found in environmental sources, the use of *E. coli* as a fecal indicator has been challenged as being unreliable; but as no suitable substitute has been proposed, it continues to be used as an indicator of insanitation worldwide.

E. coli is a member of the family Enterobacteriaceae and the genus is composed of Gram negative, aerobic, facultatively anaerobic, non-sporeforming rods. They have the ability to ferment a variety of sugars, but the fermentation of lactose with production of acid and gas is characteristic, as it is for other members of the coliform group. *E. coli* can be further classed into biotypes based on the IMViC tests (indole, methyl red, Voges-Proskauer, and citrate), where biotype I and II have ++−− and −+−− reactions, respectively. Since the majority of *E. coli* are indole positive, biotype I are much more prevalent. Other biochemical traits include ß-glucuronidase activity, but absence of urease, phenylalanine deaminase and H_2S production. Serological typing of *E. coli* is based on 173 somatic (O), 56 flagellar (H) and 80 capsular (K) antigens, so is very complex. But serotyping continues to be useful for strain classification and especially in the identification of some of the notable pathogenic serotypes such as O157:H7.

E. coli is often regarded as harmless, but when intestinal barriers are compromised, especially in debilitated or immunosuppressed hosts, even typical *E. coli* may cause infections, so they are known as opportunistic pathogens. There are, however, *E. coli* groups which have acquired virulence factors and have the ability to cause diarrheal disease in

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healthy humans. These are known as pathogenic *E. coli* or diarrheagenic or enterovirulent *E. coli* and there are six major groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent *E. coli* (DAEC). Another group, Shiga toxigenic *E. coli* (STEC), is comprised of a large group of strains that produce Shiga toxin (Stx), but not all appear to be pathogenic to humans. EHEC is a subset of STEC and comprised of pathogenic strains. Pathogenic *E. coli* are usually grouped based on distinct epidemiological and clinical features and/or unique virulence factors, so the identification of strains in each group entails testing for the specific virulence determinants. An exception, however, is EHEC O157:H7, which is widely recognized by its serotype.

This chapter examines general characteristics, disease, and epidemiology of pathogenic *E. coli* groups. It also includes discussions on methods used for detection and characterization, as well as preventive measures to inactivate and control these pathogens in foods. Of the pathogenic *E. coli* groups EHEC, and especially O157:H7, has been implicated most in outbreaks worldwide; hence, extensive research and method development has focused on this group. Lastly, EAEC has seldom been associated with large outbreaks, although an unusual EAEC strain that produced Stx caused a large outbreak in Germany in 2011, so the importance of horizontal virulence gene transfer among pathogenic *E. coli* and the emergence of atypical strains is also discussed.

14.2 Illness and epidemiology

Almost all infections with pathogenic *E. coli* begin with the colonization or attachment to the intestinal mucosa, followed by elaboration of virulence factors, that causes the symptoms or gastrointestinal illness which are characteristic for that group. The symptoms of pathogenic *E. coli* infection can range from asymptomatic or mild diarrhea in healthy adults to severe complications, such as hemolytic uremic syndrome (HUS), that can result in fatalities, especially in the young, the elderly and in immunocompromised host. The public health importance of pathogenic *E. coli* also varies as some groups are well recognized pathogens and cause foodborne diseases worldwide, whilst others only cause sporadic cases of gastrointestinal illness in mostly underdeveloped countries and often via unknown food sources. A summary of some properties and illness associated with the various pathogenic *E. coli* groups is shown in Table 14.1 and discussed below.

14.2.1 EPEC

EPEC was first identified as a pathogen in 1955, and it is most commonly associated with infantile diarrhea in children under 2 years of age. EPEC infantile diarrhea is prevalent in developing countries, and it sometimes surpasses in frequency to diarrheal infections caused by rotavirus. In developed countries or those with good hygiene standards, EPEC has become less significant and only causes sporadic infection. A variety of foods, including raw beef and chicken, cold pork, meat pie and coffee substitute drink, have been implicated in EPEC outbreaks worldwide but water is also a common vehicle of infection. The use of contaminated water in the preparation of infant formulas is suspected as a source of EPEC infection in children. The infectious dose of EPEC for infants is unknown; but data from volunteer feeding studies showed that, in healthy adults, it is estimated to be 10^6 organisms. After ingestion of the contaminant, the average incubation prior to the onset of illness is

Table 14.1 Some Properties and Symptoms of Pathogenic *E. coli* Groups

Traits/ Symptoms	ETEC	EPEC	EHEC	EIEC	EAEC	DAEC
Toxin (s)[a]	LT/ST	–	Stx (VT)	–	EAST1	–
Invasive	–	low	low	+	–	–
Adhesin	fimbrae	intimin	intimin	non-fimbrae	fimbrae	fimbrae
Stool	watery	watery, bloody	watery, very bloody	mucoid, bloody	watery, mucoid	watery
Fever	–	low	–	+	low	–
Major serotypes	O128c, O6, O8, others	O26, O111 others	O157:H7, O26, O111 others	O28c, O29 others	O44, O104 O3, others	O11, O75 others
I_D[b]	10^8	10^6	$10^{1\text{-}2}$	10^6	unknown	unknown

[a]LT – labile toxin; ST – stable toxin; Stx (VT) – Shiga/Vero toxin; EAST1 – ST-like toxin.
[b]I_D – Infectious dose.
Table modified from P. Feng and S.D. Weagant, FDA BAM, Chapter 4A (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070080.htm)

36 hr, but can range from 17 to 72 hr. The predominant symptoms of EPEC infection are diarrhea with watery stools that sometimes can be bloody, with little or no fever. The illness is usually over in a few days; but in severe cases, it can last up to 14 days. EPEC infections are best treated by preventing dehydration arising from the diarrheal symptoms. Some antibiotics have been found to be effective, but antibiotic-resistant EPEC strains are known to exist. Infection or exposure to EPEC does appear to build up immunity, which may account for the lower incidences of EPEC infections in older children.

14.2.2 ETEC

ETEC is best known as the causative agent of traveler's diarrhea; but it is also an important diarrheal pathogen in infants. ETEC infections are endemic in many developing countries in the tropics or in areas with poor hygienic standards, and infections are especially prevalent in the warm, wet months. The onset of illness is usually 26 hrs after ingestion but it can range from 8 to 44 hrs. It is characterized by sudden onset of watery diarrhea, without blood or mucus, and it is rarely accompanied by fever or vomiting. Illness is usually self limiting, mild and brief, lasting only a few days; but in some severe forms, infections can persist for up to 19 days and resemble cholera with up to five or more daily passages of rice water-like stools that result in severe dehydration. Antibiotic treatment is usually not required in ETEC infections but it seems to be effective in reducing the duration and severity of the illness. ETEC infections are usually not transmitted by person-to-person contact but some hospital infections have occurred, probably caused by insanitary conditions. Consumption of contaminated food or water accounts for most ETEC outbreaks. In 1975, a large outbreak affecting 2000 people was traced to sewage-contaminated water at a national park. Contaminated well water in Japan and water supply aboard cruise ships have also been implicated in ETEC outbreaks. Foodborne outbreaks of ETEC have occurred in restaurants and at food catered events and have implicated Brie, curried turkey mayonnaise, crabmeat, deli food and salads. ETEC is often found in feces of asymptomatic carriers so humans are the most likely source. In most cases, foods are contaminated with ETEC via infected food handlers or by the use of contaminated water during food preparation. The infectious dose for ETEC is high and data from volunteer feeding studies estimate it to be 10^8 cells.

14.2.3 EHEC

The principal illness caused by EHEC is hemorrhagic colitis (HC), which is characterized by acute abdominal cramps and bloody diarrhea. In some cases, the diarrhea symptoms can be very severe, consisting of all blood and occurring every 15–30 minutes. Other symptoms include vomiting but seldom accompanied by fever. The incubation before onset of illness is usually 3–4 days but can range from 1 to 9 days and the illness can last from 2–9 days. Approximately 3–7% of the HC cases may progress to more serious complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) that can be life-threatening, especially in children and the elderly. HUS is characterized by acute renal failure, with a mortality rate of 3–5%; but many survivors suffer permanent disabilities, including renal insufficiency and neurological deficits. TTP more commonly afflicts adults and has similar effects as HUS, but it can also affects the central nervous system; so may be accompanied by fever and other neurological disorders. EHEC infection are treated mostly by administering fluids and salts to prevent dehydration, but in severe cases or HUS, dialysis and blood transfusions may be required. Antibiotic therapy has had mixed results in treating EHEC infections and, in some cases, may increase the risk of developing HUS. One speculation is that Stx is a major virulence factor causing the illness and that antibiotics are lysing the bacteria to release more Stx into the host.

There are estimated to be over 300 STEC serotypes, but not all appear to be pathogenic for humans. STEC have been found in fresh meat, poultry, seafood, produce and other foods not implicated in illness, and have even been isolated from the feces of healthy humans so the health significance of STEC as a whole is uncertain. EHEC, on the other hand, is a subset of STEC and comprises pathogens of which O157:H7 is the type strain most often implicated in infections worldwide. However, some non-O157 EHEC are emerging as important pathogens and increasingly causing illness worldwide. For example, O26, O111, O121, O145, O103 and O45 (referred to as the 'big 6') have been found to be the non-O157 serotypes most frequently isolated from clinical infections in the US, while the EU has focused on O157, O26, O111, O103 and O145 serotypes as those of concern. This list of priority serotypes not only vary geographically but can also change as other serotypes emerge as important pathogens. Non-O157:H7 EHEC infections are often transmitted via food or water but can also be spread by contact. Infections by non-O157 EHEC varies from mild, non-bloody diarrhea to HC and can also progress to HUS. In some countries, they surpasses O157:H7 in frequency of infection and account for most of the HUS cases reported.

Serotype O157:H7 was first identified in 1975, but was not recognized as a foodborne pathogen until the hamburger outbreak in Western US in 1982. Since then, it has been fully established as a major foodborne pathogen that continues to cause the majority of the sporadic and EHEC outbreaks worldwide, leading to speculations that it may be more virulent than other EHEC strains. Serotype O157:H7 has been isolated from deer, sheep and other animals, but cattle remain the primary reservoir. Hence, undercooked contaminated ground beef or beef products still account for many of the infections. Other infections have been caused by contaminated drinking or recreational water, raw milk and person-to-person and animal contacts. O157:H7 outbreaks have also implicated unexpected foods such as unpasteurized apple cider, yogurt, mayonnaise, salami, cookie dough and raw milk cheeses. Many of these are acidic foods that have a pH of ~3.6, which was generally regarded as being bacteriostatic and safe for consumption. But O157:H7 has been shown to tolerate low pH and persist in acidity for extended periods. More recently, the trend to healthier diets has greatly increased the consumption of fresh fruits and vegetables, which also resulted

in increases in O157:H7 infections caused by bagged salads, spinach and sprouts. One speculation is that produce is contaminated in the field and since it are processed in vast quantities, a single contaminated plant can spread the contamination to the entire batch. Furthermore, as these products are widely distributed, the scope and impact of the outbreaks have also been large. The infectious dose of O157:H7 is estimated to be very low and in the range of 10 to 100 cells.

14.2.4 EIEC

The bacillary dysentery symptoms of EIEC closely resemble shigellosis. The symptoms of EIEC infections include chills, fever, headache and abdominal cramps, but it is characterized by profuse, watery diarrhea that in some cases contains traces of blood. Symptoms usually begin 11–18 hrs after ingestion; but may vary from a few hours up to 48. EIEC are most often implicated in outbreaks, but sporadic infections also occur and it remains an important cause of endemic diarrhea in South America, Mexico and in some Eastern European countries. EIEC was first recognized as a pathogen in 1947, when it caused an outbreak that infected 47 children after they consumed canned salmon that most likely was contaminated after opening. It has since caused many outbreaks worldwide, implicating water, imported cheese, potato salad, vegetables and other foodstuffs. EIEC infections are infrequent in the US but one of the largest outbreaks occurred in Texas in 1992, when 370 people were infected after consuming guacamole at a catered event. There is no known animal reservoir for EIEC so humans are suspected to be the primary source. Also, carriers and asymptomatic infections seem to be rare; hence, EIEC contamination of foods is most likely via ill food handlers. Person-to-person spread of EIEC infection has been reported to occur. EIEC closely resembles *Shigella* in pathogenesis, but the infective dose is higher than the 10–100 cells estimated for *Shigella*. A human feeding study showed that as much as 10^8 EIEC are required for infection, but most likely it is around 10^6.

14.2.5 EAEC and DAEC

EAEC was first described in 1987 and is now a recognized pathogen that causes persistent diarrhea (≥14 days in duration) in various countries worldwide. The symptoms of EAEC infections are watery diarrhea without blood, afebrile or low-grade fever, and little or no vomiting. But there are exceptions, as infected infants have been reported occasionally to exhibit bloody stools and mucoid, liquid green stools. Prior to the German outbreak in 2011, EAEC mostly caused sporadic infections with only a few restaurant- and hospital-associated outbreaks. For example, a foodborne outbreak of EAEC in England infected 133 persons; the source of infection was traced to a meal in a restaurant but the food was not identified. EAEC was also implicated in a few outbreaks of persistent diarrhea in a hospital nursery in Serbia and also in a hospital malnutrition ward in Mexico, which resulted in a few fatalities. In most of these, the illness lasted only 3–9 days, but a few cases persisted for 18–20 days. The sources of infection in these hospital outbreaks were not determined, but humans appear to be the only known source of EAEC and asymptomatic carriers also seems to be prevalent.

In the summer of 2011, an unusual strain of EAEC caused a large outbreak in Germany that infected over 4100 people, with ~850 cases of HUS and 54 fatalities, making it the largest HUS outbreak ever recorded. The source of infection was epidemiologically linked to sprouts, but the pathogen was not isolated from the seeds. The causative pathogen,

identified as serotype O104:H4, was genetically an EAEC strain, but it was unusual in that it also produced Stx2. The *stx* gene that encodes for Stx resides on bacteriophages, which are fairly abundant in human sewage. Hence, one speculation is that this O104:H4 strain had been infected by a *stx2*-encoding bacteriophage and acquired the ability to produce Stx2 via horizontal gene transfer. Since this strain exhibited both EAEC and EHEC traits, some have designated this strain as an enteroaggregative hemorrhagic *E. coli* (EAHEC).

The role of DAEC as a diarrheal agent was uncertain, as studies from Chile, Brazil and Thailand showed little correlation between the presence of DAEC in stools and illness. However, surveys from Mexico and Bangladesh showed that DAEC was more prevalent in stools of diarrheal patients as compared to healthy controls. Also, a study of hospitalized patients in France showed that DAEC accounted for many of the diarrheal cases where no other gastrointestinal pathogens were found. A more recent report from Brazil showed that DAEC is an important diarrheal pathogen, especially in children under a year old. DAEC infections cause watery diarrhea without blood, but it has been shown that vomiting may be more significantly linked to DAEC infections than diarrhea. Only a limited number of DAEC infections have been reported and no food vehicles have been identified. The infection dose for both EAEC and DAEC has not been determined.

14.3 Detection of the organism, toxin and pathogenicity

The strains that comprise the pathogenic *E. coli* groups are phenotypically diverse, hence group-specific microbiological methods do not exist. But some serotypes, like O157:H7, do exhibit unique phenotypes and these have been useful to develop serotype-specific microbiological methods. Advances in technology have introduced many molecular techniques which have enabled the development of very specific assays. Some of those that target unique markers can be strain or serotype-specific, while those that target virulence genes or factors can even be group-specific. This section examines some of the microbiological and molecular methods, including serology and virulence testing, that are used for isolating and characterizing pathogenic *E. coli*.

14.3.1 Microbiological methods

Except for EIEC, most pathogenic *E. coli* ferment lactose and tolerate elevated incubation temperatures so behave as typical *E. coli* on most probable number (MPN) assays or on media like EMB. However, extensive screening and virulence analysis will be required to identify the pathogenic groups. Also, the elevated temperature often used in *E. coli* tests and the detergent, sodium lauryl sulfate, used in MPN can cause the loss of virulence plasmids, which can complicate the virulence testing needed for identification.

A method for isolating pathogenic *E. coli* from foods is described in the *FDA Bacteriological Analytical Manual* (BAM), where foods are pre-enriched in brain heart infusion broth for 3 hr at 35 °C to resuscitate stressed cells, then enriched in tryptone phosphate broth at 44 °C for 20 hrs, followed by plating on L-EMB or MacConkey (MAC) agar for differential isolation. For EIEC, Hektoen Enteric (HE), Salmonella-Shigella, and MAC may be used; but, HE and MAC appear least inhibitory for EIEC. It should be cautioned, however, that this is a general method and is not specific for any pathogenic group. It is also labor intensive, as extensive screening of isolates by serotyping and virulence analysis will be required to identify the respective pathogenic groups.

Due to the notoriety of EHEC worldwide, most method development has focused on this pathogenic group. EHEC strains are also very diverse, but there are unique traits within some serotypes. For instance, unlike most *E. coli*, O157:H7 strains do not ferment sorbitol (SOR) or exhibit ß-glucuronidase (GUD) activity. Similarly, O26 serotype strains do not ferment rhamnose, so these traits have been used to develop differential media. The Sorbitol MacConkey agar (SMAC) for O157:H7 strains was developed in 1986 and is now available commercially. SMAC uses MAC agar base but with the sugar SOR instead of lactose. Bacteria that ferment SOR produce acid, which changes the color of the phenol red pH indicator and appears as red colonies, while O157:H7 strains, which do not ferment SOR, produce colorless, neutral grey colonies. A similar concept is used in the rhamnose MacConkey (RMAC) agar for O26 stains. SMAC is very useful in clinical testing, where prompt plating of a patient's bloody stools onto SMAC is very effective in isolating O157:H7. However, it is not selective so has had limited use in food testing, where background flora often found in foods masked O157:H7 colonies. Also, there are other enteric bacteria that do not ferment SOR and resemble O157:H7 colonies on SMAC. For food testing, SMAC was modified by adding potassium tellurite (2.5 µg/ml) and the antibiotic cefixime (0.05 µg/ml) to make it more selective. Known as TC-SMAC or CT-SMAC, it effectively inhibited the growth of other bacteria and is a fairly selective plating agar for O157:H7. It should be cautioned, however, that not all strains of O157:H7 are resistant to tellurite. Atypical strains, such as the pathogenic, sorbitol fermenting O157 (SFO157) strains that were first found in Germany, are sensitive to potassium tellurite and will not grow on CT-SMAC. Similarly, some EHEC of serotype O26, O111 and O145 are resistant to tellurite, but many other EHEC serotypes are sensitive so will not grow on tellurite-containing media. There are also many specialty media like Rainbow, BCMO157, Chromagar and others, that use chromogenic or fluorogenic substrates to test SOR, GUD, ß-galactosidase or combinations of these. Specialty media are useful, as O157:H7 and selected other EHEC serotypes can be distinguished by colony color but these specialty agars can be costly.

Despite the added selectivity of plating media, the complexities of food matrices require that foods still need to be enriched prior to plating for isolation. Enrichment media often used for O157:H7 are modified EC (mEC) or modified TSB (mTSB) that are supplemented with novobiocin or acriflavin. Another medium, the EHEC Enrichment broth (EEB), developed for the analysis of carcasses, uses the antibiotics vancomycin, cefixime and cefsulodin to inhibit Gram-positive bacteria, aeromonads and *Proteus* species, respectively. EEB is selective for O157:H7, especially in foods that contain high levels of normal flora. But, in less contaminated foods, the level of cefixime may need to be reduced or it can inhibit the growth of O157:H7. Another useful enrichment is modified buffered peptone water that contains lactose to favor the growth of enterics, pyruvate to allow resuscitation and acriflavin, cefsulodin and vancomycin for selection. This enrichment broth, mBPWp+ACV, used at 42 °C, was effective in selecting for O157:H7 and other EHEC serotypes from various foods. It should be cautioned, however, that the effectiveness of any enrichment media can vary with foods so a medium found to be effective for one type of food may not be applicable to other foods.

Once an isolate has been obtained, it still needs to be identified and characterized to determine its pathogenic group. Bacteria are most often identified by biochemical traits and this testing has been greatly simplified by the use of miniaturized biochemical kits like API20E or the automated VITEK assays that can quickly identify the isolates as *E. coli*. However, serotyping, and especially virulence assessment, can be complex procedures that are labor intensive and in the past have required using tissue culture cells.

14.3.2 Molecular methods

Molecular techniques have greatly facilitated characterization and virulence testing of pathogenic *E. coli*. These range from simple latex agglutination assays that are useful for quick serotyping of strains to molecular tests that use polymerase chain reaction (PCR) to amplify serotype or virulence-specific genes.

14.3.2.1 *Serotyping*

Pathogenic *E. coli* are grouped based on virulence factors or clinical manifestations, so serotyping is not a reliable tool for identifying pathogenic *E. coli*. An exception is O157:H7, where typing for the O157 and the H7 antigens is crucial in the definitive identification of this pathogen. Still, serotyping continues to be useful in epidemiological investigations and as some non-O157 EHEC serotypes are emerging as important foodborne illness worldwide, serotyping isolates have become more critical.

Serotyping the various *E. coli* O and H antigens has been greatly simplified by the use of latex agglutination (LA) kits. Most LA kits are used for testing for O157:H7; however, kits for non-O157 EHEC serotypes are also becoming available (Table 14.2). In LA, a colony from an agar plate is used to make a suspension in a drop of antibody coupled latex beads reagent. If the bacteria have the specific antigen, the antibody-latex agglutinates with the bacteria to form visible clumps within minutes. LA assays are rapid and simple to perform, but it is important to follow kit instructions and use proper controls. The use of a heavy inoculum can also cause false reactions and also there are *E. coli* strains that will non-specifically autoagglutinate with sera to yield false reactions.

Most LA kits for O157:H7 only detect the O157 antigen, so merely indicate that the isolate has the O157 antigen and is not indicative that the isolate is O157:H7. The O157 serogroup is large and, aside from O157:H7, there are many other O157 strains that are not of H7 flagellar type; these often do not have virulence genes and are non-pathogenic. Also, due to similarities in the lipopolysaccharide layer, many polyclonal anti-O157 sera can cross-react with isolates of *E. hermanii*, *Salmonella* group N, *Citrobacter* and others; therefore, it is important that O157 isolates are further tested for the H7 antigen. A few LA kits also include anti-H7 latex (Table 14.2), but it should be cautioned that bacteria are sometimes non-motile and do not express the H antigen; hence, they will need to be induced for motility before H typing. Also, anti-H7 should not be used independently of the anti-O157 reagent, as false-positive H7 agglutinations can also occur, and there are *E. coli* that carry the H7 antigen but are not of the O157 serotype.

14.3.2.2 *Virulence factors and pathogenicity testing*

Pathogenesis of *E. coli* usually begins with attachment to the intestinal epithelial cells, followed by the manifestation of other virulence traits like toxin production, invasion, etc. The ability to attach or adhere is usually tested by tissue culture cells and it remains a useful assay to differentiate adherence patterns of EPEC, EAEC and DAEC. Similarly, cell culture and animal models were used to test for the various toxins and other virulence factors produced by pathogenic *E. coli*. However, much of this testing is now commonly carried out using molecular tests that are easier, sensitive and designed for specific testing needs. For instance, assays that target Stx or *stx* are group-specific and detect all STEC, while those that target unique markers, like the +93 *uidA* single nucleotide

Table 14.2 Partial List* of Available Sera for Serotyping *E. coli* Strains

Serotype	Assay	Format[a]	Company
O (various)		Sera	Denka Seiken, Japan
		Difco sera	BD, Franklin Lake, NJ
		Sera	Oxoid (Remel), Lenexa KS
		Sera	Staten Serum Institute, Denmark
		Sera	Mast Group, UK
O103	DryspotSeroCheck	LA[a]	Oxoid (Remel), Lenexa, KS
O111	O111-F	LA	Denka Seiken, Japan
	DryspotSeroCheck	LA	Oxoid (Remel), Lenexa, KS
	Bactrace	Serum	KPL, Gaithersburg, MD
	E. coli O111		Mast Group, UK
O128	DryspotSeroCheck	LA	Oxoid (Remel), Lenexa, KS
O145	DryspotSeroCheck	LA	Oxoid (Remel), Lenexa, KS
O26	O26-F	LA	Denka Seiken, Japan
	DryspotSeroCheck	LA	Oxoid (Remel), Lenexa, KS
	E. coli O26	LA	Mast Group, UK
	Bactrace	Serum	KPL, Gaithersburg, MD
O91	DryspotSeroCheck	LA	Oxoid (Remel), Lenexa, KS
O104		Sera	Staten Serum Institute, Denmark
O157 & H7	RIM	LA	Remel, Lenexa, KS
O157	Dryspot	LA	Oxoid (Remel), Lenexa, KS
	Prolex	LA	Pro-Lab, Round Rock, TX
	Wellcolex	LA	Remel, Lenexa, KS
	O157-AD	LA	Denka Seiken, Japan
	RapidChekO157	LA	SDIX, Newark, DE
	O157 Rapid Test	LA	Microgen, UK
	ANI *E. coli* O157	LA	ANI Biotech, Finland
	E. coli O157	LA	Mast Group, UK
	Bactrace	Serum	KPL, Gaithersburg, MD
H (various)		sera	Denka Seiken, Japan
			Staten Serum Institute, Denmark
			Mast Group, UK
H7	Wellcolex	LA	Remel, Lenexa, KS

*Table modified from: Feng, P. 2007. Rapid Methods for the Detection of Food Borne Pathogens – Current and Next Generation Technologies. In Food Microbiology Fundamental and Frontiers, eds 3rd ed. Doyle and Beuchat, pp. 911–934. Washington DC: ASM Press.
[a]LA – Latex agglutination.

polymorphisms (SNP) in O157:H7, are serotype-specific. Those assays that target virulence genes or proteins use formats that include antibody-based enzyme-linked immunosorbent assays (ELISA), lateral flow devices and various real-time PCR (rt-PCR) platforms (Table 14.3).

Molecular assays are very useful for strain characterization and virulence assessment but, when coupled with enrichment steps, they have been used to screen foods for the presence of pathogenic *E. coli*. When used in screening, a negative result is often accepted; but a positive is regarded only as presumptive and needs to be confirmed. Also, since these methods use different technologies and formats, their efficacy and efficiency may vary with foods so it is important that they are validated prior to use in food testing. It should also be cautioned that a positive genotypic test, such as to *stx*, only indicates that a bacteria that carries *stx* sequences is present, but does not indicate that the cell is viable or Stx is made. Bacteria are known to carry genes that are not expressed due to genetic defects or physiological factors. Lastly, many assays use the multiplex format to detect multiple targets in one reaction. However,

Table 14.3 Partial List* of Commercial Assays for Detecting Virulence Factors and Genes in Pathogenic *E. coli*

Target	Trade Name	Assay format[a]	Manufacturer
Shiga toxin (Stx) 1 & 2	Premier EHEC	ELISA	Meridian, Cincinnati, OH
	RidaScreen Vtx	ELISA	rBiopharma, Germany
	BioStar Shigatox	ELISA	Iverness Med., Louisville, CO
	VerotoxinStool antigen	ELISA	IVD Research, Carlsbad, CA
	Verotox-F	RPLA	Denka Seiken, Japan
	ImmunoCardSTAT	LFD	Meridian, Cinncinati, OH
	VTEC	RPLA	Oxoid, Hampshire, UK
	Duopath	LFD	Merck, Germany
	VTEC Screen	LA	Denka Seiken, Japan
stx genes	Assurance GDS	rt-PCR	BioControl, Bellevue, WA
	GeneDisc STEC	rt-PCR	GeneSystems, France
	Mericon VTEC	rt-PCR	Qiagen, Valencia, CA
	SureFood STEC	rt-PCR	r-biopharm, Germany
	IQ Check	rt-PCR	Bio-Rad, Hercules, CA
stx, O157	Assurance GDS	rt-PCR	BioControl, Bellevue, WA
stx, eae, O157, O145, O111, O103, O26, O104:H4	GeneDisc STEC	rt-PCR	Pall, Port Washington NY
stx, eae, O157, O26, O103, O45, O111, O121, O145.	IQ Check	rt-PCR	Bio-Rad, Hercules, CA
stx, eae O157	GeneGenEHEC	rt-PCR/hyb	Sylab, Germany
stx, eae, O157, H4, *iha* (O104)	GeneGenEHECplus	rt-PCR/hyb	Sylab, Germany
Stable toxin (ST)	COLIST	ELISA	Denka Seiken
	E. coli ST	ELISA	Oxoid
Labile toxin (LT)	VET	RPLA	Denka Seiken
	Phadebact	unknown	BactusAB, Sweden

[a]ELISA – enzyme-linked immunosorbent assay; LA – latex agglutination; RPLA – reverse passive LA; LFD – lateral flow device; rt-PCR – real time PCR; hyb – hybridization.
*Table modified from: Feng, P. 2007. Rapid Methods for the Detection of Food Borne Pathogens – Current and Next Generation Technologies. In Food Microbiology Fundamental and Frontiers, eds 3rd ed. Doyle and Beuchat, pp. 911–934. Washington DC: ASM Press.

when used in testing food enrichments, which contain a mixed pool of bacteria, it is possible that not all the targets detected may be coming from the same cell.

14.3.2.3 *EPEC*

EPEC pathogenesis begins with localized adherence to epithelial cells via a fimbriae called bundle-forming pilus (BFP). This triggers signal transduction activity by the genes on the locus for enterocyte effacement (LEE) pathogenicity island, which includes *tir* and *eae* that encode for the translocated intimin receptor and intimin, respectively. The secreted Tir protein binds to the epithelial cell and serves as a receptor to intimin to allow the adherence of EPEC cells, resulting in attachment and effacement (A/E) lesion. A typical EPEC is a strain that has *eae* and exhibits the A/E phenotype and has the EPEC adherence factor plasmid that carries the *bfpA* gene that encodes for BFP. There are also many atypical EPEC strains that have *eae* but not *bfpA*, and some of these have also been implicated in human illness.

The BFP protein is essential for the initial localized adherence of EPEC. This phenotype is often tested with the HEp-2 adherence assay, which is also the standard test for

differentiating strains of EPEC (localized adherence, LA), from EAEC (aggregative adherence, AA) and DAEC (diffusely adherent, DA). The presence of the EAF plasmid can be tested by PCR that are specific for *bfpA* or to other markers on the EAF plasmid.

The intimin protein is essential for EPEC pathogenesis, but it is also a virulence factor of many EHEC strains. The intimin of EPEC and EHEC are 83% homologous and more diverse in the receptor binding regions at the C terminus. There are estimated to be 30 *eae* alleles (ξ, ß, γ, etc.) that can be found in various EPEC and EHEC serotypes. There are many *eae*-specific PCR assays and those that target conserved *eae* regions can detect most alleles, while others target specific alleles. For example, O157:H7 strains produce γ–intimin, so a γ–*eae*-specific PCR will detect O157:H7 but also a few other serotypes like O145 and EPEC O55:H7 that also carry γ–*eae* allele.

14.3.2.4 *ETEC*

ETEC strains are characterized by the production of enterotoxins and fimbrial colonization factor antigens (CFA), all of which are plasmid encoded. ETEC pathogenicity resembles that of *Vibrio cholerae*, where bacteria attach to the intestinal mucosa via CFA, then produce heat-labile (LT) and/or heat-stable (ST) enterotoxin that causes watery diarrhea. LT is a large 86 kDal protein which is easily inactivated at 65 °C for 30 min. There are two serologically distinct LT types. LT-I is important in causing illness in man and animals and the two closely-related variants are designated LTh-I for human or LTp-I for pig origin. LT-II, is produced mostly by animal isolates of *E. coli* and not usually associated with illness. The ST is a small peptide of ~2 kDa and is stable to heating at 100 °C for 30 min. There are also two distinct ST types, where STa is produced by ETEC and a few other pathogens and include the human (STh) or pig (STp) variants. STb is produced only by ETEC, mostly by isolates from pigs.

The LT and ST enterotoxins used to be assayed in vivo, but antibody kits, such as an ELISA for ST, are now available (Table 14.3). LT shares 80% homology in protein sequence with the cholera toxin of *V. cholerae*, so assay kits will often react with both toxins. The genes encoding for LT and ST have been sequenced so PCR assays can be used to test isolates for the presence of LT and ST genes.

14.3.2.5 *EHEC*

EHEC pathogenesis begins with attachment to the intestinal cells, via *eae*-encoded intimin followed by the production of Shiga (Stx) or Vero cytotoxins (Vtx) which cause cell death by interfering with 23S rRNA to inhibit protein synthesis. Some EHEC strains like O113:H21 and O91:H21 do not have *eae* but still cause HUS. These *eae*-negative EHEC strains have been found to have other putative attachment factors like the STEC agglutinating adhesin (Saa), but the role of these proteins in virulence has not been fully established. Similarly, the large EHEC plasmid, like pO157, carry *ehxA* that encodes for enterohemolysin, which is produced by many EHEC strains but also by generic *E. coli*, so its role in pathogenesis is uncertain.

Stx was first identified in *Shigella dysenteriae* Type I and it produces only one type of shiga toxin. In *E. coli*, there are two toxin types, Stx1 and Stx2, and both are phage encoded. The production of Stx is characteristic of STEC and strains can produce either or both Stx, but Stx alone appears to be insufficient to cause illness. Clinical evidence suggests that Stx2 appears to be the more important, as EHEC strains that carry *stx2* and *eae* are most often implicated in severe illness like HUS. The Stx1 of *E. coli* is nearly identical to that

produced by *S. dysenteriae* Type I, but there are three subtypes (Stx1, Stx1c and Stx1d) in *E. coli*. Stx1c is the most common subtype among STEC strains from sheep and it has not been found in O157:H7 strains, so the health risk of some of these subtypes is uncertain. The Stx2 of *E. coli* only shares 55% homology to Stx1, and there are seven known Stx2 subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g). Thus far, Stx2a, Stx2c and Stx2d look to be pathogenic for humans. Some of the other subtypes are produced by animal or environmental STEC strain, so their health risk is uncertain. It is estimated that there are over 300 STEC serotypes, but many of these do not have other virulence factors, have not caused illness and can be found in feces of healthy adults. Hence, the biggest challenge in EHEC testing is to identify those STEC serotypes that are pathogenic and will cause illness in humans.

Testing for *stx* or Stx is critical in the detection of EHEC and STEC strains. Stx used to be assayed by observing for its cytotoxic effects on HeLa or Vero cells; but anti-Stx antibodies and *stx*-specific PCR assays are now available. Similarly, the A/E lesion caused by intimin can now be assayed by PCR to *eae* instead of having to use HEp-2 or HeLa cell monolayers (see EPEC section above).

There are several commercial kits that use anti-Stx antibodies to test for Stx1 and Stx2. Some are simple lateral flow devices or reverse passive latex agglutination assays that can differentiate the two toxins and the latter can also determine the titers of the toxins produced. Most antibody assays, however, are sandwich ELISA where anti-Stx or globotriaosyl ceramide (Gb3), the cellular receptor for Stx, are used to capture Stx, followed by detection using an enzyme-conjugated anti-Stx antibody (Table 14.3). These assays only detect Stx, so are not specific for O157:H7 or any other EHEC serotype, and also the reactivity of the antibodies to Stx subtypes varies greatly among the kits.

Many *stx*-specific PCR assays have been published and at least a dozen are commercial kits. Most of these use various rt-PCR formats and many are multiplex assays that can detect multiple targets, including other virulence factors as well as serotype-specific markers (Table 14.3). But there are limitations in dye markers and instrument capabilities so that, when testing for large numbers of targets, a series of sequential multiplex assays may need to be used in order to detect all the targets. Multiplex assays are very useful for characterizing pure culture isolates, but are less efficient in food testing, as the targets detected may not be within the same cell. Also, interference by food matrices, the presence of inhibitors and normal microbial flora require that foods are enriched in selective broth prior to testing with rt-PCR assays. An example of screening food enrichments using rt-PCR is described in the FDA BAM, Chapter 4A. Section K.

14.3.2.6 *EIEC*

EIEC closely resembles *Shigella* and both share similar virulence traits, namely the ability to invade epithelial cells. The invasion phenotype is encoded by the pInv plasmid, but its expression appears to also require regulatory genes that reside on the chromosome. The size of pInv can vary from 120 mDal in *S. sonnei* to 140 mDal in EIEC and other *Shigella* species; but the invasion gene on the plasmid is identical. EIEC pathogenesis begins with cellular invasion via endocytic vacuoles. Once internalized, the vacuoles lyse, the bacteria multiplies intracellularly and spreads laterally to other cells. The invasion phenotype used to be assayed in vivo but now are often tested with *inv*-specific PCR assays. Since the *inv* genes of *Shigella* and EIEC are identical, *inv*-specific assays will detect both, so the isolates may also need to be identified as *E. coli* by biochemical reactions.

14.3.2.7 *EAEC and DAEC*

The pathogenesis of diarrheal illness for both of these pathogenic *E. coli* groups is not well understood. However, since the groups are defined based on cellular adherence patterns, the HEp-2 assays is the standard phenotypic test to distinguish between the localized adherence (LA) of EPEC, aggregative adherence (AA) of EAEC and the diffuse adherence (DA) pattern of DAEC. The aggregative adherence of EAEC to HEp-2 cells is mediated by an adhesin, called aggregative adherence fimbriae I (AAF/I). Adherence results in increased mucus secretion, which forms a biofilm to entrap the bacteria onto the intestinal epithelium. The AAF/I factor is encoded by a large plasmid and regulated by *aggR*, which is also a central regulator gene of several virulence functions. EAEC also produce a cytotoxin and a ST-like enterotoxin called EAST1. It is hypothesized that EAEC adheres to intestinal mucosa via AAF/I and elaborates the toxins, which causes diarrhea and damage to the mucosa. PCR assays specific for these genes have been developed and *aggR*-specific PCR assays were used in the identification of the German O104:H4 strains during the outbreak.

The pathogenesis of DAEC group is highly complex. One group of DAEC strains elaborates afimbrial adhesins, encoded by the *afa* gene cluster and these may also exhibit a mannose-resistant adhesin, designated as Dr adhesin. DAEC strains that produce Afa/Dr are most often associated with urinary tract infections. DAEC strains that more frequently cause infantile diarrhea elaborate a fimbrial adhesin, designated F1845 that causes the DA pattern on HEp-2 cells. About 75% of DAEC strains produce F1845, which is encoded by a cluster of five genes (*daaA* to *daaE*) and the genetic loci seems to be both chromosomal and plasmid encoded, depending on strains. The *daaC* gene encodes for a component essential in F1845 fimbrae expression and *daaC*-specific PCR assays have been useful in identifying diarrheagenic DAEC strains.

14.3.3 Horizontal gene transfer and variants of Pathogenic *Escherichia coli*

Most assays target specific virulence factors or, in the case of O157:H7, unique traits like the absence of SOR fermentation and GUD activity, and the presence of O157 and H7 antigens. However, reliance on a few traits for differentiation can be risky, as other enteric, like *E. hermanii* and *Hafnia*, also do not ferment SOR or exhibit GUD and can resemble O157:H7 colonies on media that detect these traits. To compound the problem further, phenotypic variants of O157:H7 that do not express typical traits have been isolated worldwide. For instance, a GUD-positive variant of O157:H7 was isolated from a HC patient in the US in 1994. At the time, the isolation of such a strain was thought to be a rare occurrence until a GUD-positive O157:H7 strain was implicated in a large outbreak in Hokkaido, Japan. The *E. coli* GUD is encoded by *uidA* and studies showed that GUD-negative O157:H7 strains had the entire *uidA* gene, but also had a dinucleotide GG insertion that caused a frame shift mutation, which abrogated GUD expression.

The O157 serogroup is comprised of a large, diverse group of O157 strains and, aside from O157:H7, it also includes many that do not have the H7 antigen and are not pathogenic. Hence, serotyping for both the O157 and the H7 antigens is crucial in the identification of O157:H7. This task, however, is complicated by the existence of both motility and serotypic variants of O157:H7. The mechanism for motility in *E. coli* is under complex regulatory control and affected by environmental factors; hence, strains may be non-motile (NM) due to physiologic or genetic factors. The isolation rate for O157NM strains increased from 6%

in 1990 to 47% in 1996 and since these strains can't be typed for the H7 antigen, their health risk is uncertain as some may be NM variants of O157 non-H7 strains that are not pathogenic. Characterization of various O157NM strains showed that some strains can be induced back to motility and, furthermore, all O157NM strains that produced Stx turned out to be of the H7 serotype and therefore will cause disease. Similarly, an O rough strain of O157:H7 that did not express the O157 antigen was first isolated from beef in Asia. This variant strain was genetically identical to O157:H7, had all the virulence genes and even had gene sequences for the synthesis of the O157 antigen; but the antigen was not expressed. Further analysis showed that the absence of O157 antigen expression was due to an IS629 insertion within the *gne* that was essential in O antigen biosynthesis. O rough strains of O157:H7 was also thought to be rare until another strain with similar IS629 insertion in *gne* was isolated from a HC patient, indicating that these strains are pathogenic. These motility and serotypic variants of O157:H7 are not easily identified by routine serological assays.

Some phenotypic variants of O157:H7 are also known to carry multiple atypical traits. For instance, the SFO157 strains that were first isolated from HUS patients in Germany are genetically related to O157:H7, but are highly unusual strains that not only ferment SOR, but also have GUD activity and are NM. SFO157 strains could not be induced back to motility, suggesting the existence of genetic defects. Studies determined that the absence of motility in SFO157 strains was due to a 12 bp deletion in the master regulator gene *flhD*, that controlled motility and other functions. SFO157 only produces Stx2, but is highly pathogenic and has caused more HUS cases than O157:H7. SFO157 strains are found in many countries in Europe but have yet been found in the US.

Lastly, many of the virulence genes carried by pathogenic *E. coli* reside on mobile genetic elements, such as plasmids and bacteriophages, which can be lost, acquired or transferred. This can result in variants that no longer express trait phenotypes or unexpected strains that express virulence factors. In fact, the O157:H7 serotype is postulated to have emerged from its closest genetic relative, EPEC O55:H7, via horizontal transfer of various genes and virulence markers. A good example of this genetic transfer is the ability to produce Stx. The genes encoding for Stx1, Stx2 and some of the subtypes reside on phages, which may be induced and lost in adverse conditions or even during routine culturing, resulting in Stx-negative strains. Strains of EHEC and O157:H7 that do not produce Stx have been isolated from both environmental and clinical sources. Conversely, it is also possible for bacteria to acquire *stx*-bearing phages and gain the ability to produce Stx. For example, Stx2-producing strains of *Citrobacter freundii* and *Enterobacter aerogenes* have been found and implicated in human illness. Similarly, *S. dysenteriae* Type I used to be the only *Shigella* species that produced shiga toxin, but strains of *S. flexneri* and *S. sonnei* that produce Stx have also been isolated. A more recent example is the O104:H4 serotype strain that caused the large German outbreak in 2011. This strain produced Stx2, a trait of EHEC but, genetically, it was a strain of EAEC which causes persistent diarrhea in underdeveloped countries, but is seldom implicated in major foodborne incidents. One speculation is that this O104:H4 EAEC strain somehow acquired the stx_2 phage and thereby the ability to produce Stx2. As to the source of the *stx* phage, it is uncertain, but sewage has been found to contain high titers of *stx*-bearing phages, so this may have been the possible reservoir.

Plasmids are extra-chromosomal, mobile elements that are well known to carry antibiotic resistance genes, as well as many virulence markers. Among pathogenic *E. coli*, the *ehxA* gene that encodes for enterohemolysin in EHEC and both the LT- and ST-encoding genes produced by ETEC are plasmid borne. Plasmids can be transferred among bacteria via conjugation. For example, *E. coli* strains that produced Stx1, a trait of STEC, but also had

the ST gene that is characteristic of ETEC have been isolated from fresh produce. Since both *stx* and *ST* genes are on mobile elements, it is uncertain if the STEC strain had acquired the ETEC plasmid or perhaps the ETEC strain was infected by the *stx*-phage.

14.4 Physical methods for destruction of the organism (and toxin)

Pathogenic *E. coli* generally has similar growth and survival characteristics as generic *E. coli*; hence are inactivated by similar disinfectants and physical control measures. Due to the importance of O157:H7 in foodborne illness, many survival and inactivation studies have been carried out using this pathogen. Strains of O157:H7 seeded into ground beef can survive up to nine months in frozen storage at –20 °C. In meats, including poultry, *Salmonella* and *Campylobacter* are usually inhibited by 2–4% NaCl; but O157:H7 will tolerate up to 8% salt and its survival and growth at low temperatures may even be enhanced by some of the commonly-used food additives. However, pathogenic *E. coli* strains are not particularly heat resistant and thermal inactivation studies showed that O157:H7 in ground beef had a D-value of only 9.6 seconds at 64.2 °C and was even more easily inactivated than *Salmonella*. Cooking or pasteurization of foods therefore remains one of the most effective measures to control pathogenic *E. coli* in foods.

Although the organism may be heat sensitive, the toxins produced by EHEC and some by ETEC are more stable. Studies showed that purified Stx1 can withstand heating for 60 min at 75 °C and up to 5 min at 80 °C, thus suggesting that the toxin can survive the cooking conditions prescribed for ground beef. There is, however, little evidence that Stx are produced in foods. One study showed that 306 ng/ml and 452 ng/ml of Stx1 are produced in ground beef and milk, respectively, if these products are held at 37 °C for 48 hrs and with good aeration. These parameters, however, are seldom encountered in normal milk and beef handling and storage conditions. Moreover, there is little evidence that ingestion of preformed Stx in foods will causes illness. The fact that O157:H7 has a very low infection dose (10 to 100 cells) further suggests that illness occurs as a result of ingesting the organism.

Like other bacteria, pathogenic *E. coli* are relatively sensitive to irradiation and can be eliminated from foods by low-dose treatments. Irradiation is most useful in products like spices that are difficult to treat to reduce microbial load; hence, it remains one of the most effective and practical means to process many of the spices that we consume. The potential to use irradiation to reduce the microbial load of other difficult to process products like fresh produce is being explored. Studies showed that a dosage of 2–3 kGy of gamma irradiation is usually sufficient to decontaminate raw meats and poultry of all foodborne pathogens. But the effectiveness and the irradiation dosage required may vary depending on the type of the product and even the temperature of the product during irradiation (i.e. fresh, refrigerated or frozen) may have an effect.

14.5 Prevention/control measures

Pathogenic *E. coli* is transmitted via the fecal-oral route; hence, most outbreaks are caused by the consumption of contaminated food and water. A large variety of foods has been implicated in infections of EPEC, ETEC, EIEC and EHEC worldwide. Little is known about the sources of EAEC and DAEC but these two pathogenic groups are thought to be

disseminated mostly by contact. Also, up until the recent German EAEC outbreak in 2011 that implicated sprouts, few food vehicles have been associated with EAEC and DAEC infections. Transmission of secondary infections via animal or person-to-person contact is well documented to occur with O157:H7 and also occurs with some of the other pathogenic *E. coli* groups. A number of infections have been disseminated via contact transmission and this is especially common in nursing homes and in daycare centers, where good personal hygienic practices are not always observed. Incidences of contact transmission can be greatly reduced by emphasizing good personal hygiene and proper hand-washing techniques. Children or patients with symptoms of diarrhea should be closely monitored to prevent cross-contamination of others.

Reservoirs for strains of many pathogenic *E. coli* groups remain uncertain; hence, infected patients and human carriers are suspected to be the sources of these pathogenic *E. coli*. On the other hand, EHEC, and especially O157:H7, has been isolated from intestinal tracts of cattle, sheep, deer and other animals, but cattle remain the primary reservoir. Carcasses are most likely contaminated with O157:H7 through contact with intestinal contents during slaughter or via cattle hides that have been found to harbor the pathogen. Hence, intervention strategies such as the use of trisodium phosphate, hot acid sprays have been used to reduce the level of O157:H7 from carcasses.

Meat and meat products that are only surface contaminated with EHEC are easily killed by cooking. But if the contaminated meats or trimmings are used to make ground beef, the pathogen is mixed into the product; it can persist in frozen ground beef for months and is not killed unless the meat has been thoroughly cooked. Hence, the health risks associated with the consumption of undercooked ground meats persist.

The tradition of using acidity to control microbial growth and to preserve the shelf life of foods has been practiced by all cultures for centuries. Examples of these include yogurt, cheeses, pickles and fermented meats. Some foodborne pathogens, however, have been shown to be acid-resistant and this capability is thought to be an important trait of pathogens to enable their passage through the gastric barrier to cause disease. Bacterial acid-resistance properties are genetically encoded and *E. coli* can be induced to acid-resistance by exposure to low pH conditions. Acid-resistance traits also seem to be linked to other factors, as acid-resistant bacteria also exhibit increased tolerance to other stress factors like antimicrobial agents, heat, UV light and irradiation.

The acid-resistance properties of most pathogenic *E. coli* groups are unknown, but EIEC and EHEC are well known to resist acidity. The ability of O157:H7 to survive in moderate acidic conditions is especially well documented as studies showed that it can survive up to 56 days at pH ≥4.0. In terms of food safety, the traditional belief was that bacteria cannot grow in foods with pH <3.6, but many acidic foods with pH at or below 3.6 have been implicated in EHEC and O157:H7 outbreaks, including apple cider, juices, mayonnaise, yogurt and fermented sausages. The acid-resistant properties of O157:H7 have also enabled it to survive normal food-processing conditions. For instance, ground beef contaminated with O157:H7 has been shown to survive the acidic and curing conditions used to make fermented sausages and this product has been implicated in several outbreaks of O157:H7 and other EHEC worldwide. Similarly, milk can become contaminated with O157:H7 if it comes in contact with manure during milking or handling. *E. coli* contaminated in milk are easily killed by pasteurization, but the consumption of raw milk remains risky. Raw milk is commonly used to make cheeses, where the acidity resulting from lactose fermentation and the subsequent curing and aging processes are relied upon to keep pathogen growth under control. In the US, most cheeses are made with pasteurized milk, but raw milk is allowed to

be used to make some types of semi-hard cheeses, provided that they have been aged for 60 days to reduce microbial load. Even so, raw milk Gouda cheeses have caused O157:H7 outbreaks. These examples show that some of the traditional food-making processes may be inadequate and that acidity alone is not effective in controlling EHEC, hence additional intervention strategies are needed.

Many recent outbreaks of EHEC and O157:H7 have been linked to the consumption of fresh produce such as lettuce, spinach and sprouts. Produce can become contaminated in the field or from improper sanitation, handling or processing. The use of compost, sewage-contaminated water for irrigation and droppings from wild animals and birds have all been suspected as sources of pathogen contamination in fresh produce. Washing and rinsing whole produce prior to consumption can reduce bacterial load but not eliminate the risk entirely; some studies suggest internalization of pathogens in plants. Furthermore, the effectiveness of washing products like sprouts and fresh-cut produce that are often deemed as ready-to-eat appears to be even more difficult when bacteria attaches to the exposed wound areas of shredded vegetables. Modified atmosphere packaging (MAP) is a well-known means of controlling microbial growth in foods and some have explored the potential of using MAP to control the growth of pathogenic *E. coli* in produce. However, fresh produce seeded with O157:H7 and stored at various temperatures and combinations of mixtures of CO_2, O_2 and N_2 levels showed that MAP had no inhibitory effect and O157:H7 grew at 10 °C and 20 °C and survived refrigerated storage at 5 °C. Similarly, another study showed that the use of 3% O_2 and 97% N_2 had little or no effect on O157:H7 populations and that the type of salad vegetables and the product holding temperatures were relevant factors that affected pathogen survival and proliferation.

Improper food handling, poor personal hygiene and infected and/or asymptomatic food handlers can contaminate foods and cause pathogenic *E. coli* infections. Several restaurant outbreaks of O157:H7 have been suspected or traced to mishandling, resulting in cross-contamination of raw meats with mayonnaise sauces or salad bar items. To prevent potential transmission of pathogenic *E. coli* from carrier to foods, consumers at home and food handlers at institutions or restaurants should know how to properly handle and store foods and to thoroughly wash hands with hot soapy water prior to food preparation. Also, food handlers that exhibit gastrointestinal symptoms should not be preparing meals, serving foods or handling clean serving plates and utensils. To reduce cross-contamination between foods, counter tops, cutting boards, utensils and dishes should be cleaned and washed after contact with raw meats and salad vegetables. Also, cooked foods should not be placed back on unwashed plates that previously held raw meat, poultry or seafood, where it can come into contact with raw juices. Although all of these are useful, safe food-handling practices, proper cooking remains the most effective means to minimize pathogenic *E. coli* infections. Since the 1993 hamburger outbreak in the Northwestern US, the federal guidelines have been modified to require that ground meats are cooked to 160 °F and also recommend that meat thermometers are used to ensure that the ground meat has been cooked to the proper temperature. Similarly, except for some fresh squeezed juices which have to comply with Federal Juice HACCP regulations, most juices produced now are pasteurized. The problems of pathogenic *E. coli* associated with fresh produce are, however, much more difficult to resolve. These products, which are constantly in high demand by health-conscious consumers, are regarded as ready-to-eat and are consumed without further treatment. Furthermore, they are mass produced and broadly distributed, so that any incident of contamination can have large health risk impacts and consequences.

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15 *Cronobacter* spp. (formerly *Enterobacter sakazakii*)

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15.1 Introduction

Cronobacter (formerly known as *Enterobacter sakazakii*) is a genus of Gram-negative, facultatively anaerobic, oxidase-negative, catalase-positive, rod-shaped bacteria of the family *Enterobacteriaceae*. These organisms are rare opportunistic pathogens and can cause life-threatening infections in immunocompromised individuals, predominately neonates (infants less than a month old) and elderly adults. Clinical syndromes of *Cronobacter* infection include necrotizing enterocolitis (NEC), bacteraemia and meningitis. It has been reported that the case fatality rates range between 40 and 80% with long-term complications that can include delayed neurological development, hydrocephalus and permanent neurological damage. *Cronobacter* species can be detected in a variety of different environments, such as households, livestock facilities, food-manufacturing operations, and powdered infant formula (PIF) production facilities. A non-exhaustive range of food sources where the presence of *Cronobacter* isolates has also been detected include dairy-based foods, dried meats, water and rice. Contaminated PIF has been epidemiologically linked with several clinical cases and outbreaks in hospital neonatal intensive care units.

15.2 Classification

Cronobacter species were originally referred to as yellow-pigmented *Enterobacter cloacae*. In 1980, it was reclassified as a new species, *Enterobacter sakazakii*. which consisted of 15 phenotypically distinct biogroups. A 16th biogroup was later added in 2006. Phylogenetic studies using partial 16S ribosomal DNA (rDNA) and *hsp60* sequences suggested that this genus might require reclassification. Later, and following further extensive polyphasic analysis, a new genus *Cronobacter* was proposed in 2008. Redesignation was supported by optical mapping along with other data that confirmed the revision of this taxon. Currently, seven species (including *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*,

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Table 15.1 Distribution of Bio-groups among the Genus *Cronobacter*

***Cronobacter* species**		**Bio-groups**
Cronobacter sakazakii sp. nov.		1, 2–4, 7, 8, 11 and 13
Cronobacter malonaticus sp. nov.		5, 9 and 14
Cronobacter turicensis sp. nov.		16
Cronobacter muytjensii sp. nov.		15
Cronobacter condimenti sp. nov.		1
Cronobacter universalis sp. nov.		Separate genomospecies
Cronobacter dublinensis sp. nov.	*Cronobacter dublinensis* subsp. *dublinensis* subsp. nov.	12
	Cronobacter dublinensis subsp. *lausannensis* subsp. nov.	10
	Cronobacter dublinensis subsp. *lactaridi* subsp. nov.	6

C. dublinensis, *C. condimenti* and *C. universalis*) are recognized and these comprise the 16 biogroups that are described in Table 15.1.

Enterobacteriaceae can also be classified according to the nature of the O-antigens they express. The O-antigen is located on the outer surface of Gram-negative bacteria as a component of the lipopolysaccharide (LPS) structure and is responsible for serological diversity. For *Cronobacter* species, ten O-serotype gene clusters have now been identified based on the long-range amplification of the *rfb*-encoding locus (located between *galF-gnd* in Gram-negative enteric bacteria) followed by *MboII*-mediated restriction fragment length polymorphism (RFLP) analysis. The structural composition of several O-serotypes have also been described. Interestingly, two of these O-serotype gene clusters were shared among *C. sakazakii* and *C. muytjensii*, as well as *C. malonaticus* and *C. turicensis* strains.

15.3 Isolation and identification

Cronobacter are found naturally in the environment. They have been cultured from a range of dry foods, including PIF, powdered milk, spices, herbal teas, and starches. The bacterium has also been found in other locations in the environment, including wastewater. Interestingly, these bacteria can survive in desiccated conditions.

Cronobacter can grow readily on various nutrient culture media, including restricted infant formulas, non-selective agar and chromogenic plating media such as Tryptic Soy Agar (TSA), Lysogeny broth agar (LB), Druggan-Forsythe-Iversen agar (DFI agar, which is renamed as choromogenic *Cronobacter* isolation agar (CCI agar)), and *Enterobacter sakazakii* chromogenic plating medium (ESPM). In general, it has been shown that *Cronobacter* can grow between approximately 5.5 and 47 °C with two different colony types being noted and referred to as types A and B. Type A colonies are described as *either dry or mucoid*, *crenated (notched or scalloped edges), and rubbery when touched with a loop*, whilst type B colonies are described as possessing *a typical smooth colony appearance, easily removed with a loop*. It is now thought that these two colony morphotypes are phase variants expressing the rugose-smooth phenotype (discussed below). The rugose phenotype in other enteric organisms has been shown to facilitate resistance to desiccation, killing by antimicrobial compounds such as hypochlorite and an increased ability to form and persist within biofilms.

The earliest isolation and detection method developed for *Cronobacter* species was described by Muytjens et al. in 1988. The U.S. Food and Drug Administration (U.S. FDA, originally provided an isolation and identification protocol that was launched in 2002; with a revised protocol being subsequently published in 2009) and the International Organization for Standardization (ISO, 2006 ISO/TS 22964) developed technical standard protocols for the isolation and detection of *Cronobacter* species from PIF (Table 15.2).

On occasions when *Cronobacter* species occur in a food matrix, they may be present in low numbers and almost certainly in an injured state. Therefore, it is important to culture these cells initially in a suitable medium, which can resuscitate them. A nonselective broth, such as modified Buffer Peptone Water (mBPW), incubated at 35–37 °C, can be used for this step. However, a selective enrichment broth that can inhibit the growth of Gram-positive and other competing Gram-negative bacteria would provide an advantage, in terms of a reduction in the background flora. The time duration required for the pre-enrichment of any PIF sample varies from a minimum time period of 6 h to a maximum overnight period (ranging from 18 to 24 h), followed by selective enrichment and subsequent isolation using selective agars. Typical colonies are confirmed using a selective agar and/or a suitable real-time PCR assay, with the final identification based on either biochemical and/or molecular characterisation. In the revised US-FDA protocol there is one enrichment step, which is then followed by a molecular method that can be used for quick confirmation. This approach eliminates two days from the detection procedure compared to the original protocol.

15.4 Epidemiology and infection

Based on reports from epidemiological investigations and in vitro mammalian tissue culture studies, *Cronobacter* species may elaborate a variable virulence phenotype. Only *C. sakazakii*, *C. malonaticus* and *C. turicensis* have been linked with neonatal infections. Currently, there is limited information available on the mechanisms involved in pathogenesis, although there are several potential markers including *ibeA, ibeB, yijP* and *ompA* that have been previously identified from genome sequencing.

The first putative *Cronobacter* virulence factor reported was an enterotoxin-like molecule described by Pagotto et al. in 2003. In 2008, Townsend et al. reported that *Cronobacter* could attach to intestinal cells in vitro and survive within macrophages, based on conventional tissue culture assay. More recently, Franco et al. demonstrated that the *cpa* gene, which codes for an outer membrane protease in *Cronobacter sakazakii*, activates plasminogen and mediates serum resistance.

The outer membrane protein A, encoded by the *ompA* gene, is currently the best characterized virulence marker. This has been shown to be required for binding of the bacterium to human brain microvascular endothelial cells. The *ompA* gene is present in all *Cronobacter* strains and in *E. coli* K-12, which has been linked with the invasion-related cascade leading to neonatal meningitis. More recently, it was reported that the disruption of tight junctions significantly enhanced the adherence of *C. sakazakii* to Caco2 cells in culture and that the same marker was required for basolateral invasion. At the epithelial cell surface

C. sakazakii infection results in damage of these cells, following the recruitment of greater numbers of dentritic cells, compared with macrophages and neutrophils. Using a NEC mouse model, these effects were shown to be mediated through OmpA and involved inducible Nitric Oxide synthase (iNOS).

Table 15.2 Detection Protocols for *Cronobacter* in PIF

Procedure	FDA (original)	ISO/TS 22964	FDA (Revised)
Pre-enrichment	Make 1:10 (w/v) of sample in distilled water, incubated overnight at 36 °C	Make 1:10 (w/v) of sample in BPW, incubated at 37 °C for 18 ± 2 h	Make 1:10 (w/v) of sample in BPW, incubated at 36 °C for 6 h
Selective enrichment	Transfer 10 ml pre-enrichment to 90 ml EE broth, incubated overnight at 36 °C	Transfer 100 µl pre-enrichment to 10 ml mLST/vancomycin medium, incubated at 44 °C for 24 ± 2 h	
Selection/ Isolation	Make an isolation streak and spread plate from each EE broth onto VRBG Agar, incubated overnight at 36 °C	Streak from the cultured mLST/ vancomycin medium one loopful on the chromogenic agar in Petri dishes, incubated at 44 °C for 24 ± 2 h	Centrifuge 40 ml samples, 3,000 g, 10 min and resuspend pellet in 200 µl PBS; Spread 100 µl onto chromogenic media, incubated overnight at 36 °C
Confirmation	Pick five presumptive positive colonies and streak onto TSA, incubated overnight at 25 °C	Select five typical colonies and streak on TSA agar, incubated at 25 °C for 48 ± 4 h	Pick two typical colonies from each chromogenic media confirmed with real-time-PCR, API 20E, Rapid ID 32 E
Identification	Yellow colonies are confirmed with the API 20E test kit	Select one yellow colony from each TSA plate for biochemical characterisation	
Detection Time	5 days	6 days	3 days

Cronobacter infections are rare and are most likely to be under-reported, especially in developing countries. Thus the epidemiology of *Cronobacter* species is incomplete and poorly understood. Bowen and Braden first described the epidemiology of 46 clinical cases of *Cronobacter* infection reported between 1961 and 2005, including 12 infants presenting with bacteraemia, 33 with meningitis, and one with a urinary tract infection. Infants presenting with bacteraemia were of higher birth weight (2454 g), had a gestational age of 37 weeks, with infections occurred at 6 days, compared with those infants presenting with meningitis. Meningitis was reported to have a high mortality rate of 42% and more than 74% of the survivors suffered chronic neurological and developmental complications. Friedemann analysed 120–150 neonatal *Cronobacter*-confirmed infections based on data published between 2000 and 2008. The overall lethality of the 67 invasive infections noted was 26.9%. Lethality of *Cronobacter* meningitis, bacteraemia and NEC was calculated to be 41.9% ($P < 0.0001$), <10% and 19.0% ($P < 0.05$), respectively. Two key risk factors were identified, a longer gestational age at birth and parentage not from Europe. These are significant factors relevant to neonatal *Cronobacter* meningitis based on a logistic regression model.

Mother-to-baby transmission through the birth canal in newly-born infants is suspected as a potential source of *Cronobacter* infection. However, the route of exposure and the incubation period remain to be clarified. Nonetheless, a clear link between infected patients consuming contaminated PIF and isolates cultured from unopened (same-batch) cans has been described. It has also been reported that plant material may be a natural habitat for *Cronobacter* species. Therefore, although PIF is regarded as an epidemiologically-linked food source for this pathogen, environmental or extrinsic sources of contamination cannot be ruled out. Moreover, reports on *Cronobacter* infections in immunocompromised adults suggest other potential sources of contamination, such as the home environment or transient carriage in adult caretakers, among others. It was estimated that the annual incidence rate among the low birth weight infants (i.e., weight <2500 g; children <12 months of age) was 8.7 per 100,000 infants in the United States; and similarly the incidence rate in a second study was estimated to be 9.4 per 100,000 among very low birthweight infants (i.e., weight <1500 g).

Additionally, the occurrence of *Cronobacter* species infections reported in adults has increased among the elderly who have experienced strokes that affected their ability to swallow (dysphagia). These individuals require reconstituted powdered protein supplements as part of their daily diet. This is a feature that can be expected to become more common in the future due to the increasing age profile of the world's population, and as trends for consumption of synthetic, dehydrated formulas for such individuals increase.

15.5 Detection protocols

15.5.1 Conventional bacteriological culture

A conventional culture-based protocol for *Cronobacter* species was first developed by Muytjens et al. in 1988. The U.S. FDA recommended testing protocol and the ISO/TS 22964 method are now widely used for the detection of *Cronobacter* species (Table 15.2).

Selective media for *Cronobacter*, including Leuschner-Bew agar, Druggan-Fosythe-Iversen (DFI, newly remaned CCI) agar, Oh-Kang agar, ESPM agar and HiCrome™ *Cronobacter* spp. agar (Sigma-Aldrich, Switzerland), have been developed, and are based on

the α-glucosidase and β-cellobiosidase enzyme activities in *Cronobacter* strains. Moreover, selective agar for Gram-negative bacteria, such as violet red bile agar (VRBA), MacConkey agar and desoxycholate agar, can also be used for the isolation of *Cronobacter* from foods.

However, despite the availability of selective agar media, some of these formulations were shown to inadequately support the growth of some *Cronobacter* isolates and other related species, including *Enterobacter helveticus*, *Enterobacter pulveris* and *Enterobacter turicensis*, which are often found in the same ecological niches.

A chromogenic medium denoted as *Cronobacter* enrichment broth (CEB) was developed as a one-step pre-enrichment and enrichment protocol, which facilitated a two-day culture method for the detection of *Cronobacter* species in PIF. A cationic-magnetic-bead capture technique was also utilized to improve the sensitivity of detection for *Cronobacter* from PIF.

15.5.2 Immuno-based detection protocols

Immuno-based assays are convenient methods that can be applied for the detection of selected bacteria. Monoclonal antibodies are used in these types of detection platforms. For example, the VITEK® immuno-diagnostic assay system (VIDAS®) was developed as a commercial protocol for the detection of *Salmonella*, *Escherichia coli* O157:H7, *Listeria* species, *Campylobacter jejuni* and the enterotoxins of *Staphylococcus* species. The VIDAS® *Salmonella* protocol has been validated and certified by regulatory organisations as an approved method of analysis in foods. Early-stage research on a VIDAS®-based *Cronobacter* platform has demonstrated the potential application of this protocol for the detection of *Cronobacter* from PIF and related foods.

15.5.3 Molecular-based detection protocols

Molecular detection techniques are regarded as useful tools to support and extend our understanding of the epidemiology of any given bacterium. For example, real-time PCR assays can be designed to detect one or more genes unique to a bacterium including *Cronobacter*. Some of these target genes are shown in Table 15.3. As an example. the *rpoB* gene-based PCR assay was developed to distinguish among the seven *Cronobacter* species. Of note, *C. malonaticus* and *C. sakazakii* could not be accurately identified, using this original approach, without the need for a second confirmatory PCR assay.

Yan et al. developed a PCR- and array-based biomarker for the characterisation of *Cronobacter* in 2011. In this study, 126 biomarkers (including putative virulence factors) were used to differentiate *Cronobacter* from other foodborne pathogens.

Pulsed-field gel electrophoresis (PFGE) is considered a gold standard for the epidemiological investigation of foodborne pathogenic bacteria. Mullane et al. applied PFGE to fingerprint *Cronobacter* species recovered from a PIF production line and the environment. Moreover, a PulseNet International-validated PFGE protocol is expected to be available shortly. In 2008, a second subtyping method, multilocus variable-number tandem-repeat analysis (MLVA), was developed to subtype geno- and pheno-typically diverse *Cronobacter* isolates.

Genome sequencing has also facilitated these molecular detection methods by providing a range of novel target genes. In 2009, Kuhnert et al. described a multilocus sequence analysis (MLSA) method applied to *Cronobacter* which included the *recN, rpoA* and *thdF* genes. Later in 2009, El-Sharound *et al*. applied *recN* gene sequence analysis for the detection of *Cronobacter* species recovered from dried milk and related products in Egypt.

Table 15.3 Gene Targets Useful for the Detection of *Cronobacter* Species

Genus loci	Gene targets
ribosomal DNA (rDNA)	16S rRNA 23S rRNA $tRNA_{Glu}$ FISH
1,6 α-glucosidase	*gluA*
MMS operon	*dnaG*
Zinc-containing metalloprotease	*zpx*
Outer membrane protein A	*ompA*
Species loci	Gene targets
rfb (O-antigen)	*wehC* [CsakO:1] and *wehI* [CsakO:2] *wzx* [CsakO:3; CturO:1;Cmuy O:1; and Cmal O:1 and O:2]
β-subunit of RNA polymerase	*rpoB*
Other gene targets RNaseP *infB* (initiation factor)	

Another sequence-based approach, multilocus sequence typing (MLST) was developed using seven housekeeping genes – *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA* – which provide sequence types that can discriminate between all *Cronobacter* species. A highly stable sequence type (denoted as ST4) within *C. sakazakii* was reported to be responsible for several neonatal infections. However, this scheme has, so far, not been applied for use in any ongoing epidemiologic investigation.

High-throughput genomic analysis has been used to facilitate the correct identification of bacterial species. These approaches can be used for comparative purposes in order to rapidly and simultaneously investigate the presence/absence of all annotated genes or coding sequences or/and polymorphisms that may contribute to a specific morphology or physiology. Microarray-based comparative genomic indexing analysis of the *Cronobacter* genus identified species-specific genes that could be evaluated as candidate markers for inclusion in a molecular-based detection protocol. Next generation sequencing has also been used for the comprehensive analysis of *Cronobacter* genomes in order to better understand the pathogenicity and evolution of the genus in tandem with the characterisation of virulence genes. While this approach might reveal species-specific genomic information, it can be expected to provide much more detail necessary to extend our understanding of the biology of this bacterium.

15.6 Genomes of the genus *Cronobacter*

Genome sequencing technology is becoming cheaper and more accessible than it has ever been. The competition between companies providing next-generation sequencing technology has helped improve quality and the speed at which data are delivered. This has opened up a new field through which to study *Cronobacter* species and, consequently, whole genome sequencing data for three *C. sakazakii* strains, ATCC BAA-894, ES15, SP291 and a

C. turicensis z3032 (LMG 23827) strain, have now become available. Chromosomal and plasmid annotation of these sequences can help to provide a comprehensive understanding of the link between geno- and phenotype such as virulence, persistence and resistance characteristics. Strain variability at the genome level provides a challenge to fully understand the species and how it may adapt in particular environments.

Cronobacter sakazakii ATCC BAA-894 was the first fully sequenced genome within the genus. These data revealed a single chromosome of 4.37 Mb (57% GC) along with two plasmids, denoted as pESA2 and pESA3 (31-kb, 51% GC and 131-kb, 56% GC respectively). The chromosome contained 4392 genes and the source of this isolate was a batch of contaminated PIF consumed by infants in a neonatal intensive care unit. During the investigation, the PFGE profile of BAA-894 was indistinguishably matched to the clinical isolates recovered. These genomic data provided a robust starting point from which to begin whole genome comparisons. A 387,000 probe oligonucleotide tiling DNA microarray was designed using this information and then used to carry out a comparative genomic hybridization (CGH) experiment against five other *C. sakazakii* strains and representatives from four other *Cronobacter* species: *C. turicensis*, *C. muytjensii*, *C. malonaticus* and *C. dublinensis*. A core subset of 2404 (59.9% of 4382 unique annotated genes) genes were identified in common across all five *C. sakazakii* strains investigated and 1899 (43.3%) genes were found to be common to all five *Cronobacter* species. Along with investigating the core genome content, this study identified 21 genes unique to *C. sakazakii* including the pilin FilmA protein, the porin papC and the chaperone PapD, proteins for the phosphotransferase system, a putative sialic acid transporter, N-acetylneuraminate lyase and RelB from a toxic/antitoxin system.

In 2011, Stephan et al. published the complete genome sequence of *C. turicensis* LMG 23827, also known as z3032, which caused fatal infections in two newborn infants in Zürich in 2005. Similar in size to *C. sakazakii* ATCC BAA-894, this former genome was 4.38 Mb (57% GC), and contained three plasmids: pCTU1 – 138-kb (56% GC); pCTU3 – 54-kb (50% GC); and pCTU2 – 22-kb (49% GC). Arising from the origin of the strain, virulence characteristics were the main interest in this isolate, and these authors identified 22 proteins with homology to those annotated with the keyword 'virulence' and 223 with virulence- and disease-related proteins. Some of these determinants included genes for iron acquisition, a type IV secretion system, flagella and effector proteins.

A third complete genome *C. sakazakii* ES15, an isolate cultured from ground whole grains, was published in 2012. The chromosome was 4.27-Mb (57% GC) in size, with 3,916 genes and 2 propahges. No plasmid(s) was found in this isolate. Using the KEGG database, the metabolic/biosynthetic pathway analysis highlighted the complete sets of genes for glycolysis and the tricarboxylic acid (TCA) cycle, as well as for flagellum assembly, which confirmed the facultative aerobic and motile nature of this bacterium. Interestingly, a relatively high number of ABC transport and phosphotransferase systems (PTS) were present in this genome, indicating efficient nutrient uptake transporters. An outer member protein A (OmpA) previously shown to play a role in invision into brain microvascular endothelial cells (BMEC) , was identified in this genome. However, a component of copper/silver resistance cation effux system, IbeB, was absent in this genome, when compared to *C. sakazakii* ATCC BAA-894.

The most recently sequenced isolate was *C. sakazakii* SP291, a persist and thermotolerant strain isolated from PIF manufacturing environment. This genome consisted of a 4.3-Mb chromosome (56.9% GC) and three plasmids denoted as pSP291-1, [118.1-kb (57.2% GC)], pSP291-2, [52.1-kb (49.2% GC)] and pSP291-3, [4.4 kb (54.0% GC)]. Phenotypic microarray (PM) data highlighted two interesting functional categories, comprising of genes related to the bacterial stress response and resistance to antimicrobial and toxic compounds.

The complete sequence of eight available plasmids suggested two closely related groups, donated as *plasmid group 1*, containing pSP291-1, pESA3, and pCTU1; along with *plasmid group 2*, consisting of pSP291-2 and pCTU3. Genes shared in *plasmid group 1* consisted of a complete ABC transporter, including the ATP-binding component, the periplasmic substrate-binding module and the permease element, the RepFIB-like origin of replication gene *repA*, two plasmid-borne iron acquisition systems (*eitCBAD* and *iucABCD/iutA*), as well as the *Cronobacter* plasminogen activator *cpa* gene, despite of a 17-kb type 6 secretion system (T6SS) locus identified previously in pESA3, a 27-kb region encoding a filamentous hemagglutinin gene (*fhaB*), its specifc transporter gene (*fhaC*), and associated putative adhesins (FHA locus) identified in pCTU1, as well as a histone acetyltransferase HPA2 and related acetyltransferases protein along with an uncharacterized protein ImpH/VasB in pSP291-1. In *plasmid group 2*, fifteen heavy metal (copper, cobalt, zinc, cadmium, lead, and mercury) resistance genes, an osmosensitive K+ channel histidine kinase protein (KdpD), and a virulence-associated protein VagC were shared in both plasmids. However, a putative glutathione S-transferase protein, a LysR family transcriptional regulator, a putative phage-associated acyl carrier protein, a S-adenosylmethionine: tRNA ribosyltransferase-isomerase protein, permeases of the major facilitator superfamily and abortive infection protein were present in pSP291-2 only.

15.7 Controls in manufacturing environment

Real-time surveillance of *Cronobacter* in the PIF production environment is a useful first step in an attempt to reduce the bacterial load in this setting and control the potential for subsequent dissemination. Mullane et al. investigated *Cronobacter* in a powdered milk protein manufacturing facility. These data showed that the correct installation and maintenance of air filters could play an important role in reducing the dissemination of *Cronobacter*. Data from ongoing surveillance studies clearly showed that a *Cronobacter sakazakii* isolate can persist for long periods in the modern production environment and in this case it is thought to have adapted to environmental stresses, such as desiccation and high temperatures (60 °C). An understanding of the molecular mechanisms associated with such characteristics may be helpful as a means to develop strategies to effectively eliminate them.

Walsh et al. characterized the variability between environmental and clinically-derived strains of *Cronobacter sakazakii* in infant milk formula and ingredients. Environmental strains were more persistent in dry ingredients, while clinical strains were more thermotolerant, which could be an indication of the pathogen's ability to produce extracellular polysaccharide (EPS). However, the features contributing to the thermotolerant phenotype observed remain unclear and require further detailed experimental investigation.

Using a bacterial artificial cloning approach in *Escherichia coli* and subsequent screening of transformants for fluorescence on calcofluor plates, Grimm et al. identified cellulose as an extracellular matrix component present in the biofilm of a *Cronobacter* clinical isolate. This study suggested that the exopolysaccharide composed of cellulose might cause rugosity due to its overexpression; while the role of other bacterial exopolysaccharides in rugosity cannot be ignored. Understanding the contribution made by these phenotypes at a molecular level might improve the prospect of developing strategies designed to eliminate *Cronobacter* from the manufacturing environment.

15.7.1 Biocides to control *Cronobacter*

In the modern food industry, biocides and chemical-based disinfection protocols are widely used to support food safety and hygiene measures. Commercially-available biocide formulations are effective in killing *Cronobacter* species when used as recommended by the manufacturer. Mean minimum inhibitory concentration (MIC) values, however, can vary for different biocide formulations. When tested on surface-dried bacterial cells and bacteria contained in a biofilm, the biocide formulations exhibited a reduced killing effect on *Cronobacter*, which suggested that biofilm or surface dried-associated phenotypes play a role in the persistence of *Cronobacter* in the manufacturing environment. Therefore, control strategies within this environment should be considered to eliminate the development of biofilms and surface-dried responses in *Cronobacter* species.

Natural biocides as food additives have also been considered as an alternative means to control *Cronobacter*. It was reported that the combination of lactic acid and copper sulfate could be used to eliminate *Cronobacter* in the PIF industry setting. This approach may become more relevant as the use of biocides in manufacturing environments may lead to selective pressure on the bacterium, which could result in the emergence of a tolerant phenotype.

Studies have investigated the effect of sublethal exposure of *Cronobacter* to both biocide formulations and active biocidal agents. Interestingly, no alteration in the tolerance to selected agents was identified. However, the potential for cross-resistance to clinically important antibiotics has given rise to concerns among healthcare professionals, and the continuous monitoring of cleaning regimes in place within food manufacturing industries should be considered and constantly reviewed.

15.7.2 Natural antibacterial compounds to control *Cronobacter*

Natural antibacterial compounds, such as essential oils, polyphenols and prebiotics, are known to be effective against *Cronobacter*. Essential oils derived from plants have been shown to inhibit foodborne pathogens including *Cronobacter*. Compared to artificial preservatives, these oils have the potential for widespread use in the food industry. As an example of this, 'trans'-cinnamaldehyde (TC), a component of bark extract from the cinnamon plant, can effectively inhibit the formation and inactivate *Cronobacter* in biofilms. TC also reduced *Cronobacter* tolerance to desiccation, acid and osmotic stresses and enhanced the killing effect of heat treatments. TC was reported to down-regulate genes involved in biofilm formation and many important stress regulators, such as *rpoS*, *phoP/phoQ* and *ompR*. Furthermore, TC was also found to inhibit the expression of proteins involved in active transport across the membrane, flagellar biosynthesis, along with other genes. Moreover, the ability of TC to disrupt proteins involved in the survival of *Cronobacter* indicates that further study on the potential of TC as a control mechanism for *Cronobacter* infections may prove useful. These insights also highlight the need for developing appropriate protocols to guide the use of essential oils for pathogen control in the food-manufacturing environment.

Polyphenols are usually found in plants. They are a natural plant-associated defense mechanism designed to protect against infection. Red muscadine (*Vitis rotundifolia* Michx) juice is a rich source of polyphenols and this displayed strong antimicrobial activity against *Cronobacter*, with tannic acid showing the greatest effect.

Prebiotics are nondigestible food ingredients that stimulate the growth and activity of beneficial bacterial in the digestive system. Several prebiotics, such as polydextrose (PDX) and galacto-oligosaccharides (GOS), have been studied for their ability to inhibit bacterial

adherence to host cells in vitro. The combination of these two prebiotics was shown to inhibit *Cronobacter* adherence, based on data from tissue culture experiments. Therefore, as food grade natural agents, prebiotics may have a promising future use as a tool for the control of *Cronobacter* infection.

15.8 Future prospects

Future attention to improve the control *of Cronobacter* should focus on those aspects that were originally suggested in the 2004 FAO/WHO meeting report.

1. Manufacturers should implement an effective HACCP environmental monitoring programme that encompasses the microbiological quality of raw materials, with specific zoning along the entire processing chain through to the final product. This will minimize the entry of *Cronobacter* into the PIF and limit the growth/persistence of this pathogen in PIF products and within manufacturing facilities.
2. Greater collaboration between researchers and government agencies will help to provide assistance in solving *Cronobacter*-related issues. Governments and intergovernmental bodies should set target directives for *Cronobacter* to guide food manufacturers towards improved control in the quality of their PIF products and further reduce the risk of *Cronobacter* infection.
3. Improved PIF product labels will serve to communicate important information to consumers and will create awareness of the correct method to be used for reconstituting PIF products.
4. Education of healthcare professionals will enhance their understanding of the issues and assist caregivers to ensure that PIF is prepared, handled and stored properly. Assist developing countries in establishing effective measures to minimize risk of *Cronobacter* infection.
5. Researchers and public health officials should develop a better understanding of the ecology, virulence and other characteristics of *Cronobacter* as a means of developing effective ways to reduce contamination in reconstituted PIF.
6. Researchers and public health officials should investigate and report sources and vehicles of contamination and establish laboratory-based domestic and international networks such as the integrated food safety system (IFSS), as underpinned by the United States Food Safety and Modernization Act of 2011 (US–FSMA) which will support and maintain the Pathogen-Annotated Tracking Resource Network system (PATRN) as a prototype model system.
7. Researchers and public health officials should develop effective and rapid *Cronobacter* detection protocols for the PIF industry.

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16 Aflatoxins and *Aspergillus flavus*

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16.1 Introduction

Mycotoxins are defined as natural products produced by fungi that evoke a toxic response in higher vertebrates and other animals when fed at low concentrations. Mycotoxins could also be toxic to plants or other microorganisms, but are not classified as antibiotics of fungal origin. Biological conversion products of mycotoxins are also called mycotoxins. Fungi produce numerous organic compounds called secondary metabolites, such as mycotoxins, which are not required for the growth of the producing fungus, and are produced after cessation of an active growth phase. Mycotoxins are members of a class of low molecular weight, organic compounds derived primarily from amino acids, shikimic acid or malonyl CoA and are generally produced in the mycelia of filamentous fungi, but can accumulate in specialized structures of fungi such as conidia or sclerotia as well as in the environment surrounding the organism. Fungal species that produce mycotoxins are very diverse, with some mycotoxins being produced by a single fungal species or even by specific strains of a fungal species or a number of fungal species. The toxic effects of mycotoxins are as diverse as the fungal species that make these toxins. Some mycotoxins have acute toxic effects while others more often are toxic after long-term exposure (chronic effects). Mycotoxicology is the study of the mycotoxins and their corresponding toxic effects on animals and humans (mycotoxicoses). Over 300 mycotoxins have been identified, but only a few, such as aflatoxins, implicated in mycotoxicoses involving humans have been studied in detail.

Aflatoxins, produced primarily by the fungi *Aspergillus flavus* and *A. parasiticus* on agricultural commodities, are by far the best-characterized class of mycotoxin. It is generally accepted that modern mycotoxicology began in 1960. After 10,000 turkeys died when fed a toxin-contaminated peanut meal ('Turkey X' disease), it was discovered that the causative agent was aflatoxin, produced by *Aspergillus flavus*, in the peanut meal. It was also determined that moldy corn affected the earlier deaths of livestock, namely horses in Illinois (1933–1934 where 5000 horses died) and swine (1500 lost in southeastern United States). These observations provided the impetus to associate fungal toxins with illness in animals.

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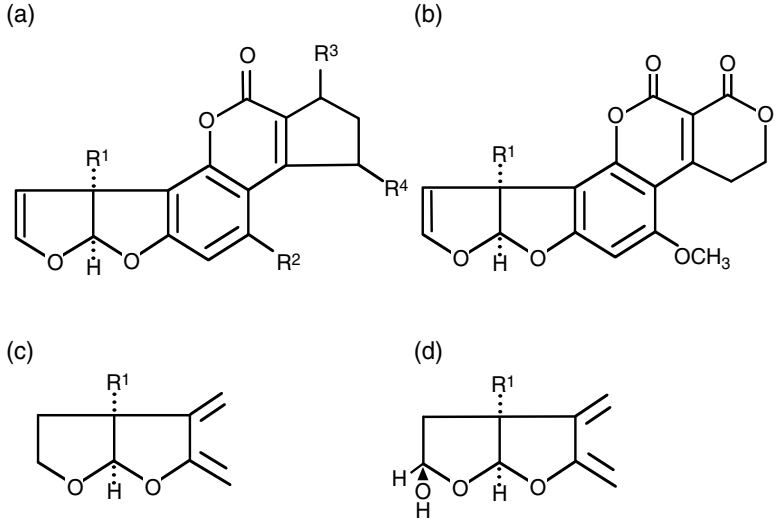
16.2 Aflatoxins

16.2.1 Chemical structure and biosynthesis

Aflatoxins are a group of polyketide-derived bis-furan-containing dihydrofuranofuran and tetrahydrofuran moieties (rings) fused with a substituted coumarin (Fig. 16.1). The four major aflatoxins, B_1, B_2, G_1 and G_2, were originally isolated from *A. flavus*, hence the name *A-fla-toxin*, with the B toxins fluorescing blue under UV light and the G toxins fluorescing green. Subsequently, additional aflatoxins have been classified and 16 structurally-related toxins have been characterized. Other significant members of the aflatoxin family, M_1 and M_2, are metabolites of aflatoxin B_1 (AFB_1) originally isolated from bovine milk. Recently, it has been shown that M group of aflatoxins could also be secondary metabolites of *A. flavus*.



Aflatoxins	Structure	R^1	R^2	R^3	R^4
B_1	A	H	OCH_3	=O	H
M_1	A	OH	OCH_3	=O	H
P_1	A	H	OH	=O	H
Q_1	A	H	OCH_3	=O	OH
R_0	A	H	OCH_3	OH	H
R_0H_1	A	H	OCH_3	OH	OH
B_2	AC	H	OCH_3	=O	H
B_{2a}	AD	H	OCH_3	=O	H
M_2	AC	OH	OCH_3	=O	H
G_1	B	H	–	–	–
G_2	BC	H	–	–	–
G_{2a}	BD	H	–	–	–
GM	BC	OH	–	–	–

Figure 16.1 Chemical structures of aflatoxins.

The aflatoxin biosynthetic pathway has been deciphered in significant detail following the discovery of the structure of these toxins, by the identification of the chemical intermediates in the pathway, the major biochemical steps as well as the corresponding genetics of aflatoxin B_1 (AFB_1) biosynthesis. Starting with the polyketide precursor, acetate, there are at least 23 enzymatic steps in the AFB_1 biosynthetic pathway. AFB_2, G_1, and G_2 are synthesized from pathways that diverge from the AFB_1 pathway. The genes for almost all the enzymes have been cloned and a regulatory gene (*aflR*) coding for a DNA-binding, Gal4-type 47-kD protein has been shown to be required for transcriptional activation of all the structural genes, with another gene *aflJ* being a co-activator. Genetic studies have also shown that all the AFB_1 pathway genes are clustered within a 75-kb region of the fungal genome. Additionally, it has been determined that a number of developmental regulatory factors are important for turning on the expression of genes required for aflatoxin biosynthesis. These proteins, such as LaeA, VeA, VelB and VosA, could be responsible for positioning AflR at the AF cluster genes in the nucleus, as well as for changing the chromatin conformation in the biosynthetic cluster part of the chromosome so that AflR and global transcriptional regulatory factors can turn on the transcription of genes in the Af gene cluster. AflR expression is induced by simple sugars (enhanced) and acidic pH; while alkaline pH, organic acids and aldehydes are inhibitory. Other nutritional or environmental factors, including signaling factors such as cAMP-dependent protein kinase A and light, also play a role in gene expression.

16.2.2 Toxic effects

Aflatoxins have been shown to be mutagenic, teratogenic and hepatocarcinogenic in experimental animals. AFB_1 is the most toxic of this group of toxins and the order of toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$. Aflatoxin M_1 is at least 10-fold less toxic than AFB_1, but its presence in milk is of concern in human health. AFB_1 is also one of the most carcinogenic natural compounds known; therefore, extensive research has been done on its toxicity and biological effects.

Acute toxic effects of aflatoxins in humans have been documented in many occurrences in different parts of the world. In 1974, in western India, 108 deaths were reported from among 397 people affected by consuming corn highly contaminated with aflatoxin. More recently, in Kenya in July 2004, over 125 deaths were reported from consumption of aflatoxin-contaminated maize. In the USA, such occurrences are rare due to careful screening of foods, but poisoning in dogs has been reported from aflatoxin-contaminated dog food.

The liver is the target organ for chronic toxicity, i.e., long-term dietary exposure to low doses of aflatoxins; with the susceptibility of animals varying with species. Aflatoxicosis (the biological effect of aflatoxin B_1) in animal models results from activation of AFB_1 by microsomal cytochrome P-450 monooxygenase in the liver for carcinogenicity. This enzyme converts AFB_1 to a variety of metabolites of increased polarity, including AFB_1–8,9-epoxide which binds covalently to N7 position of guanine in DNA, resulting in defective repair and DNA damage, mutations and, ultimately, carcinomas in many animal species. Carcinogenesis in humans is facilitated by hepatitis B virus infections in affected subjects. This correlation is based on the established association of hepatocellular carcinoma and dietary exposure to aflatoxins in patients living in high-risk areas of the People's Republic of China, Kenya, Mozambique, the Philippines, Swaziland, Thailand, and the Transkei of South Africa. The correlation was demonstrated by measuring the presence of guanine-aflatoxin adducts in urine samples indicating the modification of a guanine residue at a mutation hot spot, the transversion of guanine to thymine at the third

base of codon 249 in the tumor suppressor gene p53 (a transcription factor involved in the regulation of the cell cycle which is commonly mutated in human cancers).

16.2.3 Sampling of contaminated commodities

As the importance of aflatoxins to human and animal health has been established, a variety of sensitive methods for sampling, detection and quantification have been developed. Appropriate sampling is an important task but sometimes difficult to obtain. Some crops such as peanuts, pistachio nuts and figs usually have few contaminated kernels that can be highly contaminated, and without adequate and sensitive sampling the level of aflatoxin in a product might be underestimated.

Procedures for sampling specific commodities have been established in the EU, the USA and other countries. For example, in the US, the minimum sample size for corn is based on the type of lot. That is, for trucks 2 pounds (~ 908 g) are required; for railcars 3 pounds (~ 1362 g) and for barges/sublots 10 pounds (~ 4540 g). However, a larger sample size may be requested. A minimum sample size of 10 pounds is required for composite type samples.

16.2.4 Methods for detection and quantification

The analytical method to determine aflatoxin content is usually selected according to the availability of equipment, supplies and technical expertise, and also to the number of samples, cost, time and ease of test. Currently, there are many traditional and rapid methods for detection and quantification of aflatoxins in food and feeds. In this chapter, we briefly describe the traditional methods and focus on the most common and rapid commercial methods available. The later tests have provided an easy, rapid and economical alternative to the difficult and time-consuming traditional methods.

16.2.5 Traditional methods

Initial methods of detection are based on grain fluorescence (BGY) under ultraviolet light. In addition, fluorescence of aflatoxins allowed development of a number of technologies for detection and quantification, from thin layer chromatography to high-performance liquid chromatography.

The traditional method for aflatoxins analyses involves three steps: sample extraction, separation and detection. Sample extraction usually involves solvent extraction of seed (using chloroform, methanol, ethanol and other), solid-phase extraction (using C18 or immune-affinity columns, see below), supercritical-fluid extraction or matrix solid-phase dispersion. Thin layer chromatography or HPLC are used in the separation process, and the detection can be done by analysis of UV absorption, fluorescence or mass spectrometry.

TLC is a simple and economical method to detect aflatoxins, but has low sensitivity. HPLC and gas chromatography/mass spectroscopy are the most precise and sensitive methods for aflatoxin quantification and identification. However, these methodologies require expensive equipment, trained personnel, and are very laborious.

16.2.6 Rapid methods for detection and quantification

Similar to the traditional methods, once the sample is collected, aflatoxin extraction and purification are required before detection. Usually, aflatoxin is extracted from a ground sample by shaking or blending with methanol/water. Removal of compounds from the

sample, which may interfere with aflatoxin detection, is necessary. This can be achieved by ready-to-use clean-up columns that enhance purification and preconcentrate the toxin prior to the detection step.

The use of commercial clean-up columns provides a rapid and easy way to separate aflatoxins from matrix interferences and recover the toxin for quantitation by TLC, HPLC, GC, mass spectroscopy, fluorometry and immunoassays. These columns are of different sizes and are packed with different matrix components suitable for testing a wide range of commodities (Table 16.1).

A newer method involves use of immuno-affinity columns which are reliable and user-friendly. These columns contain gel having immobilized antibodies that selectively bind the toxin of interest from the crude extract. The column uses a specific monoclonal antibody to selectively isolate, immobilize, and concentrate aflatoxins AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2. During this process, the column is washed to remove interfering compounds, while the toxin remains immobilized on the column. Then the purified aflatoxin could be eluted from the column using methanol for determination purposes.

Some columns remove analytical interferences from sample extracts in one step, eliminating washing and elution steps found in solid phase extraction and affinity columns. The interferences adhere to the chemical packing in the column and the purified extract, containing the analytes of interest, can be collected.

Rapid methods for detection are becoming widespread due to their sensitivity, specificity, rapidity, simplicity, and cost. These are mainly based in immunoassays, and include the lateral flow devices (immune-chromatography), and ELISA in microtiter plates or in tubes (Table 16.2). Most of the immunoassays are based on monoclonal antibody for the detection of AFB_1. Immunological analysis of AFB_1 in many cases provide a measurement for total aflatoxins because of the cross-reactivity of the antibody for all four B and G aflatoxins. These systems cannot differentiate between the various aflatoxins, but detect their presence to differing degrees.

Other methodologies, such as the florisil tip, use the fluorescence of aflatoxins for detection. In the latter case, the cleaned sample is passed through a florisil tip, where the aflatoxins are collected. The florisil tip is then viewed under UV light at 366 nm where the natural fluorescence of the aflatoxins is observed as a blue/purple band on the tip. The intensity of the fluorescence on the tip is compared to a nonhazardous fluorescent card to obtain a semi-quantitative result. In general, quantitative rapid methods use microtitre readers, colorimeters, fluorometers and scanners to determine the amount of toxin present.

16.2.7 Regulatory limits for aflatoxin levels

The toxic effects of aflatoxins have resulted in statutory limits imposed by several countries for the level of toxin acceptable in human food and animal feed (Table 16.3). Generally, the limits imposed by many countries range between 5 and 20 ppb (parts of aflatoxin per billion parts of food or feed substrate, i.e., micrograms per kilogram). Consequently, contaminated commodities have to be either discarded or destroyed, putting an enormous burden on the food-related industries.

Economic losses due to aflatoxin contamination drive the need for prevention and control of aflatoxins contamination. Contaminated seed cannot be sold or used and must be discarded. This problem is primarily relevant to developed countries where food is in ample supply. In developing countries, where food may be in short supply, inadequately screened

Table 16.1 Commercial Clean-up Columns for Aflatoxin Isolation and Concentration

Name	Description	Aflatoxins	Intended use	Company
EASI-EXTRACT® AFLATOXIN	Immunoaffinity column	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2	Variety of commodities	R-Biopharm AG
AFLASCAN®	Immunoaffinity columns with florisil tips	AFB_1, AFB_2, AFG_1 and AFG_2.	Variety of commodities including nuts, maize, spices and dried fruit	R-Biopharm AG
AFLAPREP®M	Immunoaffinity columns	AFM_1 and AFM_2.	AFM_1 in milk and dairy products	R-Biopharm AG
AFLAPREP®	Immunoaffinity columns	AFB_1, AFB_2, AFG_1 and AFG_2	Variety of commodities	R-Biopharm AG
RIDA®Aflatoxin column	Immunoaffinity columns	AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1	Difficult samples such as nuts, herbs, spices, tea leaves and baby or infant food	R-Biopharm AG
NeoColumn™ For Aflatoxin	Immunoaffinity column	AFB_1, AFB_2, AFG_1 and AFG_2	Variety of commodities	Neogen Corporation
NeoColumn™ For Aflatoxin DR	Immunoaffinity column	AFB_1, AFB_2, AFG_1 and AFG_2	Corn, corn byproducts, peanuts and tree nuts	Neogen Corporation
AflaTest®WB	Immunoaffinity columns Specifically for HPLC use	AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1	Variety of commodities	Vicam
AflaTest®WB SR	Immunoaffinity columns	AFB_1, AFB_2 , AFG_1, AFG_2, and AFM_1 maximize AFG_2 recovery	Variety of commodities	Vicam
Afla B™	Immunoaffinity columns	$AFB_1$and AFB_2	Variety of commodities	Vicam
Afla M1 FL+	Immunoaffinity columns (Specifically for fluorometer)	aflatoxin AFM_1	Milk samples	Vicam
Afla M1 HPLC	Immunoaffinity columns (Specific for HPLC)	aflatoxin AFM_1	Milk samples	Vicam
AflaOchra HPLC™	Immunoaffinity columns	AFB_1, AFB_2, AFG_1, and AFG_2, and ochratoxin A	Variety of commodities	Vicam
AOZ HPLC™	Immunoaffinity columns	AFB_1, AFB_2, AFG_1, AFG_2; ochratoxin A and zearalenone	Variety of commodities	Vicam
Myco6in1™ LC/MS/MS	Immunoaffinity columns. For use with LC/MS/MS or HPLC	aflatoxins, deoxynivalenol, fumonisins, ochratoxin A, zearalenone, and T-2 and HT-2	Variety of commodities	Vicam
AflaCLEAN		AFB_1,AFB_2, AFG_1 and AFG_2	Variety of commodities	LCTech
Afla-OtaClean	Immunoaffinity columns	AFB_1, AFB_2, AFG_1 and AFG_2 as well as Ochratoxin A	Variety of commodities	LCTech
AflaStarTMFit-Immunoaffinity Columns	Immunoaffinity columns	AFB_1, AFG_1, AFB_2, AFG_2	Variety of commodities	Romer Labs
MycoSep®Columns	Chemical adsorbents	Aflatoxin	Variety of commodities	Romer Labs

Table 16.2 Commercial Kits of Rapid Methods for Detection of Aflatoxins

Format	Name	Aflatoxins	Detection limit	Intended use	Company
ELISA Microtiter well plate					
	Aflatoxin Plate Kit	AFB_1, AFG_1, AFB_2, AFG_2	Quantitative 2 ppb.	Nuts, grain and grain products	Beacon Analytical Systems, Inc.
	Aflatoxin M1 Plate Kit	aflatoxin AFM_1	Quantitative 0.3 ppt	Fresh milk and milk powder	Beacon Analytical Services, Inc.
	AflaREADTM	AFB_1	Quantitative 0.1 ppb	Variety of commodities	LCTech
	AflaREAD fast	AFB_1	Quantitative 2-100 ppb	Peanuts and maize	LCTech
	AgraQuant®Total Aflatoxin	Total aflatoxin (AFB_1, AFB_2, AFG_1 and AFG_2)	Quantitative 1, 3 or 5 ppb	Grains, cereals, nuts, animal feeds and other commodities	Romer Labs
	AgraQuant® Aflatoxin B1	AFB_1	Quantitative 2 ppb	Grains, cereals, nuts, animal feeds and other commodities	Romer Labs
	AgraQuant® Aflatoxin M1	aflatoxin AFM_1	Quantitative 18 ppt (fresh milk) 252 ppt (skim milk powder) 257 ppt (full-cream milk powder) 128 ppt (cheese)	Milk and milk products	Romer Labs
	Agriscreen®	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	(Qualitative) Screens @ 20 ppb	Variety of commodities	Neogen Corporation
	RIDASCREEN® Aflatoxin Total	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Quantitative analysis. 1.75 ppb	Cereals and feed	R-Biopharm
	RIDASCREEN® Aflatoxin B1 30/15	AFB_1	Quantitative 1 ppb	Cereals and feed	R-Biopharm
	RIDASCREEN® Aflatoxin M1 30/15	Aflatoxin AFM_1	Quantitative. 5–50 ppt	Milk, milk powder and cheese	R-Biopharm
	RIDASCREEN®FAST Aflatoxin	Total aflatoxins	Quantitative <1.7 µg/kg (ppb)	Cereals and feed	R-Biopharm
	RIDASCREEN®FAST Aflatoxin M1	Aflatoxin AFM_1	Quantitative <367 ng/l (ppt)	Milk and milk powder	R-Biopharm
	RIDASCREEN®FAST Aflatoxin SC	AFB_1	Quantitative 2 ppb	Variety of commodities	R-Biopharm
	Veratox®	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Quantitative 2 ppb	Variety of commodities	Neogen Corporation

(*Continued*)

Table 16.2 (*Continued*)

Format	Name	Aflatoxins	Detection limit	Intended use	Company
	Veratox®Aflatoxin Single Test (AST)	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Quantitative 3 ppb	Variety of commodities	Neogen Corporation
	Veratox®For Aflatoxin HS	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Quantitative 0.5 ppb	Variety of commodities	Neogen Corporation
Elisa in tubes					
	Aflatoxin Tube Kit,	AFB_1, AFG_1, AFB_2, AFG_2	Quantitative 2 ppb	Aflatoxin in corn and peanuts.	Beacon Analytical Systems, Inc.
Lateral Flow Devices					
	AgraStrip® Aflatoxin A	AFB_1, AFB_2, AFG_1 and AFG_2	(Qualitative) screens @ 10 ppb.	Variety of food products	Romer Labs, Inc
	QuickTox for QuickScan	Total aflatoxin	Quantitative 2.5 ppb	Corn	EnviroLogix
	QuickTox™ Kit for Aflatoxin - 20 ppb	Total aflatoxin (AFB_1 and AFB_2)	(Qualitative) screens at 20 ppb	Corn	EnviroLogix
	Reveal®for Aflatoxin	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	(Qualitative) screens at 20 ppb	Variety of food products	Neogen Corporation
	Reveal®for Aflatoxin M1	Aflatoxin AFM_1	Qualitative 500 ppt	Milk	Neogen Corporation
	Reveal®Q+	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Quantitative 2–150 ppb	Corn and corn products	Neogen Corporation
	Reveal®for Aflatoxin SQ	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Semi-quantitative <10 ppb	Corn and rice hulls	Neogen Corporation
	Reveal®for Aflatoxin SQ Peanut	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Semi-quantitative <10 ppb	Peanut	Neogen Corporation
	RidaQuick®Aflatoxin,	Aflatoxins	semi-quantitative ≥4 ppb	Variety of food products	R-Biopharm,
	RIDA®QUICK Aflatoxin RQS	Aflatoxins	Quantitative 4 ppb	Corn	
	ROSA®Aflatoxin	Aflatoxins	Quantitative Reader Range 0–150 ppb	Variety of food products	Charm Sciences, Inc.
	ROSA®Fast Aflatoxin Quantitative Test	Aflatoxins	Quantitative Reader Range 0–150 ppb	Corn	Charm Sciences, Inc.

	ROSA®Aflatoxin P/N Test	Aflatoxins	P/N (qualitative) Screens at 10–20 ppb	Corn	Charm Sciences, Inc
	ROSA®BEST Aflatoxin P/N,.	Aflatoxins	P/N (qualitative) Screens at 10–20 ppb	Corn	Charm Sciences, Inc
	Charm SL Aflatoxin M1	Aflatoxin AFM_1	0 to 750 ppt Reader Range Test for Milk Quantitative	Milk	Charm Sciences, Inc
	Charm II Aflatoxin M1 Tests	Aflatoxin AFM_1	0.025 ppb or 0.25 ppb	Milk	Charm Sciences, Inc
Other Fluorometric tests	FluoroQuant®Afla	Total aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2)	Quantitative 5 ppb	Corn, corn meal, popcorn, rice, sorghum (milo), wheat, soy, and corn soy blend	Romer Labs, Inc
	FluoroQuant®Afla Plus	Total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)	Quantitative 0.6 ppb–1.9 ppb.	Corn, raw blanched and unblanched peanuts, raw almonds, decorticated cottonseed, cottonseed meal	Romer Labs, Inc
	FluoroQuant®Afla IAC	Total aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2)	Quantitative not Provided	Corn, corn screenings, distillers dried grains with soluble (DDGs) and peanuts	Romer Labs, Inc

Table 16.3 Aflatoxins in Foodstuffs: Maximum Tolerated Levels in Some Representative Countries

	Tolerance (µg/kg)	
Country	**AFB_1 in food**	**AFM_1 in milk/milk products**
Austria	1	0.01–0.05
Belgium	5	0.01
Brazil	3–15	0.1–0.5
China	50	
France	5–10	0.2
Germany	5	0.01–0.05
India	30	
Japan	10	
Kenya	20	
Mexico	20	
The Netherlands	5	0.02–0.2
Nigeria	20	1
Sweden	5	0.05
Switzerland	1	0.05
United States	20	0.5

foods may be responsible for long-term health implications that are generally overlooked. However, economies of developing countries could be seriously affected when the presence of even the smallest amount of toxin in export commodities is not accepted by developed countries. Elimination of aflatoxins from the food and feed supply would sharply reduce this economic cost. Therefore, research in many laboratories throughout the world is focused on pre- and post-harvest control of these toxins.

16.3 *Aspergillus flavus*

16.3.1 Other health concerns

In addition to aflatoxins, *A. flavus* produces a number of other minor mycotoxins. These include cyclopiazonic acid, which is an indole-tetramic acid found as a natural contaminant of agricultural raw materials and animal feed. The toxicity of this compound has been demonstrated in many animal species. Five additional secondary metabolites with some toxic effects are also produced by *A. flavus* under some conditions, namely aspergillic acid, aflatrem, aspertoxin, kojic acid, and B-nitropropionic acid. *A. flavus* is also known to cause allergic and infective conditions of humans and certain other vertebrates, including allergic bronchiopulmonary aspergillosis and invasive pulmonary aspergillosis in immunocompromised and aging humans and certain other vertebrates (*A. flavus* is second only to *A. fumigaus* as the primary causative agent of aspergillosis). For these reasons, and the fact that it is found in soils across the globe, this fungus has received significant attention and been researched in great detail.

16.3.2 Biology and habitat of *A. flavus*

Aspergillus flavus is found in both temperate and in subtropical regions of the world. Agronomically, *A. flavus* could be considered a plant pathogen. But it is not a true pathogen from a plant pathology point of view because living tissue is only a minor substrate for these

soilborne filamentous fungi. Ecologically, *A. flavus* grows mainly on dead matter, and is therefore a saprophyte. It can grow on a wide variety of substrates, including decaying plant and animal debris found in the soil where it must compete with the other soil microflora. The two major factors that influence soil populations of *A. flavus* are soil temperature and soil moisture. These fungi are considered semithermophilic and semixerophytic because they can grow at temperatures of 12–48 °C and at water activity (a_w) as low as 0.80. The optimum temperature for growth is 25–42 °C. Fungal growth and conidial germination are ideal at water activity greater than 0.90, and is completely inhibited at a_w <0.75.

16.3.3 Morphological identification of aflatoxin-producing *Aspergillus flavus*

Fungal genus *Aspergillus* reproduces asexually, forming mitospores called conidiospores (conidia) on specialized structures called conidiophores (Fig. 16.2), single-celled spheres or ellipsoids. At 40× magnification under a light microscope, the spores of *A. flavus* look smooth. Some members of these genera also produce a sexual state called the teleomorph with a different name; the sexual state of *Aspergillus flavus* is *Petromyces flavus*. The sexual

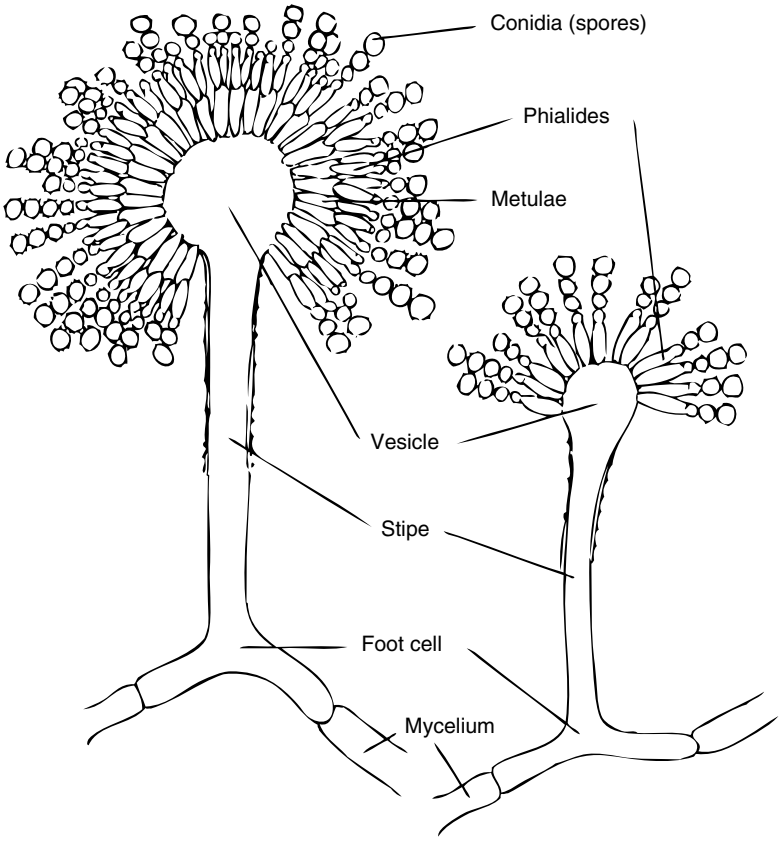


Figure 16.2 Basic morphological structure of *Aspergillus flavus* showing (a) biseriate and (b) uniseriate conidial heads.

states of section *Flavi* described to date are characterized by hard, sclerotia-like ascomata. The sclerotia of these species may develop into ascomata over time when two appropriate mating types are crossed (i.e., the species is heterothallic). Most aspergilli that produce teleomorphs are homothallic, that is, they can form ascospores with only one strain present. Sex in *A. flavus* is heterothallic meaning it must occur between individuals having the opposite mating type, either *MAT1-1* or *MAT1-2*.

After the discovery of MAT loci for populations of *A. flavus*, sexual reproduction between parents belonging to opposite mating type and a different VCG (vegetative compatibility group) resulted in production of cleistothecia and ascospores, indicating sexual crossing.

16.3.4 Diversity in *A. flavus* populations

Aspergillus flavus isolates are separated into vegetative compatibility groups (VCGs), maintaining a barrier to recombination by hyphal fusion and limiting genetic exchange across VCGs. New VCGs may arise through chance mutations at compatibility loci, but they may also occur through genetic recombination. Isolates of *A. flavus* from different VCGs can differ in enzyme production, virulence, and aflatoxin-producing ability. *A. flavus* also has been divided into small and large sclerotial morphotypes, called S and L, with isolates belonging to the S group producing numerous sclerotia (highly melanized, compacted mycelia bodies) and fewer conidia than those of the L strain. The L strain, or the 'typical' *A. flavus*, produces fewer sclerotia but more conidia when grown under the same conditions. Additionally, S and L morphotypes respond differently to pH and growth and differentiation in light and dark environments. Recent phylogenetic studies have determined that these morphotypes most likely diverged separately from an aflatoxin B- and G-producing ancestor.

16.3.5 Evolution of *A. flavus*

Recent phylogenetic analyses have provided insight into the evolution of *A. flavus*. Phylogeny based on aflatoxin biosynthetic genes, two distinct lineages of *A. flavus*, were observed: one is composed of more toxin-producing individuals while the other lineage is predominantly non-aflatoxigenic. Coalescent analysis shows these lineages to be very old and sharing an ancient common ancestor; *A. flavus* probably evolved from B- and G-producing ancestor about 5–8 million years ago. These lineages are co-evolving, with individuals from each lineage exchanging genetic material. The *A. flavus* strains that have partial aflatoxin biosynthetic gene cluster appear to share the nonaflatoxigenic lineage, and are experiencing lineage-specific gene loss over time. The *A. flavus* S and L morphotypes can be distinguished based on a distinct deletion in the aflatoxin biosynthesis gene cluster between two genes (aflF and aflU) necessary for aflatoxin G formation. Based on conservation of these deletions in the different types of *A. flavus*, it is suggested that the separation of the S and L morphotypes may have occurred about 1 million years ago.

When *A. flavus* interacts with plants, the primary source of inoculum is conidia present in the soil. The presence of sclerotia in infected tissue and in the soil in the southern US suggests that these structures play an important role in fungal survival when conditions are unfavorable for growth and propagation. *A. flavus* has developed an extraordinary ability to colonize plants and escape its normally saprophytic role. These adaptations, mainly seen in *A. flavus*, may result from an over-representation in the genome of proteolytic enzyme-encoding genes, nitrogen utilization genes and genes involved in carbohydrate metabolism.

These adaptations could be a response to changing environment such as the emergence of grasslands both in North America and Africa during interglacial periods when regions of the Earth became more temperate (about 5–8 million years ago). As a consequence, there were changes in nutrient composition of the soils, with subsequent genetic adaptations by fungi. It is proposed that when agriculture was developed, there was a loss of selection pressure for toxin and secondary metabolite production, and non-aflatoxigenic strains of *A. flavus* may have become a common variant in the population. And this process continues to this day when between 30% and up to 80% of the isolates of *A. flavus* in different regions tested lack the ability to produce aflatoxins.

16.3.6 Pre-harvest contamination

Under conditions of high temperature and low moisture, optimum for *A.flavus*, these fungi thrive and out-compete other soil and plant microflora by producing abundant conidia which are easily dispersed in the air. This allows *A. flavus* to out-compete other microflora on the seed surface, placing them in an ideal position to readily colonize both insect-injured or otherwise susceptible seeds. Under drought conditions, many of the physiological defense systems of the host plant are compromised due to high temperatures and water stress. Further, these conditions often lead to defects in the seed, which facilitates fungal invasion and breaching of the seed's structural barriers. Injury, especially that caused by insects, is very important in the epidemiology of *A. flavus* infection with abundant sporulation often observed on developing damaged seeds. Injury not only allows an easy means of entry to the fungus, but it also causes dehydration of the kernels, thus creating a more favorable environment for growth and aflatoxin production.

16.3.7 Post-harvest contamination

Aspergillus flavus can also rot improperly stored grain and contaminate the grain with aflatoxins. The two major environmental conditions for contamination, as for pre-harvest contamination, are temperature and moisture. Properly dried grain does not support growth of the fungus. Insect activity in stored products sometimes creates favorable microclimates for fungal growth and, once fungal growth starts, the water from metabolism by the fungus provides sufficient additional water for further growth and mycotoxin development.

16.3.8 Aspergillus flavus *genomics*

Initially, the two main objectives of the *A. flavus* genomics project were to understand: (i) the genetic control and regulation of genes involved in aflatoxin production; and (ii) the evolutionary process of this fungus and toxin production. First, an expressed sequence tag (EST) strategy provided rapid identification of genes potentially involved in aflatoxin contamination of crops by *A. flavus*. There were 7218 unique genes identified from more than 26,000 cDNA clones in an *A. flavus* cDNA expression library, with 34% of the genes having no homologs in existing databases. Subsequently, *Aspergillus flavus* strain NRRL 3357 (ATCC 200026), a wild type strain widely used in laboratory and field studies, has been sequenced with 5× coverage. The genome has been assembled into 79 scaffolds ranging in size from 1.0 kb to 4.5 Mb. Over 75% of the genome is represented in the 10 largest scaffolds. The estimated genome size of 36.3 Mb is similar to that for a closely-related fungus, *Aspergillus oryzae* (36.8 Mb) (www.Aspergillusflavus.org). The genome is organized into

eight chromosomes, which are predicted to encode about 12,000 proteins. The genomics of *A. flavus* has allowed a better understanding of the many secondary metabolic processes that such fungi are known to have (over 55 putative secondary metabolite clusters have been identified, with their functions currently under investigation). Genomic studies have also revealed that specific proteins which regulate fungal development also regulate toxin production, thereby providing a link between these two processes (one associated with primary metabolism and the other with secondary metabolism). Structural, functional (using whole genome microarrays), and comparative (with genomes of other Aspergilli) genomics has provided significant information about: (a) the biosynthesis of a number of secondary metabolites; (b) the ability of the fungus to survive in the field and invade the crop plant; (c) the ability of this fungus to cause human pathogenicity; and (d) the ability of the fungus to be used for bioconversion of agricultural waste.

16.4 Control of aflatoxin contamination of crops

Strategies to address food safety and economic issues employ both pre- and post-harvest measures to reduce the risk of aflatoxin contamination in food and feed. Post-harvest measures, such as adequate storage, detection and decontamination or disposal, as well as continuous monitoring of potential contamination during processing and marketing of agricultural commodities, have proved to be crucial and indispensable in ensuring food and feed safety; however, these measures do not address the issue fundamentally. The post-harvest contamination is usually the result of pre-harvest presence of fungal contamination. Therefore, research focus in the past decades has shifted from post-harvest control to a more preventive approach employing various pre-harvest control measures. Pre-harvest control includes good cultural practices such as insect control, irrigation during drought conditions, planting and harvesting dates. In addition, new biotechnologies such as (1) the use of non-toxigenic biocompetitive strains of *A. flavus* for biocontrol of aflatoxin contamination, and (2) identification of plant constituents that disrupt aflatoxin biosynthesis or fungal growth and their use in new biochemical marker-based breeding strategies to enhance resistance in crops to aflatoxin, could potentially save the agricultural industry in the US alone hundreds of millions of dollars. Study of the genetics of the aflatoxin biosynthetic pathway is important for understanding how and why this fungus makes aflatoxins and has enabled scientists to examine strategies to interrupt aflatoxin synthesis, thereby preventing aflatoxin contamination of crops. The fungal genome of *A. flavus* has been sequenced to understand the regulation of aflatoxin formation by environmental factors. This information is being used to assist in the development of host-resistance against aflatoxin contamination by studying the effects of various physiological parameters, e.g., drought stress on gene expression in toxigenic fungi. The control strategies include the following studies:

1. *Understanding pre-harvest aflatoxin contamination process*. *Aspergillus flavus* populations increase on crop debris, on senescent or dormant tissues, and on damaged or weakened crops. The source of the initial infecting inoculum and the diversity of fungal populations can be divided into two phases, one occurring during crop maturation and the other after maturation. If damage and infection occur at the appropriate stage of maturity, very high levels of contamination (>100,000 ng/g) can result. Highly-contaminated seeds, nuts, or kernels are relatively rare, but drastically influence the overall contamination level of the crop.

2. *Farming practices to control toxin contamination*. Practices that include disease and insect management in field crops, crop rotation and adequate irrigation, use of quality seed (insect resistant germplasm, e.g., maize transformed with the gene encoding *Bacillus thuringiensis* crystal protein, Bt maize), and altering the time of planting and harvest have some impact on the control of aflatoxin contamination. However, optimization of management practices to control aflatoxin contamination is not always possible due to production cost, geographic location, and the nature of the production system for the crop. In addition, even the best management practices are sometimes negated by the complex interaction of many biotic and abiotic factors that are difficult or impossible to control.
3. *Biological control of toxin contamination*. The best biocompetitive agent to control *A. flavus* in the field is atoxigenic strains of *A. flavus*, because these strains, as compared to other potential microbial biocompetitive agents, are adaptable to the same environmental conditions as are the toxigenic strains and would be biologically active at the same time as well. *Aspergillus flavus* need not synthesize aflatoxins to infect a plant, and there is no relationship between the production of high levels of aflatoxins and strain virulence. Significant reductions in aflatoxin contamination have been obtained when non-toxigenic strains of *A. flavus* inhabiting wheat kernels as a substrate are applied to corn or cotton-growing areas with a high incidence of aflatoxin contamination. The ability of non-toxigenic strains to interfere with aflatoxin contamination of various crops has had real practical value and is in use in large-scale applications in several countries, especially in the US, Africa and Asia. Applying non-toxigenic strains to agricultural fields prior to crop development may also provide post-harvest protection from contamination.
4. *Enhancing host-resistance of plants to ward off fungal attack and toxin production*. Resistance of the plant (host-resistance) can be modified by identifying biochemical markers that affect either the ability of the fungus to grow or produce toxin after invasion. Such resistance markers have been found in the host plant or non-host plants by either traditional plant breeding methods or by molecular analyses of individual plants. These factors can be transferred into the plant of concern through breeding or genetic engineering. Germplasm which plant breeders have developed from genetically mapped lines that show resistance factors have been used to identify the resistance traits at the molecular level using protein separation techniques (proteomics). Dozens of proteins inhibitory to fungal growth or toxin production have been identified by comparing protein profiles of resistant and susceptible germplasms or by following the inhibitory trait in protein fractionation. The genes corresponding to these proteins have been isolated, and the importance of these traits for resistance is being tested by specialized molecular techniques in host (gene silencing or gene expression) for their specific role in imparting resistance to corn kernels against fungal invasion. Based on the genetic engineering results, traditional plant breeding can be used for mapping resistance traits for marker-assisted breeding.

16.5 Conclusions

Aflatoxin contamination of crops (pre-harvest) and foods and feeds (post-harvest) occurs when aflatoxigenic isolates of *Aspergillus flavus* colonize the crop or commodity and produce the secondary metabolite, aflatoxin B1 under appropriate conditions of temperature and moisture. Since aflatoxins are extremely potent carcinogens, and demonstrate acute

toxicity as well, the identification and production of these compounds by the fungus, its ecological significance, the ability of the fungus to invade crops and colonize stored commodities have been extensively studied. These studies have resulted in developing various strategies for controlling aflatoxin contamination through enhancing host crop-resistance and biological control (for pre-harvest situations) and identifying proper and adequate storage conditions (for post-harvest contamination).

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17 *Fusarium* and fumonisins

Toxigenic Fusarium *species in cereal grains and processed foods*

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17.1 Introduction

Toxigenic *Fusarium* species are most often found as contaminants of plant-derived foods, especially cereal grains. As such, these molds and their metabolites (mycotoxins) find their way into animal feeds and human foods. Animals, both food-producing animals and pets, are more often affected by the toxins of these molds than are humans because of the nature of animal feed and the way it is stored and handled. Human food is generally higher in quality and more carefully protected, particularly in countries with temperate climates. However, in other areas with more tropical and subtropical climates this may not be the case, and there is evidence that grains and processed human foods may be contaminated with mycotoxins produced by *Fusarium* species. Also, exposure of food-producing animals to mycotoxigenic molds may have an impact on the human food supply by causing death of animals, reducing their rate of growth, or by the carryover of mycotoxins and their metabolites into animal products such as meats, milk and eggs. Diseases in animals caused by mycotoxins may also suggest that similar conditions might occur in humans.

Many *Fusarium* species are plant pathogens, whilst others are saprophytic, and most can be found in the soil. *Fusarium* species are most often encountered as contaminants of cereal grains, oil seeds, and beans. Corn, wheat, and products made from these grains are most commonly contaminated. However, barley, rye, triticale, millet, and oats can also be contaminated.

17.2 Characteristics of *Fusarium* toxins

Fusarium species produce several toxic or biologically-active metabolites which include a group of mycotoxins known as trichothecenes, along with fumonisins, zearalenone, moniliformin, fusarin C, and fusaric acid. Fumonisins are the main focus of this chapter, but a brief description of some other *Fusarium* toxins is also provided. Deoxynivalenol, T-2 and zearalenone toxins are the subject of another chapter in this book.

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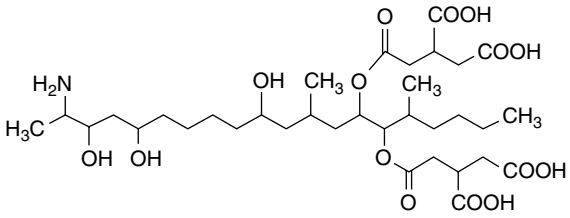

Figure 17.1 Chemical structure of Fumonisin B.

The trichothecenes are a group of closely-related compounds that are esters of sesquiterpene alcohols that possess a basic trichothecene skeleton and an epoxide group. The trichothecenes are divided into three groups: the type A trichothecenes, which include diacetoxyscirpenol, T-2 toxin, HT-2 toxin, and neosolaniol; the type B trichothecenes, which include deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, and fusarenon-X; and the type C or so-called macrocyclic trichothecenes known as satratoxins. The major producer of these toxins is *Fusarium graminearum*, which is a fungus endemic in wheat and other cereals throughout the world. *F. sporotrichioides* and *F. poae* are also species of economic importance capable of producing T-2 toxin. The production of these toxins is influenced by fungal genetic factors as well as by environmental conditions such as temperature, humidity, and substrate.

In the trichothecenes group, the toxin most commonly found in cereal grains or most often associated with human illness is deoxynivalenol (DON). This toxin is known to cause vomiting, feed refusal and reduced weight gain in pigs, as well as immunotoxic activities, gastrointestinal effects and hemorrhages in experimental animals and humans. DON is frequently detected in wheat, rye, barley, oats, other cereals, and related products. T-2 toxin, another toxin in the trichothecenes group, occurs rarely in grain in the United States, but has been associated with alimentary toxic aleukia in Russia in the 1940s and earlier and has been found in cereal grains in a number of other countries.

Zearalenone is an estrogenic compound produced by *F. graminearum* and related species. It is classified as an endocrine disrupter, causing estrogenic-like responses in animals, such as genital problems and reproductive disorders in pigs. Symptoms include swelling of the vulva in prepubertal gilts, infertility in sows, fetal death and resorption, abortions, reduced litter size and small piglets. Zearalenone is a common contaminant in cereals and other food commodities.

Fumonisins, the main focus of this chapter, are a group of related toxins that consist of a long carbon chain backbone with two tricarboxylic acid side chains and an amino group. Fumonisin B_1 (Fig. 17.1) is the most commonly occurring and most toxic of the fumonisins. These compounds resemble sphingosine, an essential phospholipid in cell membranes, and are known to disrupt its metabolism by inhibiting synthetase ceramide, blocking the synthesis of sphingolipids and causing accumulation of sphingosine. Fumonisins are the cause of leukoencephalomalacia in horses and pulmonary edema in swine. They also have been shown to cause cancer in male rats and female mice, and atherosclerotic lesions in a nonhuman primate. Fumonisins are produced by three closely-related species of *Fusarium*, *F. verticillioides*, *F. proliferatum* and *F. nygamai*. *Fusarium verticillioides* is primarily a plant pathogen that causes agricultural problems worldwide in various commodities, including corn. Fumonisins can also be found in corn-based products.

Moniliformin (Fig. 17.2), fusarin C, and fusaric acid are also toxins produced by *Fusarium* species that are of interest and concern, but have not been shown to commonly occur or be

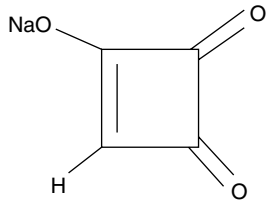

Figure 17.2 Chemical structure of Moniliformin.

specifically associated with diseases, though moniliformin has been found in corn and corn-based foods. The toxicology of these metabolites is not well documented.

17.3 Nature of human illnesses associated with *Fusarium*

Historically, various diseases or adverse effects on health have been associated with the ingestion of grains contaminated with mycotoxins, including those produced by *Fusarium* species. Examples of these conditions are presented in Table 17.1 and include alimentary toxic aleukia, Urov/Kasin-Beck disease, drunken bread, akakabi-byo, scabby grain intoxication, foodborne illness outbreaks, esophageal cancer and neural tube defects, with symptoms varying widely from headache and abdominal pain to convulsions and death. Although mycotoxins have been implicated in these human illnesses, epidemiological studies based on biomarkers are still needed. Therefore, the development of biomarkers of exposure that could be used in such studies becomes very important for a better understanding of the human health implications of mycotoxins produced by *Fusarium* species. In fact, for some mycotoxins produced by *Fusarium*, such as fumonisins and deoxynivalenol, such biomarkers have been studied. Data collected with the use of these biomarkers are valuable because they provide information regarding exposure, absorption, metabolism and elimination of these mycotoxins in humans.

17.3.1 Foodborne illness outbreaks

Outbreaks of foodborne illness have occurred in China and India that have involved corn and wheat contaminated with *Fusarium* species, deoxynivalenol, and zearalenone. A similar outbreak associated with fumonisin was observed in India after the consumption of rain-damaged moldy corn and sorghum.

17.3.2 Esophageal cancer

Fusarium verticillioides and *F. graminearum* have been associated with high rates of esophageal cancer in certain parts of the world, particularly in the Transkei region of South Africa, northeastern Italy, and northern China. In these regions, corn is a dietary staple and is the main or only food consumed. Corn and corn-based foods from these regions may contain significant amounts of fumonisins, deoxynivalenol, zearalenone and possibly other metabolites of these fungi, and fumonisins are almost always present.

Table 17.1 Human Diseases Associated with *Fusarium* Species and Toxins

Disease	Food(s)	Organism(s)	Toxin(s)	Symptoms/effects
Alimentary toxic aleukia (ATA)	Cereal grains, wheat, rye, bread	*F. sporotrichioides, F. poae*	T-2 Toxin	Burning sensation in mouth and throat, vomiting, diarrhea, abdominal pain, bone marrow destruction, hemorrhaging, death
Urov/Kasin- Beck disease	Cereal grains	*F. poae*	Unknown	Osteoarthritis, shortened long bones, deformed joints, muscular weakness
Drunken bread	Cereal grains, wheat, bread	*F. graminearum*	Unknown	Headache, dizziness, tinnitus, trembling, unsteady gait, abdominal pain, nausea, diarrhea
Akakabi-byo	Cereal grains, wheat, barley, noodles	*F. graminearum*	Unknown, possibly deoxynivalenol	Anorexia, nausea, vomiting, headache abdominal pain, diarrhea, chills, giddiness, convulsions
Foodborne illness outbreaks	Cereal grains, wheat, barley, corn, bread	*F. graminearum*	Deoxynivalenol, acetyl-deoxynivalenol, nivalenol, T-2 toxin	Irritation of throat, nausea, headaches, vomiting, abdominal pain, diarrhea
Esophageal cancer	Corn	*F. verticillioides F. graminearum*	Possibly fumonisins, deoxynivalenol, zearalenone and other toxins	Pre-cancerous and cancerous lesions in the esophagus
Neural tube defects	Corn	*F. verticillioides F. proliferatum*	Fumonisins	

17.3.3 Neural tube defects

Fumonisins have been characterized as a possible risk factor for birth defects in human populations that rely heavily on corn as a dietary staple, since studies have indicated that this toxin interferes with the utilization of folic acid, a dietary supplement used to reduce the incidence of neural tube defects (NTD). The first indications of this relationship came from observations of the prevalence of NTD along the Texas–Mexico border, particularly in the years 1990–1991 when the prevalence of NTD among Mexican–American women in this area doubled, going from 15/10,000 live births in a usual year to 27/10,000 live births in this period. This occurred in the same crop years as epizootics were attributed to exposure to fumonisins. Fumonisin levels in corn-based products during the same time period were also relatively elevated, two to three times higher (average of 1.22 ppm in cornmeal samples), compared to samples from other time periods in the same area. The same relationship between incidence of NTD and consumption of fumonisin-contaminated corn and corn products has been observed in other countries, such as rural areas of Guatemala and South Africa (rural Transkei district), as well as northern provinces of China. Besides, studies using biomarkers have suggested that fumonisin exposure, indicated by a postpartum sphinganine:sphingosine ratio in maternal serum and corn tortilla intake, led to increased risk of NTD, proportional to the dose, up to a threshold level at which fetal death may be more likely to occur.

Epidemiological studies indicate that periconceptional supplementation with folic acid can significantly reduce a woman's risk for an NTD-affected pregnancy, even though the mechanisms underlying the protective effects of folic acid are not fully understood. Studies involving fumonisins, folate, and NTD have indicated that the uptake of folate by Caco-2 cells that had been pretreated with fumonisin was reduced by up to 50%. Fumonisin also affected the placental folate transporter by altering both its endocytic trafficking and the amount of the receptor that is available for transport, via depletion of sphingolipids needed for normal receptor function. These studies have supported the hypothesis that exposure to fumonisin might be a risk factor for NTD.

17.4 Detection, isolation and identification of *Fusarium* species

Fusarium species are most often associated with cereal grains, seeds, milled cereal products such as flour and corn meal, barley malt, animal feeds, and necrotic plant tissue. These substrates may also contain or be colonized by many other microorganisms, and *Fusarium* species may be present in low numbers. To isolate *Fusarium* species from these products, it is necessary to use selective media. The basic techniques for detection and isolation of *Fusarium* employ plating techniques, either as plate counts of serial dilutions of products, or the placement of seeds or kernels of grain directly on the surface of agar media in Petri dishes, i.e., direct plating.

Several culture media have been used to detect and isolate *Fusarium* species. These include Nash Snyder (NS) medium, modified Czapek Dox (MCZ) agar, Czapek Iprodione Dichloran (CZID) agar, Potato Dextrose Iprodione Dichloran (PDID) agar, and Dichloran Chloramphenicol Peptone Agar (DCPA). The NS medium and MCZ agar contain pentachloronitro-benzene, a known carcinogen, and are not favored for routine use in food microbiology laboratories. However, these media can be useful for evaluating

samples that are heavily contaminated with bacteria and other fungi. The CZID agar has become a medium regularly used for isolating *Fusarium* from foods, because it is a good selective medium for this genus. Even though some other molds may not be completely inhibited on CZID, most are, and *Fusarium* species can be readily distinguished, but identification of isolates to species level is difficult, if not impossible, on this medium. Isolates must be subcultured on other media such as Carnation Leaf Agar (CLA) for identification. It has been reported that PDID agar is as selective as CZID agar for *Fusarium* species, with the advantage that it supports *Fusarium* growth with morphological and cultural characteristics that are the same as on Potato Dextrose Agar (PDA), which facilitates more rapid identification, since various monographs and manuals for *Fusarium* identification describe characteristics of colonies grown on PDA. Colony development by *Fusarium* on PDID and CZID agars is better than on DCPA where rates are much higher, making colony counts more difficult. DCPA modified with 0.5 µg/mL of crystal violet increases selectivity by inhibiting *Aspergillus* and *Penicillium* species but not *Fusarium* species.

Cultivation for identification is generally done in two agars: PDA for measuring colony growth rates and expression of diagnostic pigments, and CLA for development of microscopic structures. *Fusarium* species do not readily form conidia on all culture media, and conidia formed on high carbohydrate media such as PDA are often more variable and less typical. CLA is low in carbohydrates and rich in other complex naturally-occurring substances that apparently stimulate spore production and is the reason why this medium is used for observation of microscopic structures. Carnation leaves from actively growing, disbudded, young carnation plants free of pesticide residues are cut into small pieces (5 mm^2), dried in an oven at 45–55 °C for 2 h, and sterilized by irradiation. CLA is prepared by placing a few pieces of carnation leaf on the surface of 2.0% water agar. *Fusarium* isolates are then inoculated on the agar and leaf interface where they form abundant and typical conidia and conidiophores in sporodochia rather than mycelia.

Since many *Fusarium* species are plant pathogens and all are found in fields where crops are grown, these molds respond to light. Growth, pigmentation, and spore production are most typical when cultures are grown in alternating light and dark cycles of 12 h each from fluorescent light or diffuse sunlight from a window not exposed to direct sunlight. Fluctuating temperatures such as 25 °C (day) and 20 °C (night) also enhance growth and sporulation.

Historically, the identification of *Fusarium* species has been based largely on the production and morphology of macroconidia and microconidia. Identification keys, such as those proposed by Nelson et al. (1983), Samson et al. (1999), Marasas et al. (1985), and Marasas (1991), rely heavily on the morphology of conidia and conidiophores, the structures on which conidia are produced. However, more recently, the application of biological and phylogenetic species concepts to strain collections has indicated that many of the previously-described species based solely on morphological characters were in need of further division. Therefore, species in some cases are still being recognized based on a morphological species concept, but in others based on their sexual cross-fertility or similarities of DNA sequences. Therefore, the method or system used for the identification usually will depend on how much information about the isolate is needed and how the information will be used. After isolation, the organism can be cultivated and evaluated using morphological, molecular, and cross-fertility criteria, depending upon how far the identification process needs to be taken. Sometimes, a combination of the different characteristics is needed for a definitive identification.

17.5 Detection of *Fusarium* mycotoxins

If present, *Fusarium* toxins are usually found at low levels in cereal grains and processed grain-based foods. Their concentrations may range from less than nanogram to microgram quantities per gram (ppb to ppm, respectively). *Fusarium* toxins vary in their chemical structures and properties, making it difficult to develop a single method for quantitating all toxins. The basic steps involved in detection of *Fusarium* mycotoxins are similar to those for other mycotoxins. These include sampling, size reduction and mixing, sub-sampling, extraction, filtration, clean-up, concentration, separation of components, detection, quantification, and confirmation. Even though deoxynivalenol, T-2 and zearalenone toxins are the subject of another chapter in this book, the detection of these mycotoxins will be briefly discussed here, along with fumonisins.

The first problem encountered in the analysis of grains for *Fusarium* toxins is the same as for other mycotoxins, i.e., sampling. Obtaining a representative sample from a large lot of cereal grain can be very difficult if the toxin is present in a relatively small percentage of the kernels, which may be the case with toxins such as deoxynivalenol and zearalenone. On the other hand, fumonisins appear to be more evenly distributed in grain such as corn. Processed grain-based foods also may contain a more even distribution of toxins due to grinding and mixing. Once samples are collected, they are usually ground and mixed further and a sub-sample of 50 to 100 g is taken for extraction.

Like all mycotoxins, *Fusarium* toxins must be extracted from the matrix in which they are found. Most mycotoxins are more soluble in slightly polar organic solvents than water. The most commonly-used extraction solvents consist of combinations of water with organic solvents such as methanol, acetone, and acetonitrile. Following extraction, the extract is filtered to remove solids, and subjected to a clean-up step to remove interfering substances. Clean-up can be done in several ways, but the most common method used for *Fusarium* toxins is to pass the extract through a column packed with sorbant packing materials or monoclonal antibodies immobilized on Sepharose®. In recent years, the use of small, prepacked commercially-available disposable columns or cartridges such as Sep-Pak®, Bond Elut®, MycoSep®, Star™, FumoniTest, ZearalaTest, and DONTest has become more common. After the extract has been cleaned, the sample may need to be concentrated before analysis in order to detect the toxin. This may be accomplished by mild heating such as in a water bath, heating block, or rotary evaporator under reduced pressure or a stream of nitrogen. Detection and quantification of the toxins are done after they are separated from other components by chromatographic means. The most common chromatographic separation techniques used are thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC was very popular in the 1960s and 1970s, but was largely replaced by HPLC in the 1980s. Gas chromatography (GC) also has some applications, particularly for those trichothecenes that can be derivatized to volatile components. More recently, mass spectrometry has been used along with HPLC and GC to improve the limits of detection and quantitation of analytical methods.

Methods for analysis of multiple trichothecenes have been developed and they usually involve the use of gas chromatography with flame ionization detection or mass spectrometry. Other multitoxin methods have also been developed for the simultaneous detection of trichothecenes type A, B, and zearalenone by the use of HPLC coupled with tandem mass spectrometry.

When compared to other methods available, for deoxynivalenol, the use of gas chromatography provides more sensitive results than TLC, but is also more laborious. HPLC methods with detection by UV absorbance are also fairly sensitive. However, while TLC does not require much sample purification, GC and HPLC require clean-up by either solid phase extraction or immunoaffinity columns. The methods most commonly used for T-2 toxin are GC methods. Because type A trichothecenes, like T-2 toxin, lack a UV chromophore and are not fluorescent, TLC methods are unsuitable, resulting in the reliance on GC methods or HPLC methods with a different detection method, i.e., mass spectrometry. The most widely-used analytical methods for fumonisins are HPLC methods involving the formation of fluorescent derivatives. Methods have been developed using derivatizing agents, such as o-phthaldehyde (OPA), fluorescamine, and naphthalene dicarboxaldehyde. TLC methods for fumonisins have also been developed, but are used mainly for screening. The method of choice for quantitation of zearalenone is HPLC with detection by fluorescence or mass spectrometry, while TLC is still useful as a screening method. Methods for moniliformin include TLC and HPLC.

Immunoassays have been developed for *Fusarium* toxins, with enzyme-linked immunosorbent assay (ELISA) kits and lateral flow tests commercially available for deoxynivalenol, zearalenone, T-2 toxin, and fumonisins. Some of these kits and tests, their manufacturers and their limit of detection are presented in Table 17.2. Their simplicity and ease of use make it possible to handle large numbers of samples in a short amount of time and have made these tests very popular, especially for screening and semi-quantitative analysis. However, because of potential matrix interference, a full validation of ELISA kits for semi-quantitative and quantitative analysis is essential.

17.6 Occurrence and stability of fumonisins in foods

Various *Fusarium* toxins have been found naturally occurring in numerous cereal grains, but most of these grains have been destined for animal feed. However, some *Fusarium* toxins, particularly deoxynivalenol and fumonisins, have been found in processed or finished human food products. Food products that have been examined include corn meal, corn grits, corn breakfast cereals, tortillas, tortilla chips, corn chips, popcorn, and hominy corn. The most consistently contaminated products with the greatest amounts of fumonisins are those foods which receive only physical processing such as milled products, e.g., corn meal and corn muffin mixes. When fumonisin levels in corn meal obtained from Canada, Egypt, Peru, South Africa and the United States were compared, the highest levels of toxin were detected in samples from Egypt and the United States. Egyptian samples averaged 2.4 μg/g, while American samples showed mean concentrations of 1.0 μg/g of toxin in the corn meal. In another survey, the highest levels of fumonisins in corn-based foods were found in corn meal (0.5 to 2.0 μg/g) and corn grits (0.14 to 0.27 μg/g). Corn flakes, corn pops, corn chips, and corn tortilla chips were negative for fumonisins, and very low levels were found in popcorn, sweet corn and hominy (0.01 to 0.08 μg/g). In Italy, research has shown that the highest contamination levels are found in extruded corn (up to 6.1 μg/g of fumonisin B_1), followed by products such as corn grits, corn flour or polenta (levels varying from 0.4 to 3.8 μg/g for fumonisin B_1). Fumonisins have also been detected in processed corn products in Germany, Japan, Spain, and Switzerland.

When cleaning is applied prior to milling, it has been shown to cause fumonisin reductions varying from 26-69% in corn. During the dry milling process the same patterns

Table 17.2 Some Commercially-available ELISA Kits for *Fusarium* toxins

Mycotoxin	**Product name**	**Format**	Range of detection
Neogen Corporation (Lansing, MI)			
Deoxynivalenol	Agri-Screen	Microwell	1.0 ppm
Deoxynivalenol	Reveal SQ	Lateral flow	<0.5 ppm, >0.5 to <1.0 ppm, >1.0 to <2.0 ppm, >2.0 ppm
Deoxynivalenol	Veratox 5/5	Microwell	0.5–5.0 ppm
Deoxynivalenol	Veratox HS	Microwell	25–250 ppb
Fumonisin	Agri-Screen	Microwell	5.0 ppm
Fumonisin 5/10	Veratox	Microwell	0.5–6.0 ppm
Fumonisin	Veratox HS	Microwell	50–600 ppb
Fumonisin	Reveal SQ	Lateral flow	<1.0 ppm, >1.0 to <2.0 ppm, >2.0 to <4.0 ppm, >4.0 ppm
Zearalenone	Veratox	Microwell	25–500 ppb
T-2/HT-2 Toxin	Veratox	Microwell	25–250 ppb
R-Biopharm AG, USA			
Deoxynivalenol	Ridascreen®Fast	Microwell	0.2–6.0 ppm
Deoxynivalenol	Rida®Quick	Lateral flow	1.25 ppm or 0.5 ppm
Fumonisin	Ridascreen®Fast	Microwell	0.2–6.0 ppm
Fumonisin	Rida®Quick	Lateral flow	0.8 ppm or 4.0 ppm
Zearalenone	Ridascreen®Fast	Microwell	17–41 ppb
T-2 Toxin	Ridascreen®Fast	Microwell	20–400 ppb
Vicam (Watertown, MA)			
Deoxynivalenol	DONCheck	Lateral flow	1.0 ppm
Deoxynivalenol	DON-V	Lateral flow	0.2–5.0 ppm
Romer Laboratories (Union, MO)			
Deoxynivalenol	AgraQuant®	Microwell	0.2–5.0 ppm
Deoxynivalenol	AgraStrip®	Lateral flow	0.07–6 ppm
Fumonisin	AgraQuant®	Microwell	0.2–5.0 ppm
Fumonisin	AgraStrip®	Lateral flow	0.17–8.0 ppm
Zearalenone	AgraQuant®	Microwell	10–1000 ppb
T-2 Toxin	AgraQuant®	Microwell	35–500 ppb

observed with the other *Fusarium* mycotoxins are also observed with fumonisin, with the highest amounts of this toxin being found in the fines, bran and germ fractions. When the wet milling was evaluated, fumonisin also followed a redistribution pattern into the fractions of this process, with toxin being recovered from the steep water, fiber, gluten, and germ fractions, and no detectable amounts in the starch.

The effect of thermal processes on the stability of fumonisins have also been evaluated, and studies have shown fumonisin reductions of 9–15% after canning cream corn and whole kernel corn, no reductions after pressure cooking corn meal during polenta preparation, and 48% reduction after baking corn bread at 232 °C for 20 min. However, when corn muffins were evaluated, results have shown either no reduction after baking at 204 °C for 20 min or 16 and 28% reductions at 175 and 200 °C, respectively. During the process of production of corn flakes reductions of 60–70% have been reported for fumonisins, and when glucose was added to the process up to 89% reduction was observed. Studies have also indicated that corn nixtamalization reduces the fumonisin content of the final product by 99% when 1.2% lime and cooking at 100 °C for 1 h are applied.

Research on the effect of extrusion in corn grits contaminated with fumonisin has showed that greater reductions are achieved with increasing temperature and residence time when double screw extruders are used (reductions varying from 35–95%). Also, greater reductions are observed in the presence of glucose, when compared to the same extrusion conditions in the absence of this reducing sugar: 93% in the presence versus 65% in the absence of glucose at 160 °C and 40 rpm. The efficacy of extrusion in reducing the toxicity of fumonisins has also been evaluated. After extrusion of contaminated corn grits, in the presence and absence of glucose, using either single or double screw extruders, the toxicity of the final products indicated that corn grits extruded with glucose showed reduced toxicity from fumonisins when compared with unextruded or extruded corn grits in the absence of glucose, as indicated by the severity of kidney lesions and kidney weight in male Sprague-Dawley rats.

17.7 Prevention and control

The FDA/USDA Working Group advises that fumonisin levels should be less than 4 μg/g in products for human consumption, less than 5 μg/g in horse feed, less than 20 μg/g for swine feed and less than 30 μg/g for cattle feed. No other advisory or action levels have been established in the United States for fumonisins.

Preventing contamination of cereal grains with *Fusarium* species and fumonisins is very difficult because contamination occurs in the field and is subject to weather, drought and insect activity. The main preventative measure is to divert contaminated grain from being processed into human foods. As mentioned earlier, some high temperature processing systems, such as extrusion cooking, may offer some potential for reducing levels of fumonisins in processed finished foods.

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18 Other moulds and mycotoxins

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18.1 Introduction

Mycotoxins are secondary metabolites produced by certain filamentous fungi that cause a toxic effect in animals and humans. Most of the known mycotoxins are produced by some species of the fungal genera *Aspergillus*, *Penicillium* and *Fusarium*. A certain strain can produce different mycotoxins, and a particular mycotoxin may be produced by different species of moulds. If the conditions are favorable, moulds can develop and produce mycotoxins in growing crops for human and animal consumption, and also during harvest, drying and storage steps. Environmental conditions (chemical, physical, and biological parameters) affect fungal growth and mycotoxin production and, therefore, it is expected to find a heterogeneous distribution of mycotoxins in foodstuffs from year to year and according to the production process they undergo. The presence of mycotoxins in food commodities is of concern for trade, as they may lead to important economic losses.

Exposure of humans to mycotoxins occurs mainly by the ingestion of contaminated foodstuffs from vegetal or animal origin. Contamination in the last case takes place when animals are fed with contaminated feed. An alternative source of exposure is the inhalation of contaminated dusts. The diseases caused by mycotoxins in humans and animals are called mycotoxicoses. While most animal mycotoxicoses have been experimentally confirmed, human mycotoxicoses are less well understood and not as clearly defined. The toxicity of a certain mycotoxin in an organism can be classified according to the exposure dose in acute or chronic conditions. Acute toxicity refers to the ability of the compound to cause adverse effects within a short time of exposure, especially to high doses. Chronic toxicity stands for the effects of a prolonged exposure to small quantities of toxin. This last case is of special concern for human health, considering the fact that several foods of frequent consumption are susceptible to be contaminated by one or more mycotoxins.

Several mycotoxins have been identified up to the present, but those of special interest in food and feed safety are: aflatoxins (B_1, B_2, G_1, G_2, M_1); fumonisins (B_1, B_2); ochratoxin A; patulin; trichothecenes (deoxynivalenol, T-2 toxin, HT-2 toxin); and zearalenone.

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Table 18.1 Ochratoxin A, Patulin, Deoxynivalenol, T-2 Toxin and Zearalenone Producing Fungi

Genus	Mycotoxins and producing fungi
Aspergillus	**Ochratoxin A**: section *Aspergillus* (*A. glaucus*, *A. repens*, *A. sydowii*), section *Flavi* (*A. albertensis*, *A. alliaceus*), section *Circumdati* (*A. auricomus*, *A. melleus*, *A. muricatus*, *A. ochraceus*, *A. ostianus*, *A. petrakii*, *A. sulphureus*, *A. westerdijkiae*), section *Clavati* (*A. clavatus*), section *Cremei* (*A. wentii*), section *Fumigati* (*A. fumigatus*), section *Nigri* (*A. awarori*, *A. carbonarius*, *A. foetidus*, *A. japonicus*, *A. lacticoffeatus*, *A. niger*, *A. sclerotioniger*, *A. tubingensis*, *A. usamii*, *A. vadensis*), section *Terrei* (*A. terreus*), section *Usti* (*A. ustus*) and section *Versicolores* (*A. versicolor*). **Patulin**: *section Clavati* (*A.clavatus*, *A. giganteus*, *A. longivesica*).
Penicillium	**Ochratoxin A**: subgenus *Penicillium*, section *Viridicata* (*P. nordicum*, *P. verrucosum*). **Patulin**: subgenus *Penicillium*, section *Penicillium (P. caprobium*, *P. clavigerum*, *P. concentricum*, *P. dipodomyicola*, *P. expansum*, *P. formosanum*, *P. gladioli*, *P. glandicola*, *P. griseofulvum*, *P. marinum*, *P. sclerotigenum*, *P. vulpinum*) and section *Roqueforti* (*P. carneum*, *P. paneum*).
Fusarium	**Deoxynivalenol**: section *Discolor* (*F. culmorum*, *F. graminearum*). **T-2 toxin**: section *Discolor* (*F. sambucinum*), section *Elegans* (*F. oxysporum*), section *Gibbosum* (*F. acuminatum*, *F. armeniacum*), section *Martiella* (*F. solani*), and section *Sporotrichiella* (*F. langsethiae*, *F. poae*, *F. sporotrichioides*). **Zearalenone**: section *Arthrosporiella (F. semitectum)*, section *Discolor (F. cerealis*, *F. crookwellense*, *F. culmorum*, *F. graminearum)*, section *Gibbosum (F. equiseti)*, and section *Liseola (F. verticillioides*).
Others	**Ochratoxin A**: *Neopetromyces muricatus*, *Petromyces alliaceus*. **Patulin**: *Paecylomyces saturatus*, *Byssochlamys nivea*.

International agencies have studied the problem of mycotoxins in food in order to set guidelines regarding the limits of contamination in food and the tolerable intakes of the toxins. Thus, the International Agency for Research on Cancer (IARC) has investigated the carcinogenic potential of most of these toxins, and the Codex Alimentarius Commission has set the maximum levels in foodstuffs for aflatoxins, ochratoxin A, and patulin. Other countries have set the maximum levels in foodstuffs for an important number of mycotoxins in such products. Opinions regarding tolerable intakes of mycotoxins have been published by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Agency (EFSA), as values of Provisional Tolerable Weekly Intake (PTWI) or Provisional Tolerable Daily Intake (PTDI).

In this chapter, we focus on the mycotoxins ochratoxin A, patulin, zearalenone, and some important trichothecenes, like deoxynivalenol, and T-2 toxin. Ochratoxin A and patulin are mainly produced by species of *Aspergillus* and *Penicillium*, while zearalenone and the trichothecenes are mainly produced by species of *Fusarium* (Table 18.1). Other important mycotoxins such as aflatoxins and fumonisins, have been the subject of other chapters of this book.

18.2 Ochratoxin A

18.2.1 Introduction

Ochratoxins were discovered in 1965 by van der Merwe et al. as mycotoxins produced by *Aspergillus ochraceus* Wilhelm. Except for ochratoxin α, the ochratoxins comprise a polyketide-derived dihydroisocoumarin moiety linked via the 7-carboxy group to

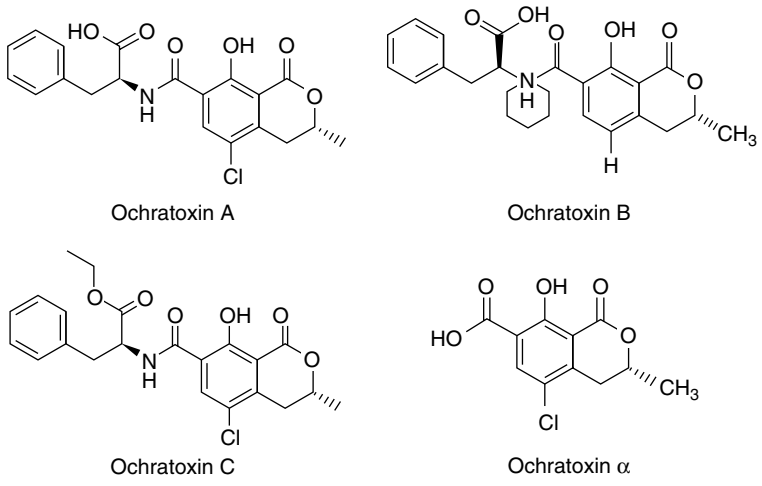


Figure 18.1 Chemical structure of main ochratoxins.

L-β- phenylalanine by an amide bond. Ochratoxins mainly consist of ochratoxin A (OTA), its methyl ester, its ethyl ester (also known as ochratoxin C), the 4-hydroxyochratoxin A, the ochratoxin B and its methyl and ethyl esters, the ochratoxin α (OTα), where the phenylalanine moiety is missing, and the OTα dechloro analog, the ochratoxin β (Fig. 18.1).

OTA, the most toxic and frequent of the ochratoxins, is a weak, organic acid, very stable, colourless crystalline compound with an intense green fluorescence under UV light in acid medium and blue fluorescence in alkaline conditions. OTA is structurally similar to the amino acid phenylalanine. The particularity of OTA is due to its high stability. It has been shown that it possesses a resistance to acidity and high temperatures. Thus, once foodstuffs are contaminated, it is very difficult to totally remove this molecule.

18.2.2 Producer fungi and ecophysiology

OTA is produced by some species of fungi belonging to different sections of the genus *Aspergillus*, such as the *Aspergillus Flavi*, *Circumdati*, *Clavati*, *Cremei*, *Fumigati*, *Nigri*, *Terrei*, *Usti* and *Versicolores* sections (Table 18.1). Among them *A. carbonarius*, *A. niger*, *A. ochraceus*, *A. steynii*, *A. tubingensis* and *A. westerdijkiae* are specially important. OTA is also produced by species of the genus *Penicillium* (*P. nordicum*, *P. verrucosum*), *Petromyces* (*P. alliaceus*) and *Neopetromyces* (*N. muricatus*). *P. verrucosum* is the major OTA-producing fungus in northern Europe, while *A. ochraceus* is more important in warmer climatic zones.

Temperature and water activity (a_w) are the key environmental factors that affect both the rate of fungal spoilage and the production of mycotoxins, although other parameters like light, substrate aeration, inoculum concentration, microbial interactions, mechanical damage, insect infestation and presence of preservatives are other influences. In general, the ochratoxigenic species are not aggressive pathogens, but are often well adapted to substrates with low a_w, and they can easily colonize grapes, cereal grains or oilseeds stored under inappropriate conditions.

Scientific studies have focused mainly on the influence of environmental factors on fungal growth and OTA production by *P. verrucosum* and *A. ochraceus*, since they are recognized as the main producer species. However, more recently, many scientists worldwide have studied the influence of a_w and temperature on growth and biosynthesis of OTA by other ochratoxigenic

species belonging to *Aspergillus* section *Nigri* (black aspergilli) such as the *A. niger* aggregate and *A. carbonarius*, mainly as they were related to the presence of OTA in grapes, musts and wines. On the other hand, although *A. westerdijkiae* and other related species belonging to section *Circumdati* have been found only sporadically in a wide range of stored commodities, including cereals, they could be the cause of significant concentrations of OTA.

Many studies have confirmed the impact of abiotic factors on growth of *A. ochraceus* and *P. verrucosum* on different culture media or food matrices. For instance, minimum a_w level required for growth of OTA-producing *A. ochraceus* strains ranged from 0.77 to 0.85 a_w on synthetic agar media, whereas growth was found at a minimum a_w level of 0.80–0.85 a_w on cereal grains or green coffee beans. With regard to black aspergilli, minimum a_w for growth reported in most studies ranged between 0.90 and 0.93 a_w. Interestingly, the literature shows that under most combinations of temperature and a_w tested, *A. niger* aggregate grew more rapidly than *A. carbonarius*. The reproducibility of these optima for growth indicates that *A. niger* aggregate has a higher optimum temperature for growth than *A. carbonarius* and possibly also takes greater advantage of high water activities for rapid growth. On the other hand, *P. verrucosum* has been classified as a xerophilic fungi capable of growing at 0.80 a_w, although there is a strong influence of the type of substrate on minimum a_w level for fungal growth and, possibly, on the amount of toxic metabolites produced.

In general, temperature and a_W range for OTA production is narrower than that for fungal growth, both in culture media and food. It has been observed that high levels of a_w (0.960 and 0.995) favored OTA production by *A. carbonarius* and *A. niger* aggregate strains. On the other hand, it has been reported that significant levels of OTA could be produced at 4 °C by *P. verrucosum* and at a_w levels as low as 0.86, although the highest production of OTA by this fungus occurred between 0.93 and 0.98 a_w at 10–25 °C. It has been also described that alternating temperature cycles (20/30, 20/37 and 25/42 °C) and photoperiod have an effect on growth and OTA production of *A. carbonarius* on synthetic nutrient medium. The different temperature regimes assayed affected significantly both the mycelial growth and the OTA production, with the best growth and OTA production being the 20/30 °C cycle.

18.2.3 Molecular tools for detection of ochratoxigenic fungi

Molecular studies are now providing useful data, which help clarify the identification and taxonomy of ochratoxigenic fungi. Such studies involve RFLPs of both nuclear and mitochondrial DNA, PCR-based techniques and phylogenetic analysis. However, to date, there is limited information about the genes involved in the OTA biosynthesis, but it is well known that a polyketide synthase (PKS) is required.

Among the ochratoxigenic fungi, different PKS genes involved in OTA biosynthesis have been identified in *A. ochraceus*, *A. westerdijkiae*, *A. carbonarius*, *P. nordicum* and *P. verrucosum*. Some of these genes have been used as a target to develop accurate methods which allow a rapid, sensitive and specific identification and quantitation of OTA-producing strains. Recently, the whole genome of an *A. niger* strain has been sequenced and several secondary metabolite gene clusters have been described.

18.2.4 Occurrence of ochratoxin A in foods and feeds

Worldwide, natural occurrence of OTA has been reported in a large variety of foods. OTA contamination has been detected in cereal grains (barley, wheat, maize and oats), green coffee beans, peanuts, sorghum, olives, dried fruits, spices, wine and grape juice, beer,

Figure 18.2 Fungal spoilage of fruits. A bunch of grapes contaminated by ochratoxigenic black aspergilli (left) and a patulin producer strain of *Penicillium expansum* contaminating an apple (right).

liquorice, mixed feeds, cheese, poultry and pig meats. Cereals normally account for 50–80% of the average consumer intake of OTA, followed by wine (approx. 15%) and coffee (approx. 12%).

Natural occurrence of OTA in maize and maize-based products is a worldwide problem. Maize kernels are a good substrate for mould infection and production of mycotoxins. *A. niger* is frequently isolated from maize and a high incidence of *A. carbonarius* has been also reported. Both species could be a source of OTA in maize and other food products in both tropical and subtropical zones of the world.

In grapes, *Aspergillus* spp. belonging to *A.* section *Nigri* develop particularly in damaged grapes during ripening, although they may occur and form OTA on grapes from veraison to harvest (Fig. 18.2). OTA concentrations tend to increase with grape maturity. Berry damage is the primary factor affecting the disease development and OTA accumulation in berries. The damage may be due to birds, insects, infection by other fungi, or rain. OTA is much more commonly detected in dessert wines, followed in decreasing order of contamination by red, rosé and white wines.

OTA has been extensively documented as a contaminant of green coffee beans, in levels ranging from 0.1 to 80 µg/kg, while levels of OTA in roasted coffee have ranged from 0.05 to 23.5 µg/kg. Most remarkably, OTA has been detected in the final coffee brew prepared by common methods.

18.2.5 Absorption, distribution, excretion and biotransformation

After oral ingestion, OTA is rapidly absorbed and reaches the systemic circulation, where it is extensively bound to serum albumin and other macromolecules. The unbound fraction is as low as 0.02% in humans. In many species, including monkeys and humans, the major route of excretion is renal elimination, whereas in rodents biliary excretion seems to prevail. Both biliary excretion and glomerular filtration play important roles in the plasma clearance of OTA. Biliary excretion and entero-hepatic recirculation of OTA-glucuronides may account for the interindividual and interspecies variability of kinetic parameters observed in various kinetic studies.

It has been suggested that, in most species, OTA is absorbed from the stomach as a result of its acidic properties (pK_a: 7.1), but it has been demonstrated that the small intestine is the major site of absorption, with maximal absorption in the proximal jejunum.

Once OTA has been absorbed, the concentrations of the toxin and its metabolites in tissues and plasma depend on the length of feeding, the dose, whether the OTA is naturally occurring or crystalline, the route, the degree of serum binding, the half-life of OTA, and the duration of an OTA-free diet. These factors are important in assessing the natural occurrence of residues in animal tissues. It has been demonstrated that OTA accumulates in organs such as kidneys, liver, muscle, lung, heart, intestine, testicles, spleen, brain, skin, and also in the fat, in different proportions, according to the dose and frequency of administration.

After a single oral dose, the maximum serum concentrations of OTA occurred within 10–48 h in pigs and rats, at 2–4 h in ruminant calves, after 1 h in rabbits, and after 0.33 h in chickens. Maximum concentrations in tissues were found within 48 h in rats.

OTA is hydroxylated in the liver only to a minor extent, resulting in the epimers 4-R- and 4-S-hydroxyochratoxin A. In some animal species also 10-OH-OTA production has been described. Several data indicate the formation of pentose and hexose conjugates of OTA, which could also be detected in the urine of OTA-treated animals. In addition to hepatic metabolism, OTA is hydrolysed to the non-toxic OTα at various sites. In rats, detoxication by hydrolysis to OTα is a function of the bacterial microbiota of the caecum. The enzymes responsible for hydrolysis to OTα in cows and rodents are carboxypeptidase A and chymotrypsin. Studies with rat tissue homogenate showed that the duodenum, ileum, and pancreas also have a high capacity to carry out this reaction, whereas the activity in the liver and kidney was low, and it was non-existent in rat hepatocytes and rabbit and rat liver. Incubation of the contents of the four stomachs of cows indicated effective hydrolysis of OTA to OTα by the ruminant protozoa, whereas sheep also have a good capacity to detoxify OTA before it reaches the blood.

18.2.6 Toxicity of OTA in man and animals

Kidneys are probably the main target organ for OTA. Renal lesions are characterized by karyomegaly and necrosis of tubular cells and thickening of the tubular basement membranes. The observed effects are dose- and time-dependent. There is increasing evidence that renal toxicity caused by OTA is associated with cellular oxidative stress.

Besides nephrotoxicity, OTA has immunosuppressive properties resulting in higher susceptibility to infections, and it is teratogenic and carcinogenic (Group 2B IARC). Furthermore, OTA interferes with blood coagulation and carbohydrate metabolism and inhibits protein synthesis by competing with phenylalanine in the phenylalanyl-tRNA synthase-catalyzed reaction.

The acute toxicity of OTA is relatively low, although large species differences in sensitivity are seen with oral LD_{50} values ranging widely in different species. Oral LD_{50} values have reported 0.2 mg/kg bw in dogs, 1 mg/kg bw in pigs, 3.3 mg/kg bw in chicken, and 46–58 mg/kg bw in mouse. Dogs and pigs have been reported to be the most sensitive species. Effects of acute poisoning such as multifocal haemorrhages in various organs and fibrin thrombi in the spleen, brain, liver, kidney and heart have been reported following single dose administration. Nephrosis, hepatic and lymphoid necrosis, and enteritis with villous atrophy have also been observed in the test species. At present, there are no documented cases of acute toxicity reported in humans.

The subchronic and chronic effects of OTA are of greatest concern. OTA has been shown to be nephrotoxic, hepatotoxic, genotoxic, teratogenic and immunotoxic to several species

of animals and carcinogenic in mice and rats, causing tumours of the kidney and liver. OTA also exerts toxic effects on the reproductive system.

Studies on the genotoxicity of OTA remain controversial, as conventional mutagenicity tests gave negative results, whereas results obtained with ^{32}P-postlabelling techniques suggested the existence of DNA adducts. Gene mutations or sister chromatid exchange have been demonstrated in some studies in modified test systems using subcellular fractions and primary cells as activating systems, while others failed to demonstrate these effects. Increasing evidence suggests that the various genetics effects seen in in vitro and in vivo studies are compatible with the hypothesis of DNA damage induced by oxidative stress rather than indicating a direct interaction (adduct formation) of OTA with cellular DNA. Furthermore, chemical analysis failed to identify specific DNA adducts of OTA or its metabolites.

18.2.7 Human exposure assessment

OTA has a half-life on plasma of about 35 days in humans and, due to its binding to plasma proteins, its enterohepatic circulation, and its renal reabsorption, the blood concentration of OTA is considered to represent a convenient biomarker of exposure during the previous weeks. Human exposure, as demonstrated by the occurrence of OTA in blood, urine and in human milk, has been observed in various countries in Europe.

A highly significant relationship has been observed between Balkan endemic nephropathy (BEN) and tumours of the urinary tract, particularly with tumours of the renal pelvis and ureters. Similarly, in Tunisia and Egypt, a chronic interstitial nephropathy of unknown etiology (CINI), that shows marked similarities with BEN, has been described. CINI is slowly progressive, insidious, non-inflammatory, and it is becoming a leading cause of renal failure and death in the fourth or the fifth decade of life. Studies performed so far have shown high rates of blood OTA contamination among nephropathy patients.

To characterize the mycotoxin hazard in humans a 'safe dose', such as the provisional tolerable daily intake (PTDI) is estimated. Several international organizations have established different PTDI levels for OTA and, in some cases, provisional tolerable weekly intakes (PTWI). So The Nordic Working Group on Food Toxicology and Risk Evaluation established in 1991 a PTDI of 5 ng OTA/kg bw/day, values confirmed by the Scientific Committee on Food of the European Union in 1998. However, in 2006 the EFSA established a PTWI of 120 ng OTA/kg bw/week (that is, approximately a PTDI of 17 ng OTA/kg bw/ day), whereas the JEFCA reduced this value to a PTWI of 100 ng OTA/kg bw/week (approx. 14 ng OTA/kg bw/day).

Nevertheless, studies developed in different European countries have shown that the mean dietary intakes rarely exceed the 5 ng OTA/kg bw/day level. The European Commission estimated the mean dietary intake based on plasma concentration of OTA from six countries (Germany, Italy, Norway, Spain and the UK) and the resulting value was 0.67 ng/kg bw/day, far below the proposed PTDI. On the other hand, in Spain OTA daily intakes were estimated based on OTA plasma concentrations, and on food consumption and contamination data, obtaining mean values between 1.69 and 1.96 ng/kg bw/day, respectively.

18.2.8 Effect of food processing and detoxification

OTA is a moderately heat stable molecule that can survive most food processing operations, and therefore it appears in final and derived products. The main foods for which the fate of OTA has been studied are cereals, coffee and, to a lesser extent, wine.

In a general way, it can be stated that OTA is relatively stable once formed, but under certain conditions of high temperature, acidic or alkaline conditions or in the presence of enzymes degradation can occur. For example, this molecule can resist three hours of high pressure steam sterilization at 121 °C, and even at 250 °C its destruction is not complete. Gamma irradiation (up to 7.5 Mrad) of OTA in ethanol does not cause any degradation.

In cereal grains, OTA levels can be reduced initially by cleaning to remove dust and broken grains. However, the reduction is small and probably depends on the condition of the grain when received. Removal of the surface layers by abrasive scouring or polishing and milling to remove outer layers for white flour production lowers OTA levels, since the mycotoxin tends to be concentrated in the outer bran layers of cereals, which, on the other hand, are destined for animal feed.

Although OTA is relatively heat stable, the thermal processes can exert different effects, depending on the temperature reached. For example, OTA is usually stable during bread baking, with no loss or reduction of its concentration. However, baking of biscuits resulted in about two-thirds of the toxin being destroyed or immobilized. The higher diminishing of OTA content in biscuits can be explained by the higher temperature reached compared to bread, and for the lower water content.

During roasting of coffee, degradation of OTA occurs only to some extent. Concentrations over 20 µg/kg have been reported in commercial roasted coffee, and several reports concerning the roasting impact on OTA content in coffee beans have shown a large range of OTA reduction levels from 0–12% to 90–100%. Such variability can be related to the different analytical conditions, the roasting process or the heterogeneity in toxin distribution.

With regard to OTA contamination in wines, it has been described that red wines have a higher concentration of OTA than white wines, mainly due to the maceration process with the OTA-contaminated berry skins. On the other hand, the increasing alcohol concentration in must, during the early stage of alcoholic fermentation, may facilitate OTA extraction in the must. However, the further steps in the winemaking process result in reduction of the overall toxin concentration. Wine clarification also has a reducing effect on OTA content, since it is adsorbed in the suspended solids and later removed. Racking is a major factor for OTA reduction in the winemaking process. Fining agents in wine making may contribute to OTA reduction, depending on the agent and its concentration added to the wine. Storage of wine bottles and ageing are considered stages that reduce final OTA concentration.

In beer production, significant OTA reductions (40–89 %) have been detected in the grist during mashing, most probably due to proteolytic degradation. Another 16% can be eliminated with the spent grains. Regarding fermentation, OTA losses vary in the range 2–69%.

18.2.9 Legislation

The European Union has established several regulations for OTA in different kinds of foodstuffs. The levels vary from 0.5 µg/kg in infant foods, 2 µg/kg in wine and grape juice, 3–5 µg/kg in cereals, 5–10 µg/kg in coffee, 10 µg/kg in dried vine fruits, 15–30 µg/kg in spices, 20 µg/kg in liquorice root (ingredient for herbal infusion) to 80 µg/kg in liquorice extract (for use in food, in particular beverages and confectionary). With regard to raw materials for animal consumption and feedstuffs, the EU recommends not to exceed values between 25 to 250 µg/kg, depending on the destination of the feed.

The EU has not set any limit for contamination of animal products. However, some countries have appropriate regulation about this subject. In Romania, the maximum allowed level

for meat is 5 µg/kg. The Danish Veterinary and Food Administration set the guidelines for the control of OTA in pigs. Organs condemnation is enforced for OTA levels in kidney between 10 and 25 µg/kg, and entire carcase condemnation for levels above 25 µg/kg. In Italy, the maximum admissible value for OTA in pork meat and derived products is 1 µg/kg. On the other hand, Canada has set maximum recommended levels for OTA in feedstuffs for pigs (0.2–2 mg/kg) and poultry (2 mg/kg).

18.3 Patulin

18.3.1 Introduction

Patulin was first isolated by Birkinshaw et al. in 1943 from *Penicillium griseofulvum* and *P. expansum*. After the discovery of penicillin by Fleming and the emergence of strains resistant to this antibiotic, screening studies were conducted to find new substances of fungal origin that had antibiotic properties. Preliminary results of this compound were very satisfactory because of its antibiotic properties against Gram-positive and Gram-negative bacteria, but clinical studies showed that it was toxic to humans and animals, and therefore this compound was added to the list of mycotoxins.

Chemically, patulin (4-hydroxy-4H-furo[3,2c]pirano-2(6H)-one) is relatively noncomplex in nature. It contains a five-membered cyclic lactone like penicillic acid and ascladiol (Fig. 18.3). This mycotoxin is soluble in water, alcohols, acetone, ethylacetate and chloroform. It is somewhat unstable in polar solvents such as water and methanol and loses its biological potency in alkaline conditions.

18.3.2 Producer fungi and ecophysiology

Patulin is produced by several species belonging to *Penicillium*, *Aspergillus*, *Paecilomyces* and *Byssochlamys* genera. Among the *Aspergillus* species, the number of patulin-producing species is limited to three of the *Clavati* section: *Aspergillus clavatus*, *A. giganteus* and *A. longivesica* (Table 18.1).

For the *Penicillium* genus, a recent overview determined 14 patulin species: *P. carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. formosanum, P. glandicola*, *P. gladioli*, *P. griseofulvum*, *P. marinum*, *P. paneum*, *P. sclerotigenum*, *P. vulpinum*. Only six species (*P. expansum*, *P. griseofulvum*, *P. dipodomyicola*, *P. carneum*, *P. paneum*, and *P. sclerotigenum*) have been found in food. Species associated with food products are of interest because they represent a food safety concern. The rest of the *Penicillium* species are associated with plants and soil.

With regard to *Paecylomyces* and *Byssochlamys*, *B. nivea* and some strains of *P. saturatus* produce patulin.

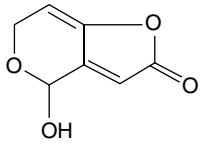


Figure 18.3 Chemical structure of patulin.

Among the patulin-producing species, *P. expansum*, causing blue mould, is one of the most common foodborne fungi on pome fruits (apples and pears), and it causes between 70% and 80% of decay in stored fruit. This species has the peculiarity that the percentage of strains capable of producing patulin is very high, in some cases almost 100% of the isolates tested, and shows a high variability in patulin production among isolates. When apples invaded by *P. expansum* are used to elaborate apple products, these products will be likely contaminated with patulin. In addition, this species can produce patulin in other fruits like pears, apricots, kiwis, plums and peaches.

Given that *P. expansun* is a spoilage agent in high water activity fruits, temperature is the key environmental factor that affects both the rate of fungal spoilage and the production of patulin, although other parameters like inoculum concentrations, microbial intractions, mechanical damage, presence of preservatives and atmosphere storage may influence.

P. expansum is a psychrophilic fungus that grows well at 0 °C and even can grow at –2/–3 °C. Regarding patulin production, the optimum temperature is 25 °C, although it can produce this mycotoxin at 4 °C. Thus, apple storage at room temperature (deck storage) may lead to strong patulin accumulation in apples in short periods before processing begins.

18.3.3 Molecular tools for detection of patulin producer fungi

The molecular genetics of patulin biosynthesis are not fully understood, unlike other regulated mycotoxins (aflatoxins, trichothecenes and fumonisins), although the chemical structures of patulin precursors are now known. The biosynthetic pathway consists of approximately 10 steps, as suggested by biochemical studies. A cluster of 15 genes involved in patulin biosynthesis was reported in 2009.

The genes involved in patulin biosynthesis were used to develop PCR primers. Two real-time (RTi) PCR protocols were developed for identification of patulin producer strains, both based on the TaqMan and SYBR Green techniques. So, a primer pair F-idhtrb/R-idhtrb and the probe IDHprobe were designed from the isoepoxydon dehydrogenase (idh) gene. The functionality of the developed method was demonstrated by the high linear relationship of the standard curves constructed with the idh gene copy number and Ct values for the different patulin producers tested. The ability of the developed SYBR Green and TaqMan assays to quantify patulin producers in artificially inoculated food samples was successful, with a minimum threshold of 10 conidia g^{-1} per reaction. The developed methods quantified with high efficiency fungal load in foods and could be considered as an appropriate tool to detect patulin-producing moulds in food products.

18.3.4 Occurrence of patulin in foods

Although this mycotoxin can be detected in several raw materials within the food industry, apples and their respective by-products are of greatest concern regarding patulin accumulation (Fig. 18.2). Whilst a variety of other food sources and products have demonstrated patulin and/or contamination with patulin-producing fungi, the frequency of these events is much less than that of the apple industry.

Patulin levels in apple juice are generally below 50 µg/l. However, apple juice can occasionally be heavily contaminated, and constant efforts are therefore needed to minimize exposure to this mycotoxin by avoiding the use of rotten or mouldy fruit.

18.3.5 Absorption, distribution, excretion and biotransformation

After oral ingestion, patulin is absorbed at a moderate rate. In a pharmacokinetic study carried out in rats, in seven days, the toxin was recovered in faeces and urine in 49% and 36% respectively. Most of the excretion occurred within the first 24 hours. Approximately 1–2 % of the dose was recovered as $^{14}CO_2$. After the seven days, 2–3% of the dose was recovered in soft tissues and blood. The major retention sites of patulin were erythrocytes and blood-rich organs (spleen, kidney, lung and liver).

18.3.6 Toxicity of patulin in man and animals

Patulin has a strong affinity for sulfhydryl groups. This affinity explains its inhibition of many enzymes. It has been demonstrated in animals that organs affected by patulin intake include kidney, liver and intestine. Acute symptoms of patulin consumption can include agitation, in some cases convulsions, dyspnoea, pulmonary congestion, edema, hyperemia, ulceration, intestinal inflammation and vomiting. When the toxin is ingested through contaminated food, the intestine is the first organ in contact, and the intestinal epithelia cells are targets for this mycotoxin. Chronic health effects of patulin include genotoxicity, immunotoxicity, and neurotoxicity in rodents, whilst its effects on humans are not clear yet. The IARC concluded that there was inadequate evidence for carcinogenicity of patulin in experimental animals. No evaluation could be made of carcinogenicity of patulin in humans.

18.3.7 Human exposure assessment

Joint JECFA has established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 µg/kg bw/day. The studies carried out in different countries like Spain, France and Sweden resulted in exposure ranges of patulin daily intake far from this value, with negligible differences between adults and infants. Highest intake levels were estimated in a French total diet study with an average patulin intake of 0.018 µg/kg bw/day for adults and 0.03 µg/kg bw/day for children aged 3–14 years. A Swedish study showed an intake for mean consumers of 0.008 µg/kg bw/day for children and 0.004 µg/kg bw/day for adults. A Spanish study showed an intake for mean consumers of 0.008 µg/kg bw/day for children and 0.005 µg/kg bw/day for adults.

18.3.8 Effect of food processing and detoxification

The *P. expansum* spores are ubiquitous soil inhabitants. These spores can contaminate apples in pre-harvest and may be transmitted post-harvest to infection courts via water handling systems. Once contaminated, the fruit can be infected through wounds. A fruit infected by *P. expansum* is highly likely to contain patulin. Thus, several post-harvest treatments are applied to minimize the growth and patulin production. Cold temperature storage, controlled atmosphere and fungicide treatments in post-harvest are the most used. The combination of controlled atmosphere, cold and biocontrol agents may be an alternative method that reduces the use of fungicides and prevents the emergence of resistant strains. It is also more environmentally friendly.

No patulin was detected in healthy apple tissue but it was high in the decayed area. Since patulin production is associated primarily with infected rotten tissue, patulin control is

possible in the juice industry by using healthy fruits, sorting damaged and rotten fruits before processing. In addition, the waiting time before fruits are processed is critical to reduce the patulin content in the juice. Washing fruit or removing mouldy tissue immediately prior to pressing will not necessarily remove all the patulin present in the fruit since some may have diffused into apparently healthy tissue. Once in the juice, the toxin is relatively temperature stable at the low pH of the juice. So, short-term treatments at high temperature (150 °C) produce an approximately 20% reduction in patulin concentration.

Fermented products like cider will not contain patulin because the alcoholic fermentation of contaminated juices destroys the toxin. Ascorbic acid has been reported to cause the disappearance of patulin from apple juice.

18.3.9 Legislation

Since 1995, many countries have regulated patulin, mostly in fruit products such as apple juice. The vast majority of countries with regulations or guideline levels for patulin in food have set these at the same level (50 µg/kg). European regulation 1881/2006 sets a maximum level of 50 µg/kg for fruit juices and derived products, 25 µg/kg for solid apple products and 10 µg/kg for juices and foods destined for babies and young infants. The U.S. Food and Drug Administration (FDA) has established 50 µg/kg as the action level for patulin in apple juice, apple juice concentrates and apple juice products.

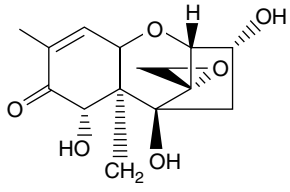
18.4 Trichothecenes and zearalenone

18.4.1 Introduction

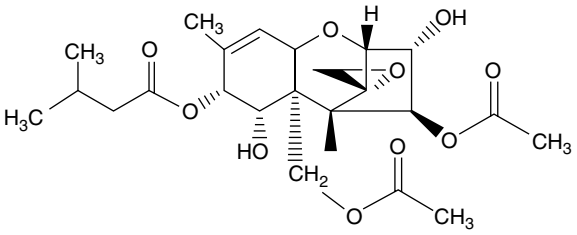
The *Fusarium* spp. produce several mycotoxins which pose a health risk to humans and animals through food and feed prepared from contaminated crops. Since the discovery of *Fusarium* anamorphic species by Link in 1809, there has been a growing interest in *Fusarium* species, a number of cosmopolitan necrotrophic pathogens of cereals, particularly important in agricultural practice on all continents. *Fusarium* head blight (FHB) is considered at the moment as the most important wheat disease in numerous countries, because of both the significant reduction in yield and grain quality and its direct relationship with the grain contamination with mycotoxins, mostly deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA).

The mycotoxins produced by the *Fusarium* spp. include the trichothecenes, ZEA, moniliformin, fumonisins and the enniatins. Trichothecenes constitute the largest group of mycotoxins produced by *Fusarium* spp. in cereal grain. These can be divided into four groups: type A–D. The type B trichothecenes are the most common trichothecenes and include DON and the closely-related NIV. Type A trichothecenes includes T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (DAS), and are considered to be more toxic to humans and animals than type B trichothecenes.

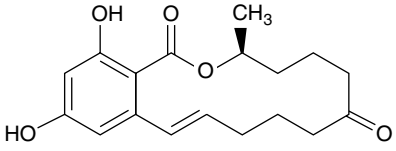
The trichothecenes are all nonvolatile, low-molecular-weight sesquiterpene epoxides. Trichothecenes have a tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring in common, and the 12,13-epoxy ring is responsible for the toxicological activity. On the basis of the absence or presence of characteristic functional groups, the trichothecenes are classified into four types (A to D). Type A trichothecenes do not contain a carbonyl group at the C-8 position. In type B trichothecenes, a carbonyl group is present at the C-8 position. The structure of ZEA consists of a resorcinol moiety fused to a 14-member macrocyclic lactone ring, which includes a trans double bond, a ketone and a methyl branch (Fig. 18.4).



Deoxynivalenol



T-2 toxin



Zearalenone

Figure 18.4 Chemical structure of deoxynivalenol, T-2 toxin and zearalenone.

18.4.2 Fungal producers and ecophysiology

DON is mainly produced by *Fusarium graminearum* and *F. culmorum which* are also producers of ZEA, which occurs commonly together with DON and similar derivates. *Fusarium cerealis*, *F. equiseti* and *F. semitectum* are also considered ZEA producers. The mycotoxins T-2 and HT-2 are produced primarily by *F. langsethiae* and *F. sporotrichioides*; also, a few isolates of *F. poae* have been reported as HT-2 and T-2 producers (Table 18.1).

F. culmorum, *F. graminearum* and *F. poae* are common pathogens of wheat and barley. *F. culmorum* and *F. poae* are common pathogens of wheat and barley in the cooler maritime regions of the world such as the UK, while *F. graminearum* tends to be the predominant *Fusarium* species pathogenic to these cereals in hotter regions of the world such as the USA. *F. graminearum, F. culmorum* and *F. equiseti* may be isolated from infected maize. Three species, i.e., *F. avenaceum*, *F. culmorum* and *F. graminearum* are considered as being the most prevalent in Europe, while several other species are less important.

Host and climatic factors influence the growth, survival, dissemination and, hence, the incidence of *Fusarium* fungi. The influence of climatic factors on *Fusarium* diseases is complicated by the fact that *Fusarium* fungi can cause disease individually or in complex infections, and there are numerous reports on how species differentially respond to different environmental variations, particularly temperature and humidity. Also, host susceptibility to fungal disease is directly influenced by temperature and osmotic stress. Temperature, humidity, light intensity and wind are the critical climatic factors affecting the production and dispersal of asexual conidia and sexual ascospores of *Fusarium*. On the other hand, germination, growth and competition between *Fusaria* are dependent upon the availability of nutrients and environmental factors such as temperature, pH, humidity, aeration and light.

Mycotoxin production in grain can begin in the field and continue throughout storage. Mycotoxin production is dependent mainly on both well-defined ranges of temperature and a_w. But, in turn, the optimum climatic conditions for mycotoxin production in infected grains depend on the substrate, *Fusarium* species and isolate. The influence of temperature and a_w on mycotoxin production by *Fusarium* fungi is probably not entirely direct but rather a function of the influence of these parameters on fungal growth. In vitro experiments show

that optimal growth of *F. graminearum* and *F. culmorum* occurs at 24–28 °C and −10 to −20 bars (water potential), and at 20–25 °C and −8 to −14 bars, respectively; *F. poae* grows also optimally at 20–25 °C. DON production by these species takes place optimally under warm and humid conditions (25–28 °C, $a_w = 0.97$); optimal conditions for ZEA production involve warm (17–28 °C), or temperature cycles (e.g., 25–28 °C for 14–15 days; 12–15 °C for 20–28 days) and humid ($a_w = 0.97$ or 90% RH) environment. Finally, T-2 production by *F. poae* occurs optimally under moderately warm and humid conditions (20–25 °C, $a_w = 0.990$).

18.4.3 Molecular tools for detection of mycotoxigenic Fusaria

Quantitative PCR assays were developed for identification of *Fusarium* species involved in FHB, based on both the TaqMan and SYBR Green techniques, and have been utilized increasingly in epidemiological studies on FHB. Lately, molecular characterization of fungi relies on sequence comparison of the internal transcribed spacer (ITS) region due to the high copy number of rRNA genes and the high degree of variation even between closely-related species. This choice may be explained by the low evolutionary process acting on such non-functional sequences. The ITS region is now perhaps the most widely-sequenced DNA region in fungi.

Detailed studies of the gene cluster involved in trichothecene synthesis have already allowed the researchers to design markers targeting variants of a number of *Tri* cluster genes; however, the differences between chemotypes do not correlate well with evolutionary trees reconstructed using sequences from the main trichothecene cluster. The amount of *Tri5* sequence is a good predictor of DON contamination.

18.4.4 Occurrence on foods and feeds

Among the trichothecenes, DON is the most frequently-occurring toxin, and is found worldwide, particularly in cereal crops such as wheat, maize, barley, oats and rye, and less often in rice, sorghum and triticale. Compared to other trichothecenes, many data have been reported on the occurrence of DON in foods and feeds, such as the EU-SCOOP Report on *Fusarium* toxins, which includes data on the occurrence of DON in food commodities from 11 European countries. DON occurrence is almost exclusively associated with cereals, and the levels of occurrence are in the order of hundreds of μg/kg upwards. DON occurs as a field (pre-harvest) rather than a storage contaminant, and almost always co-occurs with other *Fusarium* toxins. Preventive measures are difficult to implement, and even the effect of fungicide treatment on DON levels is controversial. As seasonal variations significantly influence the extent of *Fusarium* infections, levels of DON tend to vary from year to year, making it difficult to generalize to typical levels of occurrence. There is a relatively low incidence of DON in oats and rye, but it is frequent and sometimes very high levels of DON in samples of maize, wheat and barley are achieved. For these grains, the ranges of DON contamination have been reported to be 50–870, 70–1560 and 50–1200 μg/kg, respectively.

The available data show that oats can be highly contaminated with T-2 toxin and HT-2 toxin, with frequently high incidence and concentration, followed by barley. The occurrence and concentration of T-2 toxin and HT-2 toxin in oats and barley has increased across Europe since 2004. In some occasions, maize is contaminated with T-2 toxin and HT-2 toxin, usually at a moderate level, while contamination of wheat occurs very infrequently and at a low concentration level.

ZEA is almost exclusively associated with cereals (particularly maize), and the levels of occurrence are of the order of tens to hundreds of μg/kg upwards. ZEA occurs as a field (pre-harvest rather than at storage) contaminant and almost always co-occurs with other *Fusarium* toxins such as DON. Seasonal variations, as for DON, significantly influence the levels of ZEA. Studies of wheat and maize, infected with *F. graminearum* in the field, indicate that maize is invariably contaminated with both DON and ZEA, whereas wheat can have high levels of DON but usually little or no ZEA. The same situation is observed for oats, rye and barley. For maize, an incidence of 14% of samples containing >200 μg/kg has been reported.

18.4.5 Absorption, distribution, excretion and biotransformation

DON is rapidly absorbed in pigs and oral bioavailability is estimated to reach 55%. Organ distribution was measured in pigs only following a single intravenous injection of DON (1 mg/kg bw) and revealed high initial concentrations in plasma, kidney and liver. Excretion of DON occurs predominantly via urine (68% of the administered dose has been detected in urine). DON may be de-epoxidated by the microbiota of the intestinal tract with an increasing capacity from the small to the large intestine. De-epoxy DON has not been detected in blood, although it has been excreted in the urine. Moreover, the glucuronidated DON is found in blood and urine as well.

T-2 toxin is rapidly absorbed via the oral and inhalation route. Clearance of T-2 toxin itself from blood is very effective with a plasma half-life less than 20 minutes. T-2 toxin tissue concentrations are consistently high in lymphoid organs. In spleen and mesenteric lymph nodes there are detectable amounts 3 h after intra-aortal administration to pigs. In experiments with pigs orally administered, muscles and liver retained the toxin for a longer time (18 h; 0.7% and 0.29–0.43%, respectively). T-2 toxin is rapidly metabolized in different species. Metabolic transformation observed in several species in vivo and in vitro are acetylation, deacetylation (via nonspecific carboxyesterase), hydroxylation (via cytochrom P450 dependent enzymes), de-epoxidation, and glucuronide conjugation. In vivo biotransformation is mainly developed in the liver, but also in the intestine or blood plasma of rats, pigs, mice, chickens and cows. In ruminants, metabolization particularly occurs in the rumen. In various species, excretion occurs via urine and bile. In ruminants, milk contamination depends on the ruminal metabolism.

ZEA is fairly and rapidly absorbed following oral administration and can be metabolized by intestinal tissue in pigs and possibly in humans during its absorption, with the formation of α- and β-zearalenol and α- and β-zearalanol, which are subsequently conjugated with glucuronic acid. Differences between species in the metabolism of ZEA were found. α-zearalenol was the only detectable metabolite of ZEA following a single intravenous dose of 1 mg ZEA/kg bw in a pig. The cumulative recovery of ZEA plus α-zearalenol in urine and duodenal digesta after 72 hours, expressed as percentage of the total ZEA dose given, was 70% and 35%, respectively. Fourteen days after the bolus injection, ZEA and α-zearalenol concentrations in bile, liver and urine were below the limit of detection. Biliary excretion with entero-hepatic circulation occurs in rats and mice, while urinary excretion predominates in rabbits. Urinary excretion is also the main route of excretion in pigs in spite of the demonstrated entero-hepatic circulation of ZEA. Urinary excretion is also significant in humans where ZEA is mainly found as glucuronide conjugates of the parent compound and α-zearalenol.

18.4.6 Toxicity in man and animals

The toxicity of DON is relatively well investigated, and the typical dose-dependent toxic symptoms in laboratory animals as well as in farm animals comprise decreased feed intake, followed by reduction in weight gain and, at higher concentrations, vomiting and feed refusal. DON affects the immune response, and exhibits toxic effects in all animal species so far investigated, as well as in humans. Susceptibility varies considerably amongst species, but pigs are generally recognized as the most sensitive animal species. Some cases of human food poisoning by contaminated grains have been reported. Symptoms described in human patients include abdominal pain or a feeling of fullness in the abdomen, dizziness, headache, throat irritation, nausea, vomiting, diarrhoea, and blood in the stool. Acute disease outbreaks have been reported in China and India. There is no experimental or epidemiological evidence for mutagenic and/or carcinogenic properties of DON and it was classified by the IARC in Group 3 (not classifiable as to its carcinogenicity to humans). A PMTDI of 1 µg/kg bw was set.

Overall, the toxicity of T-2 toxin is extensively investigated but valid subchronic or chronic toxicity studies are lacking. Existing data reveal that T-2 toxin is a very potent toxin that primarily affects the immune and haematopoietic systems. These findings are consistently reported in several species, including mammals and poultry. Effects observed in various species after acute oral T-2 toxin treatment include nonspecific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhoea, haemorrhages as well as necrosis of the gastrointestinal epithelium, bone marrow, spleen, testis and ovary. Longer-term investigations with poultry revealed mouth lesions, lesions of the intestines, decreased weight gain and reduced feed intake and effects on the immune system. The findings in poultry are generally consistent with the effects observed in mammals. It is assumed that the observed genotoxic effects may be most likely secondary. IARC classified T-2 toxin in 1993 into carcinogenicity group 3 because there were no data available on the carcinogenicity to humans and there was only limited evidence in experimental animals. The overall evaluation of the Scientific Committee on Food (SCF) concluded that a temporary TDI (t-TDI) of 60 ng T-2 + HT-2 toxin/kg bw would protect against chronic, subchronic and reproductive effects seen up to the evaluation date.

The structure of ZEA allows its binding to mammalian oestrogen receptors. Subsequently, ZEA induces oestrogenic effects in mammals and interferes with conception, ovulation, implantation, foetal development and viability of newborn animals. The greater formation of α-zearalenol in the pig, relative to other animal species studied, may contribute to its sensitivity. Decreased fertility, increased number of resorptions, reduced litter size, changed weight of adrenal, thyroid and pituitary glands and change in serum levels of progesterone and oestradiol have been observed, but no teratogenic effects have been found. ZEA has been implicated as causative agents in epidemics of premature thelarche in girls in Puerto Rico between 1978 and 1981, and an increased incidence of early thelarche has also been reported from South-eastern Hungary. ZEA was evaluated by the IARC and, based on inadequate evidence in humans and limited evidence in experimental animals, was placed, together with other *Fusarium* toxins in Group 3 (not classifiable as to their carcinogenicity to humans). A t-TDI of 0.2 µg/kg bw was established by the SCF, whereas the PMTDI established by JECFA was 0.5 µg/kg bw.

18.4.7 Human exposure assessment

Human exposure to DON occurs predominantly via ingestion of cereals and grains. Considering the year-to-year variability of the contamination of cereals and grains with DON in Europe, an exposure of consumers close to the TDI of 1 µg/kg bw/day is possible

(14–46% for adults, 11–96% for children), as concluded by the SCF and confirmed by the recent SCOOP (Scientific cooperation on questions relating to food) task. Dietary exposure varies considerably according to traditional food supplies in different geographic regions. The WHO presented a comparative estimate of human exposure in Europe, the Far East, Latin America and the Middle East. This comparison demonstrated that in Europe, approximately 80% of the total DON intake is associated with the consumption of wheat, whereas in the Far East, wheat and rice are equally important as source of DON exposure.

The intakes of T-2 and HT-2 toxins through barley, maize, oats, rice, rye and wheat were estimated based on consumption data from the Global Environment Monitoring System (GEMS)/Food European diet programme (WHO). The total intake of T-2 toxin was estimated to be 7.6 ng/kg bw/day, wheat and barley being the major dietary sources. The SCF noted that the distribution of contamination in processed products could differ from that in raw cereals, as contamination tends to be more homogeneous after processing. Despite the limited amount of data on concentrations of T-2 and HT-2 toxins, the preliminary estimates of average contamination and dietary intake based on the GEMS/Food European diet proved to be useful.

Estimates of average dietary intakes of ZEA based on individual diet records have been presented by the FAO, indicating an exposure of 0.03 to 0.06 μg/kg bw/day, thus remaining below the PMTDI of 0.5 μg/kg bw/day set by JECFA and the t-TDI of 0.2 μg/kg bw/day established by the SCF. Data from the EU SCOOP task showed that the mean intake of ZEA, estimated from various European countries, might range from 1 ng/kg bw to 420 ng/kg bw. Bread and other cereal products were the most prominent sources of exposure. For adults, the intake interval was 2.4 to 29 ng/kg bw/day for average consumers, and 4.7 to 54 ng/kg bw for high consumers. The highest exposure estimates are for toddlers, at 9.3 to 100 ng/kg bw/day for average consumers, and 23 to 277 ng/kg bw for high consumers.

18.4.8 Effect of food processing and detoxification

Cereals usually undergo a cleaning stage before being milled. This action has been reported to reduce the *Fusarium* mycotoxins content considerably, but the outcome depends on the physical condition of the grain.

Mycotoxin concentrations in milling streams should reflect where the mycotoxins are situated in the whole grains and how the original fungi colonized the developing grain seeds. In wheat, all *Fusarium* mycotoxins were present in all fractions obtained, but were usually concentrated in the bran, shorts and germ. Also, the concentration of mycotoxin occurs in the finest particles; therefore, contamination of milling fractions is not simply due to the presence of peripheral grain tissues. In studies with maize, high levels of mycotoxins were found in the germ and bran fractions with lower levels in the grits and flour. Reduction levels of 10–90% have been reported for wheat, maize and oats and different *Fusarium* toxins. The distribution in milled cereals could be influenced by the contamination level of the original grain, and the milling process is not always effective for removal of toxins from grains.

The food processing industry uses a range of techniques such as boiling, fermentation, baking, frying and extrusion for manufacturing cereal-based foods. DON, NIV, ZEA, HT-2 and T-2 have generally been shown to be quite stable in commercial processing. Bread fermentation and baking have been recently intensively investigated, although the results were quite inconsistent. Reasons for these inconsistent results are unknown, but it is likely that the specific baking conditions including temperature, time, moisture content of the dough, type and quality of flour, and recipe influence mycotoxin stability. Average concentrations of DON in cookies, crackers and pretzels ranged from 61% (cookies) to 111% (pretzels)

compared with flour. Lesser amounts were found in doughnuts and bread: their respective DON concentrations were 44% and 30% that of flour. Mass balance estimates for DON indicated that dilution by recipe ingredients contributed to DON reductions in bread and accounted for all of the apparent reduction in doughnuts. Other studies suggested that manufacturing of bread, cakes and biscuits has little effect on DON and ZEA. The fermentation phase may induce a DON increase, probably due to the enzymatic release of native DON from bound forms occurring in the raw material. On the other hand, increasing time/temperature in the baking phase, even staying in an acceptable technological range, could reduce the native DON content (up to about 50%) in bread, but only when starting from high naturally-contaminated flour. No substantial changes of DON and its conjugate DON-3-glucoside (DON-3-Glc) occurred during the dough preparation process, i.e., kneading, fermentation and proofing. However, when bakery improvers enzymes mixtures were employed as a dough ingredient, a distinct increase of up to 145% of conjugated DON-3-Glc occurred in fermented dough. Some decrease of both DON-3-Glc and DON (10 and 13%, respectively, compared to fermented dough) took place during baking. Thermal degradation products of DON were detected in roasted wheat samples and baked bread samples.

DON and ZEA appear relatively unaffected by extrusion cooking, which involves high heat and pressure, but some added components can partially degrade these compounds. Reductions lower than 52% in DON and ZEA content have been reported during snack food production. DON concentration in pasta or noodles before cooking is significantly reduced by boiling. It is speculated that loss might be due to extraction into the cooking liquid. Alkaline cooking (nixtamalization) has been shown to effectively reduce DON in maize.

18.4.9 Legislation

European legislation states maximum levels for DON ranging from 200 µg/kg in foods for infants and young children, to 500–750 µg/kg in cereal-based food for direct human consumption, for ZEA ranging from 20 µg/kg in foods for infants and young children, to 50–100 µg/kg in cereal-based food for direct human consumption. Higher levels are set for unprocessed cereals (up to 1750 µg/kg DON, up to 350 µg/kg ZEA). With regard to raw materials for animal consumption and feedstuffs, the EU recommends not to exceed values between 0.9–12 µg/kg, and 0.1–3 µg/kg, for DON and ZEA, respectively, depending on the cereal and destination of the feed.

In the United States, the FDA has set an 'advisory level' for DON, that is, a guide level for industry which gives a safety margin for human and animal protection. This level has been set at 1 mg/kg in wheat derivatives (flour, bran and germ) for human consumption, and between 5 to 10 mg/kg for feedstuffs depending on the final destination. On the other hand, Canada has set maximum admissible levels for DON and HT-2 toxin in foods and feeds, ranging from 1–5 mg/kg to 0.025–0.1 mg/kg, respectively. Maximum recommended levels for ZEA, T-2 and DAS have been also set.

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19 Foodborne protozoa

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19.1 Introduction

Parasitic infections can be acquired by ingestion of contaminated foods and water and can cause illness in humans. Parasites are classified into several groups: the protozoa (unicellular organisms) and the helminths (multicellular organisms) which are grouped as nematoda (round worms), trematoda (flukes), and cestoda (flat worms). These are grouped based on their morphological and life cycle characteristics. This chapter focuses on the unicellular parasites: primarily coccidia and flagellate protozoans. *Cryptosporidium*, *Giardia*, *Cyclospora*, *Toxoplasma*, and *Sarcocystis* have been associated with foodborne outbreaks and are of particular significance in the US.

Protozoan parasites have been associated with human illness for many centuries. *Giardia* was found in human coprolites with a radiocarbon age of 2550 ± 80 yrs in Big Bone Cave, TN. Similarly, *Cryptosporidium* and *Giardia* have been reported in Andean coprolites estimated to be 500 to 3000 yrs old. In another additional study in Peru, *Cryptosporidium* and *Giardia* were identified in samples more than 4000 yrs old.

Cryptosporidium and *Giardia* are mostly associated with waterborne outbreaks. *Cyclospora* was described in the early 1990s; however, it may have been overlooked in earlier cases of gastrointestinal illness. Most of the reports in the developed world implicated fresh vegetables and fruits imported from endemic areas. The first *Cyclospora* foodborne outbreak was reported in the US in 1995. *Toxoplasma* has been frequently associated with consumption of uncooked pork meats; however, fresh produce contaminated with infectious oocysts may also be a significant source of *Toxoplasma* infection.

It is estimated that, in the US, foodborne toxoplasmosis is a leading cause of hospitalization (8%) and death (24%) annually. It is also estimated that, annually, the number of episodes of foodborne illness reported in association with *Cryptosporidium*, *Cyclospora*, *Giardia*, and *Toxoplasma* are 57, 616, 11,407, 76,840, and 86,686 respectively.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

19.2 Nature of the illness caused

Most foodborne protozoan parasites that dwell in the gastrointestinal tract are *Cryptosporidium*, *Giardia*, and *Cyclospora* and usually cause gastrointestinal illnesses. The clinical presentation can vary from asymptomatic to severe, but in most instances diarrhea, abdominal pain, nausea, fatigue, and vomiting are usually present during infection. Giardiasis is also characterized by foul flatulence. Anorexia is particularly noticeable in patients with cryptosporidiosis and cyclosporiasis.

Sarcocystis generally localizes in muscle tissue, causing muscular sarcocystosis. Malaise and muscular pain are characteristic with sarcocystosis. Peripheral eosinophilia and elevated serum creatinine phosphokinase levels have also been reported and are consistent with eosinophilic myositis.

Toxoplasma can infect a variety of tissues and toxoplasmosis can have a variety of presentations from asymptomatic to encephalitis and chorioretinitis. In pregnant women, miscarriage or fetal malformation can occur.

19.3 Characteristics of the agents

19.3.1 Classification

Giardia intestinalis (*lamblia* or *duodenalis*) is the most commonly-identified flagellate in food and waterborne outbreaks. *Giardia* has two forms: the trophozoite that is motile and divides by binary fission in the intestine of the infected host, and the cyst that is the environmentally-resistant stage and is excreted in the feces or stools of the infected host (Fig. 19.1).

Another group of protozoa are the coccidia, which are characterized by their ability to invade the host cells where asexual and sexual multiplication occurs. The coccidia are characterized by the oocyst size, shape, and internal structures. Based on the number of sporocysts and sporozo-

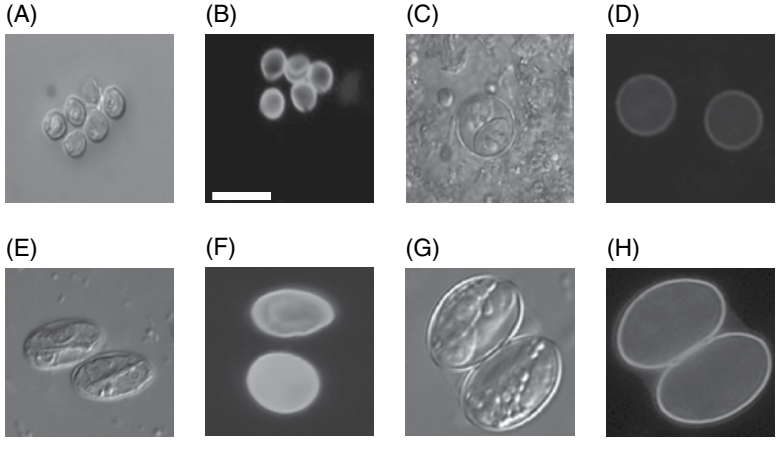

Figure 19.1 Morphological characteristics of protozoan parasites by Nomarski microscopy: (A) *Cryptosporidium parvum*; (C) *Cyclospora cayetanensis*; (E) *Giardia intestinalis*; (G) *Sarcocystis* sp. These cysts or oocysts can be observed stained using: fluorescein-tagged antibodies with (B) *Cryptosporidium parvum* and (F) *Giardia intestinalis*, or autofluorescence with (D) *Cyclospora cayetanensis* and (H) *Sarcocystis* sp. Pictures E, F, G, and H obtained from http://dpd.cdc.gov/dpdx/default.htm

Table 19.1 Taxonomical classification and morphological characteristics of foodborne protozoa

Phylum	Sarcomastigophora	Apicomplexa			
Class	Zoomastigophora	Conoidasida			
Subclass		Coccidiasina			
Order	Diplomonadida	Eucoccidiorida			
Suborder		Eimeriorina			
Family	Hexamitidae	Cryptosporidiidae	Eimeriidae	Sarcocystidae	
Genus	*Giardia*	*Cryptosporidium*	*Cyclospora*	*Toxoplasma*	*Sarcocystis*
Size	10–14 µm	4–6 µm	8–10 µm	10–12 µm	15–20 µm
Number of sporocysts	0	0	2	2	2
Number of trophozoites/ sporozoites	2 trophozoites	4 sporozoites	2 sporozoites	4 sporozoites	4 sporozoites

ites and life cycle characteristics, coccidia are classified into various species. *Cryptosporidium* has four sporozoites but no sporocysts. *Cyclospora* has two sporocysts and each contains two sporozoites. *Toxoplasma* and *Sarcocystis* have two sporocysts, each with four sporozoites (Table 19.1). These coccidia (except *Toxoplasma*) multiply inside the human intestinal epithelial cells, producing sexual stages that result in the formation of infectious oocysts. *Sarcocystis* can disseminate, infecting the vascular endothelium as it migrates throughout the body infecting other tissues, particularly muscle. *Toxoplasma* can infect not only muscle but also neural tissue and can even cross the placental barrier, thus infecting the fetus.

With the advent of molecular methods, the description of new species has results from genotyping and subgenotyping (oo)cysts from human and animal origin. Therefore, *Giardia* assemblages A and B, *Cyclospora cayetanensis*, *Cryptosporidium* spp, *Toxoplasma gondii*, *Sarcocystis hominis*, and *S. suishominis* are most commonly recognized to be of public health relevance. Of the 19 *Cryptosporidium* species that can infect humans, *C. parvum* and *C. hominis* are most frequently associated with diarrheal illness.

19.3.2 Factors affecting growth and survival

All coccidian parasites require a host to propagate and to produce the infectious stages – the oocyst. Flagellates such as *Giardia* can grow and multiply in liquid media (TYI-S-33). Although cysts can be produced *in vitro*, the viability and ability to excyst is limited. Production of cysts and oocysts can be obtained from experimental infections using animal models with both flagellates and coccidia. However, current attempts to infect animals or human volunteers with *Cyclospora* oocysts have been unsuccessful.

Cysts and oocysts are generally highly resistant to environmental conditions. *Cyclospora* and *Toxoplasma* seem to be most resistant to desiccation and to most sanitizers and water disinfectants. Cysts and oocysts are sensitive to high temperatures, but can survive freezing temperatures depending on the implicated food matrix.

19.3.3 Pathogenesis

The mechanisms of pathogenesis of parasites in humans vary significantly. The mechanisms of infection are not well understood but malabsorption is well documented in children with giardiasis leading to growth faltering and cognitive impairment when infection is prolonged.

Table 19.2 Routes of contamination and parasite multiplication in various hosts

Infection and parasite multiplication		*Giardia*	*Cryptosporidium*	*Cyclospora*	*Toxoplasma*	*Sarcocystis*
infection acquired by	ingestion of contaminated produce or water containing	Cysts	Oocysts	Oocysts	Oocysts	Oocysts, sporocysts
	Ingestion of contaminated meats containing	No	Oocysts	No	Tissue cysts	Tissue cysts
	person-to-person contamination	Yes	Yes	No	No	Yes
asexual multiplication in intestine of		Human, animals	Human, animals	Human	Felides	Human, animals
asexual multiplication in other tissues		No	Human, animals	Human	Humans, animals	Human, animals
types of tissues		No	Gall bladder, lung	Gall bladder, lung	Muscle, brain, fetus, etc	Muscle
sexual multiplication in intestine of definite host		No	Human, animals	Human	Felides	Human, animals
excretion in feces of definite host		Cysts	Oocysts	Oocysts	Oocysts (in cats)	Oocysts, sporocysts
definite host		Human, animals	Human, animals	Human	Felides	Human, animals

In coccidia, the sporozoites enter the cells of the intestinal epithelium and begin multiplying in the cytoplasm. Destruction of the intestinal epithelial cells and local inflammatory response are observed. In the case of *Cryptosporidium*, the parasitic vacuole has a unique intracellular but extracytoplasmic location (epiplasmatic). *Toxoplasma* invades other tissues, preferentially muscle, neural tissue, and in some instances will cross the placental barrier and infect fetal tissue (Table 19.2).

19.4 Epidemiology

19.4.1 Reservoirs

Giardia spp. infect the intestinal tract of humans, domestic animals, and wildlife. *Giardia* has been reported infecting humans and animals worldwide. Of all the various species of *Giardia*, only two assemblages (A and B) of *G. intestinalis* are infectious to humans and nonhuman primates (Table 19.3).

Cryptosporidium spp. infect the microvillus border of epithelial cells in the gastrointestinal and respiratory tracts of all classes of vertebrates, including humans. Humans are mainly infected with the human-specific species, *C. hominis*, and a species found in most warm-blooded animals, *C. parvum*. Although most of *Cryptosporidium* species are host-specific, currently 19 different species from genus *Cryptosporidium* have been reported to be infectious in immunocompetent and immunodeficient humans. Based on their incidence, these could be divided into three risky groups (Table 19.4).

Table 19.3 List of genotypic groups of *Giardia intestinalis* and other species, and their human infectivity

Species/assemblage	Human risk	Source of cysts
Giardia intestinalis		
Assemblage A	Yes	Human, livestock, dogs, cats, some wildlife
Assemblage B	Yes	Human, livestock, dogs, rabbit, some wildlife
Assemblage C	No	Dogs and other canids
Assemblage D	No	Dogs and other canids
Assemblage E	No	Cattle and other hoofed livestock
Assemblage F	No	Cats
Assemblage G	No	Rats
Assemblage H	No	Marine vertebrates
Giardia agilis	No	Amphibians
Giardia muris	No	Rodents
Giardia psittaci	No	Birds
Giardia ardeae	No	Birds
Giardia microti	No	Rodents

Table 19.4 List of human pathogenic *Cryptosporidium* spp.

Species/genotype	Human risk	Main source of oocysts
C. hominis§	High	human
C. parvum§	High	calves and human
C. canis	Medium/high	dog
C. felis	Medium/high	cat
C. melagridis§	Medium/high*	turkeys
C. viatorum	Medium/high*	human#
C. andersoni	Low	cattle
C. cuniculus§	Low	rabbits, human
C. fayeri	Low	kangaroo
C. muris§	Low	rodents
C. scrofatum	Low	pig
C. suis	Low	pig
C. tyzzeri§	Low	rodents
C. ubiqutum§	Low	cattle, sheep, and goats
C. wrairi	Low	guinea pig
chipmunk genotype	Low	chipmunk
horse genotype	Low	horses
monkey genotype	Low	monkeys
skunk genotype	Low	skunks

*depends on geographic location; §species/genotype infectious to laboratory mice; # natural source is unknown
Rašková V, Květoňová D, Sak B, McEvoy J, Edwinson A, Stenger B, Kváč M. Human cryptosporidiosis caused by *Cryptosporidium tyzzeri* and *C. parvum* isolates presumably transmitted from wild mice. *J Clin Microbiol.* 2013 Jan;51(1):360–2

Based on current evidence, *Cyclospora cayetanensis* infects humans exclusively. However, *Cyclospora* oocysts have been identified in feces of dogs, chickens, and ducks. No histopathological evidence of infection has supported these findings, thus the presence of oocysts in animal feces could be the product of coprophagia practiced by these animals as demonstrated in experimental studies. Epidemiological studies also resulted in a strong correlation among households that had chickens and children with *Cyclospora* infection.

Eighteen species of *Cyclospora* have been described in snakes and rodents. In addition, three other species have been reported infecting nonhuman primates: *C. papionis*, *C. colobus*, and *C. cercopiteci* infecting baboons, colobus monkeys, and green monkeys respectively. These species seem to also be highly host-specific.

The definite hosts of *Toxoplasma gondii* are cats. Sexual multiplication occurs in the epithelial cells of their intestine, resulting in the formation of oocysts that are shed in cat feces. Because *Toxoplasma* can infect most warm-blooded animals, risk of acquisition of toxoplasmosis is directly associated with consumption of uncooked meats particularly pork, sheep, and goat meat. It can also be acquired by ingestion of contaminated water and fresh vegetables and herbs.

Sarcocystis spp. requires two hosts to complete its life cycle. The intermediate host acquires the infection by ingestion of oocysts and sporocysts. In the intestine, four sporozoites are released from each sporocyst. The sporozoites cross the intestinal epithelia and migrate via the circulatory system and multiply asexually (merogony) in the endothelial cells. Several multiplication cycles can occur as the parasite migrates through the body and establishes residence in muscular tissue where the metrocytes are formed, initiating the formation of sarcocysts containing bradyzoites. When sarcocysts are ingested by a definite host, bradyzoites are released from the sarcocyst, infect the intestinal epithelial cells in the intestine and differentiate into gametocytes. Once fertilization occurs, oocyst formation starts and either oocysts or sporozoites are then excreted in the feces. The clinical presentation will vary according to the localization of the tissue cysts. Humans can serve as definite hosts for *S. hominis* and *S. suihominis*, and oocyst or sporocysts are excreted in the feces of the infected individual. *S. hominis* and *S. hirsuta* infect cattle and *S. suihominis* infect pigs. Both *S. ovifelis* of cats and *S. ovicanis* of dogs can infect sheep. Humans may serve as intermediary hosts for multiple species of *Sarcocystis*.

19.4.2 Vehicles of transmission

The life cycle of *Giardia* is completed within a single host, and fecal–oral transmission occurs. Human giardiasis can be acquired also via direct contact person-to-person or animal-to-human or other vehicles. Water is a major vehicle for many parasites, including *Giardia*. Waterborne transmission occurs when susceptible hosts ingest contaminated drinking water and by participating in recreational water activities like swimming where ingestion of contaminated water can occur. Shellfish, including oysters and clams, can concentrate cysts, and when consumed raw they pose as a potential risk of infection. Poor personal hygiene of food handlers could be another source of *Giardia* outbreaks.

When *Cryptosporidium* oocysts are excreted, they are already infectious, allowing for fecal–oral transmission. Human cases of cryptosporidiosis have been associated with consumption of contaminated water (drinking water, ice, swimming pools, recreation lakes, and paddling pools), drinks (unpasteurized milk or fruit juice) or food (vegetables, seafood – shellfish, mussels, or oysters – apple cider, and meat/meat products). Frequent source contamination can be direct contact of susceptible individuals with infected animals (pets, visits to the countryside or farms, country fairs and livestock shows) and infected individuals (family spread, nurseries, hospital, and sexual activity). Contamination by food handlers can also occur during preparation of ready-to-eat foods.

Human cases of cyclosporiasis have been associated with consumption of water, but most frequently in foods such as berries (particularly raspberries), basil, mesclum lettuce, and sweet peas that were consumed raw. Produce surveys in various endemic regions have also identified *Cyclospora* oocysts in produce and herbs that are grown close to the soil such as cilantro, black mint, parsley, etc. Because *Cyclospora cayetanensis* is considered exclusively

anthroponotic, contamination of the water (for irrigation or pesticide application) occurs with human stool samples.

Toxoplasmosis can be acquired by ingestion of raw or undercooked meats containing *Toxoplasma* tissue cysts and by ingestion of vegetables or water contaminated with cat feces containing viable *Toxoplasma* oocysts. Consumption of raw milk has also been associated to acute cases of toxoplasmosis. Waterborne transmission has also been reported. In Canada, 110 cases of acute toxoplasmosis were reported in 1995 and in 2001 in Brazil at least 426 cases of *Toxoplasma* infection implicated consumption of contaminated drinking water as the source of infection. Toxoplasma tissue cysts can be found in the meats of most farm animals, particularly swine, sheep, and goats. Humans can acquire the infection by ingestion of these contaminated meats that are not properly cooked.

Intestinal sarcocystosis is acquired by ingestion of undercooked beef or pork meat containing sarcocysts. Muscular sarcocystosis can be acquired by ingestion of contaminated water or vegetables containing oocysts or sporocyst of many *Sarcocystis* species.

Water used for irrigation and fumigation can be contaminated with animal or human feces containing *Giardia*, *Cryptosporidium*, and *Cyclospora*. Infections can be acquired by ingestion of raw fruits and vegetables exposed to this water.

19.4.3 Incidence

Giardia and *Cryptosporidium* infections have been reported worldwide and individuals of all ages are susceptible to infection. In humans, symptoms of *Giardia* infection consist of acute and chronic diarrhea, dehydration, abdominal pain and weight loss. In some cases, it may be asymptomatic. Epidemiological evidence has linked symptoms of giardiasis to some assemblages. While assemblage A is associated with clinical illness, patients with assemblage B usually have asymptomatic giardiasis.

Cryptosporidiosis has been reported worldwide in both immunocompetent and immunocompromised patient populations of all ages. *Cryptosporidium* has been more frequently associated with waterborne than foodborne outbreaks (Table 19.5). Developing countries, where children are more commonly infected than adults, have higher rates of infection than developed countries. Many developed as well as most developing countries do not have dependable surveillance systems to detect human cryptosporidiosis and many cases could be overlooked. There is a large difference in cryptosporidiosis prevalence among countries as well as the role of domestic and farm animals in its transmission.

Cyclospora cayetanensis has been described in various parts of the world in travelers returning from endemic areas and in food and waterborne outbreaks. In the US, most of the reported cases have been associated with consumption of contaminated foods containing *Cyclospora* oocysts. Epidemiological evidence has linked these outbreaks to specific fooditems. In few instances *Cyclospora* oocysts or DNA were identified in the implicated food items. Interestingly, the implicated foods in most cases were imported from countries where *Cyclospora* is endemic (Table 19.5). In the US, individuals of all ages are susceptible to the infection and the symptomology is more severe in immunocompromised patients.

Toxoplasma has three clonal lineages which have been associated with virulence and pathogenicity in humans and animals. In mice, type I is highly pathogenic whereas type II and III are considered nonvirulent. In humans, type I has been identified more commonly in congenital toxoplasmosis and in AIDS patients. In a study in Poland, 14 out of 19 cases of congenital toxoplasmosis were caused by type II, and in France eight out of eight cases of congenital toxoplasmosis were caused by type II. Contrarily, in Spain,

Table 19.5 Significant *Cryptosporidium*, *Giardia*, *Cyclospora*, *Sarcocystis*, and *Toxoplasma* foodborne outbreaks

Parasite	Year	Location	Cases (n)	What	Reference
Cyclospora	1996	Charleston, South Carolina, US	38	Fresh raspberries, potato salad	(Caceres et al., 1998)
	1996	Broward and Palm Beach, Florida, US	60	Fresh raspberries	(Katz et al., 1999)
	1996	US, Canada	1,465	Raspberries	(Herwaldt & Ackers, 1997)
	1997	Cruise ship, FL	220	nd	(Rooney et al., 2004)
	1997	US/Canada	1,012	Raspberries	(Herwaldt, 2000)
	1997	Florida, US	12	Mesclun salad	(Herwaldt, 2000)
	1998	Georgia, US	17	Fruit salad?	(Herwaldt, 2000)
	1998	Toronto, Ontario, Canada	221	Fresh raspberries	(Anonymous, 2012a)
	1999	Ontario, Canada	104	Berry dessert	(Herwaldt, 2000)
	1999	Florida, US	94	Fruits, berry	(Herwaldt, 2000)
	1999	Missouri, US	62	Chicken pasta, tomato basil, vegetable pasta salads	(Lopez et al., 2001)
	2000	Philadelphia, PA, US	54	Raspberry filling, wedding cake	(Ho et al., 2002)
	2000	Baden-Wuerttemberg, Germany	34	Salad, leafy vegetables and herbs	(Doller et al., 2002)
	2001	Vancouver, British Columbia, Canada	17	Thai basil imported from US	(Hoang et al., 2005)
	2004	Pennsylvania, US	96	Snow peas	(Anonymous, 2004)
	2004	Lima, Peru	27	nd	(Torres-Slimming et al., 2006)
	2007	British Columbia, Canada	29	Basil	(Shah et al., 2009)
	2009	Stockholm, Sweden	12	Sugar snap peas	(Insulander et al., 2010)
	2010	Ontario, Canada	210*	Basil pesto	(Anonymous, 2012b)
	2011	Atlanta, GA	>100	nd	(Anonymous, 2012c)
Giardia	1979	Minnesota, US	29	Home-canned salmon/food handler	(Smith et al., 2007)
	1985	Connecticut, US	13 of 16	Noodle salad/food handler	
	1986	Minnesota, US	88	Sandwiches/nursing home	
	1986	New Jersey, US	10 out of 25	Fruit salad/food handler	
	1990	Washington State, US	27 of 36	Ice/food handler – restaurant-based	
	1990	Connecticut, US	26	Sliced raw vegetables	(Mintz et al., 1993)

Cryptosporidium	1984	Australia	2	Raw goat milk	(Ortega & Cama, 2008)
	1985	United kingdom	1	Frozen tripe	(Ortega & Cama, 2008)
	1984	Wales, UK	19	Sausage	(Casemore et al., 1986)
	1985	Mexico	22	Milk	(Elsser et al., 1986)
	1993	Maine, US	154	Apple cider, unpasteurized	(Millard et al., 1994)
	1995	Minnesota, US	15	Chicken salad	(Anonymous, 1996)
	1995	United Kingdom	50	Milk, faulty pasteurization	(Gelletlie et al., 1997)
	1996	Connecticut and New York, US	31	apple cider, unpasteurized	(Anonymous, 1997)
	1997	Milwaukee, WI	24	nd	(Millar et al., 2002)
	1998	Washington, D.C.	148	Ill food handler	(Quiroz et al., 2000)
	2001	Sunshine Coast, Queensland	8	Unpasteurized milk	(Harper et al., 2002)
	2003	Ohio	23	Ozonated apple cider	(Blackburn et al., 2006)
	2005	Copenhagen	99 ill, 13 confirmed	Canteen salad bar	(Ethelberg et al., 2009)
	2005	Spokane, Washington, D.C.	54	Green onions	(Anonymous, 1998)
	2008	Helsinki, Finland	72 ill, 4 confirmed	Salad mixture	(Ponka et al., 2009)
	2008	Stockholm, Sweden	21	Chopped fresh parsley	(Insulander et al., 2008)
	2009	North Carolina	46	Ham from sandwich bar	(Anonymous, 2011)
Sarcocystis	1993	Malaysia	7 of 15	nd	(Arness et al., 1999)
	2011	Tioman Island, Malaysia	32	nd	(Von Sonnenburg et al., 2012)
Toxoplasma	1977	England	3	Lamb meat	(Pereira et al., 2010)
	1978	California	10	Raw goat milk	(Sacks et al., 1982)
	1980	South Carolina, Alabama	3	Raw venison	(Sacks et al., 1983)
	1993	Brazil	17	Raw mutton	(Pereira et al., 2010)
	1984	Brazil	3	Raw goat milk	(Chiari & Neves, 1984)
	1994	Korea	3	Raw boar viscera	(Choi et al., 1997)
	1995	Korea	5	Raw liver and meat from pigs	(Choi et al., 1997)
	2005	Brazil	10	Cured meat	(Pereira et al., 2010; Choi et al., 1997)

nd=no food identified

non-type II was associated with congenital cases. In cases with chorioretinitis, all three types, I, II, III, were reported.

Type II causes more than 70% of the human cases of toxoplasmosis in the US and France.

The waterborne transmission potential has also been described. In Vancouver, Canada, 110 people acquired toxoplasmosis after drinking contaminated water that contained *Toxoplasma* oocysts.

Few cases of muscular sarcocystosis have been reported, and most of them were in tropical or subtropical areas. Intestinal sarcocystosis in humans has been reported more frequently in Europe and Asia. *S. hominis* was reported in Poland, Germany, and Tibet (10.4%, 7.3%, and 21.8%, respectively). In Tibet, *S. suihominis* was reported in 7% of the population examined. In 2011, 32 cases of suspected muscular sarcocystosis were reported from travelers returning from Tioman Island (Malaysia).

19.5 Detection of organism

Environmental and food detection methods are based on (oo)cyst recovery by concentration, elution, and filtration of cysts using various laboratory procedures such as flotation, sedimentation, gradient, and immunomagnetic separation followed by microscopy or molecular characterization. Clinical specimens are usually stored under various conditions according to the detection method to perform (potassium dichromate, alcohol, water, or other innocuous or special buffer, deep-frozen).

Environmental (water, food, produce, or soil) and stool samples can be examined for the presence of *Cryptosporidium* oocysts using microscopy, molecular tools and infectivity studies, including in vitro cell cultivation. Sample storage will vary according to the examination method. *Cryptosporidium* and *Cyclospora* could be detected using conventional and molecular methods. Environmental samples (water, soil, and produce) can be examined to determine the presence of *Cyclospora* oocysts using microscopy, molecular methods and sporulation studies. When the sample is received, it can be stored in formalin but if sporulation studies, animal infectivity or tissue culture are intended to determine viability, samples should be stored in potassium dichromate, water, or other innocuous buffer. If the intent is to use molecular tools for diagnosis, it is recommended to freeze the sample.

Detection of *Toxoplasma* in environmental samples is done by identifying oocysts using microscopy or molecular assays. In animals or humans, diagnosis is done using serological tests.

19.5.1 Conventional methods

19.5.1.1 *Microscopy*

Giardia cysts are oval in shape, measure 8–12 μm long × 7–12 μm wide and have a thick retractile wall. Four nuclei are observed in the mature infectious cyst. Trophozoites have four pair of flagella and measure 9–21 μm long × 5–16 μm wide. Both *Giardia* cysts and trophozoites are diagnostic stages, but cysts are most commonly found in human stools. Cysts are identified by bright field, phase contrast, differential contrast and fluorescence microscopy using morphometric and morphological criteria. Cysts can be stained using a variety of different methods.

Cryptosporidium oocysts can be identified using a bright field, phase contrast, and differential interference contrast (Nomarski) microscopy. The oocysts (diagnostic stage) are fully sporulated, smooth, thick-walled, colorless, containing four sporozoites, and a residual

body. Oocysts of intestinal species are mostly oval measuring 4–5 µm, those which inhabit the stomach are ovoid measuring 7–9 × 5–7 µm. Oocysts can be stained using a variety of methods (e.g., fast acid staining or immunofluorescence); however, their specificity and sensitivity are not adequate for use in environmental samples. Some of the concentration methods highly increase sensitivity.

Cyclospora oocysts can be identified using a bright field, phase contrast, and differential interference contrast (Nomarski) microscopy. The oocysts are round measuring 8–10 um in diameter. The unsporulated oocyst contains a morula and when it matures the morula differentiates to form two oval sporocysts. Samples can also be examined using epifluorescence microscopy. Oocysts autofluoresce blue when using a 330–380 DM and green using an excitation filter of 450–490 DM. The intensity of fluorescence will vary according to the age and storage conditions of the sample. Oocysts can also be stained using a variety of methods; however, their specificity and sensitivity are not adequate for use in environmental samples.

Because humans are intermediary hosts for *Toxoplasma*, diagnosis can be done using serological tests including avidity assays using an ELISA, IFAT, and latex agglutination or indirect agglutination assays. Agglutination assays have been used to determine the seroprevalence of *Toxoplasma* in farm, domestic, and wild animals.

Muscular infections of *Sarcocystis* can be diagnosed by identification of sarcocysts in muscle biopsies and by serological testing. Sarcocysts in muscular tissues can be observed using hematoxylin and eosin or PAS stains when doing histopathological examinations.

Intestinal infections can be diagnosed when oocysts and sporocysts are found in the feces of infected individuals. These oocysts and sporocysts can be concentrated using routine parasitological flotation methods such as cesium chloride, zinc sulfate, percoll, etc.

19.5.2 Molecular methods

Molecular tools are more sensitive and specific than microscopy and have been developed to provide information on the species/genotypes of *Giardia*. For the purpose of accurate diagnosis, semi-nested, nested PCR combined with RFLP analyses or direct sequencing, RT-PCR targeting 18S rRNA, β-giardin, EF1-α (Elongation factor 1-alpha), GDH (glutamate dehydrogenase), and TPI (Triose phosphate isomerase) gene loci, and loop mediate isothermal amplification (LAMP) tools are used.

For *Cryptosporidium* detection, the most commonly-used technique is nested genus-specific *Cryptosporidium* PCR targeting 18S rRNA gene with direct sequencing or in combination with PCR-RFLP. In view of the high polymorphic of GP60 (also called Cpgp40/15) gene, it is most widely used for *Cryptosporidium* subtyping target (nested PCR). Some other techniques (IMS-PCR, RT-PCR, MAS-PCR) could also be used to some extent. PCR tangent on TRAPC1, COWP, and HSP70 gene loci is sufficient mostly for presence of *C. hominis*, *C. parvum*, and *C. meleagridis*.

A nested PCR targeting the 18S rRNA gene in combination with restriction fragment length polymorphism (RFLP) is frequently used to confirm the presence of *Cyclospora*. The ITS1 and ITS2 DNA detection by PCR has also been used to some extent.

Toxoplasma can also be detected using molecular tools, particularly PCR targeting the B1, SAG2, SAG3, GRA6, and β-tubulin genes.

Molecular methods are being developed to differentiate the species of *Sarcocystis* that can infect humans. The 18S ribosomal RNA gene has been the gene target for these studies.

19.5.2.1 *Viability*

Giardia cysts are very resistant to environmental and water treatment stresses. Cysts can survive several months in the cold, in surface water and in soil. Like other protozoan (oo)cysts, viability is dependent on the temperature of the environment. During freeze-thaw winter cycles, oocysts can lose viability. Trophozoites are much less resistant and they are not significant in the transmission process. *In vivo* viability tests (excystation, cultures, vital dyes in combination of microscopy or flow cytometry, and RT-PCR) are more frequently used than animal infectivity. Furthermore, excystation and animal models need a relatively high number of cysts.

Cryptosporidium oocysts are already sporulated and infectious immediately after excretion. Generally, oocysts are very resistant to unfavorable conditions, especially if kept in a wet, dark, and cold environment. Temperature is the main limiting factor for survival. *Cryptosporidium* species that harbor in the intestinal epithelium survive longer than those that infect the gastric epithelia. Both *in vitro* (oocysts excystation, vital dyes, and cell culture) and *in vivo* assay (animal and cell culture infectivity assays) for viability test have been developed. Oocysts excystation or vital dyes do not always correlate well with infectivity. On the other hand, *in vitro* assays are available for some species only.

As with all parasites, identification of the oocysts, either by conventional or molecular methods in environmental samples, does not indicate that this parasite is viable. In the case of *Cyclospora*, as of now there is no *in vivo* or *in vitro* assay, therefore sporulation (at 20–24 °C for 15 days in potassium dichromate) and electrorotation have been used as indicators of viability.

In vitro cultivation and bioassays can be used to determine viability of *Toxoplasma* tissue cysts. Feeding contaminated meat to cats will result in oocyst excretion in the cat's feces if the sample is positive. Mice fed with suspected contaminated foods and tissues (particularly brain) must be examined to determine parasite viability and infectivity. These procedures are cumbersome and expensive, and are not practical for a large number of samples.

19.6 Prevention/control measures

Thoroughly cooking or freezing meats will kill the *Sarcocystis* and *Toxoplasma* bradyzoites (tissue) inside the sporocysts. Cooking at 60 °C, 70 °C and 100 °C for 20, 15 and 5 min respectively can kill the bradyzoites. Freezing at –4 °C and –20 °C for 48 and 24 hrs respectively can also kill the parasites. Thorough washing of foods will reduce the risk of infection and boiling water will assure killing of the cysts and sporocysts.

Gamma irradiation at 0.4–0.7 kGy and high-pressure treatment using 300 MPa are effective in killing *Toxoplasma* oocysts and tissue cysts. Curing methods used in meats such as salting, smoking and fermenting can kill tissue cysts. They are temperature-sensitive and freezing for prolonged periods of time will result in parasite inactivation.

Heating at more than 70 °C for short periods of time can inactivate oocysts. Inactivation can occur when oocysts are exposed at –10 to –15 °C for more than 48 hrs, and UV radiation has also been proven effective in killing *Cryptosporidium* and *Giardia*. High-pressure treatments for 30 s at 80,000 psi can result in more than a 4 log reduction of *Cryptosporidium* oocysts. Inactivation of 98% of *Cryptosporidium* oocysts was achieved using ozone at 2.25 mg/lt. Many studies on *Giardia*, *Cryptosporidium*, and *Cyclospora* inactivation using commercial sanitizing agents have demonstrated that these are highly-resistant organisms.

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20 *Taenia solium, Taenia saginata* and *Taenia asiatica*

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20.1 Introduction

The genus *Taenia* includes many species that lodge in many vertebrates; three species develop in human beings (Fig. 20.1) who have ingested infected pig or cow meat raw or insufficiently cooked. Interestingly, these parasites usually do not cause disease to their human host. Their relevance resides in the fact that *Taenia solium* cysticerci cause neurocysticercosis, a disease of importance in public health in many developing countries, while *Taenia saginata* is cosmopolitan even in developed countries, and its study led to the recent discovery of the third species, *Taenia asiatica*.

Human taeniasis has been known since the ancieint Egyptian and Greek cultures; since Egyptians did not eat pork the species was probably *T. saginata*. Tyson (1683) discovered and described the head of tapeworms and Redi (1684) published illustrations of dogs' and cats' scolices. *T. solium* development in humans was not demonstrated until the mid-19th century: Küchenmeister fed pork containing cysticerci to convicts with death sentences and Heller gave cysticerci to a patient who died soon after. Leuckart infected a young male who was treated after expelling proglottids and Humbert and Hollenbach produced patent infections in themselves. Tapeworms, or their segments, were always found. The morality and ethics of these studies were questioned, and results were not always officially published. Nonetheless, criticism did not harm Küchenmeister's ultimate reputation, for he was acclaimed as one of the most eminent parasitologists of his time. Furthermore, attempts were made to infect rabbits, cats, dogs, pigs, sheep and monkeys with cysticerci obtained from pigs, but all were in vain and it became accepted that humans were the only definitive host of *T. solium*. In recent years, assays to induce experimental *T. solium* taeniasis were performed with similar animals, and even immunodeppressed, infections did not succeed, except in hamsters, gerbils and chinchillas, which have been used to analyze the host–parasite relationship. The development of cysticerci was studied by Yoshino in 1933 in swine that ingested gravid tapeworm segments released by him after he swallowed cysticerci. The description of the larval stage from one to 325 days of growth contains great detail and precision.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

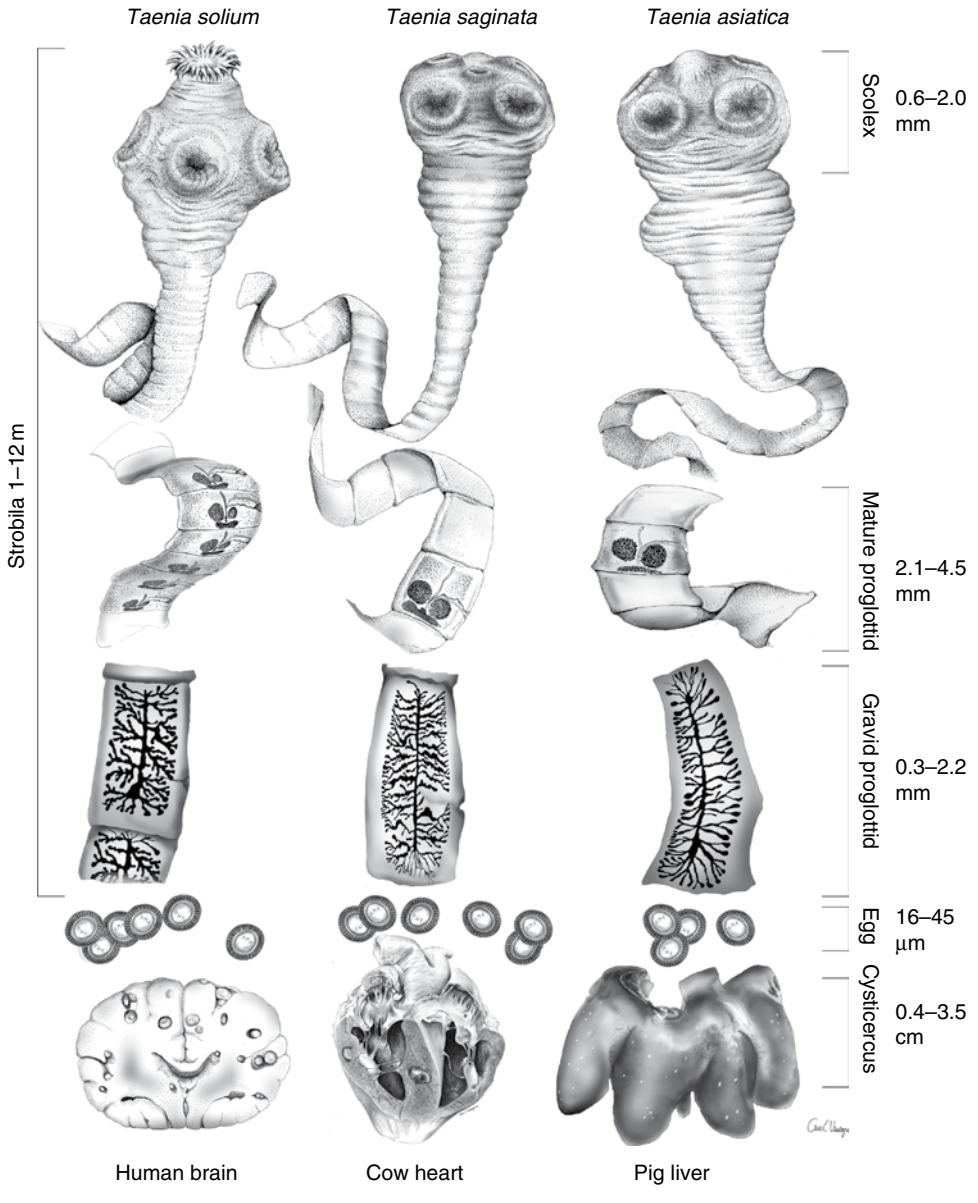

Figure 20.1 Diagram showing the three human tapeworms. *Source*: Flisser, A. et al. (2004) Portrait of human tapeworms. *J. Parasitol.* **90**, 914–916, Allen Press Publishing Services.

The successful demonstration in the 1850s of the complete life cycle of *T. solium* naturally suggested that a similar process might occur with *T. saginata*. The clue came from clinical and epidemiological observations; physicians noticed that sickly children, who had been ordered to eat raw beef in order to strengthen them, particularly those in St. Petersburg, frequently contracted infection with *T. saginata*. It was also realized that European Jews, who were proscribed from eating pork, were not afflicted with *T. solium*

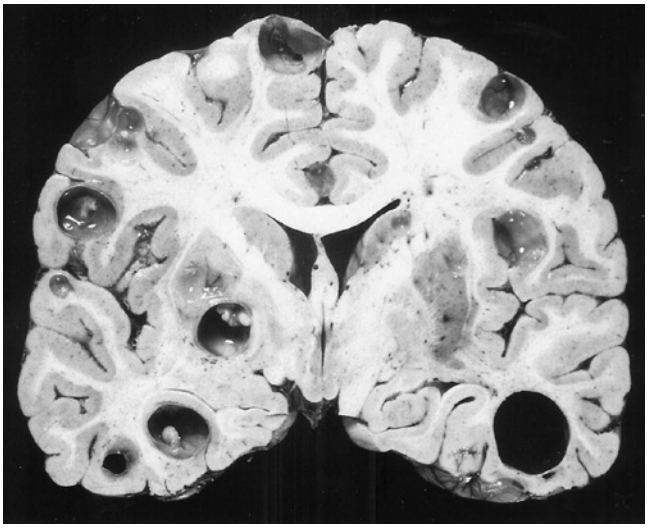

Figure 20.2 Section of human brain with cysticerci. Photograph courtesy of Dr. Maria Teresa Rabiela, formerly of the laboratory of the author, Faculty of Medicine, Universidad Nacional Autonoma de Mexico.

but acquired infections with *T. saginata*. Also travelers to various parts of the globe reported that *T. saginata* infections were more common in certain regions. The most notorious of these was Ethiopia, where almost everyone was infected and inhabitants ate mostly beef, raw by preference. A decade later, Huber put forward the hypothesis that cysticerci would be found in the tissues of cattle. This was demonstrated by Leuckart who fed a calve with 25–30 *T. saginata* proglottids and found a dozen cysticerci from the sternomastoid muscle in a biopsy performed seven weeks later; this finding was later confirmed by several other scientists.

T. asiatica was identified in the last decades when scientists from Asia and the Pacific observed people infected with adult tapeworms that seemed to be *T. saginata* but who acquired the infection after eating pork, not beef. The taxonomy of this tapeworm generated different opinions, since some considered it as a subspecies and therefore named it *T. asiatica*, while others showed that it was a different species and named it *T. asiatica*. Molecular studies confirmed it as an independent species but 'sister' to *T. saginata*.

20.2 Nature of illness in humans and animals

The most important health problem caused by *T. solium* is neurocysticercosis (Fig. 20.2). This disease arises from the development of cysticerci in the human central nervous system. Convulsive crisis and epilepsy are the most frequent clinical manifestations. Many others can occur depending on the location (parenchyma, subarachnoid tissue and ventricles), number and development or involution stages of the parasites as well as characteristics of the immune response of the host. The most severe manifestations are associated with extra-parenchymal cysticerci that can cause hydrocephalus due to mechanical obstruction of ventricular circulation of cerebrospinal fluid or to inflammatory reaction in basal cisterns. Symptoms usually occur after the cyst has initiated its degenerative process and

are due mainly to the inflammatory response they induce or to residual scarring. In contrast, living cysticerci induce minimal inflammation, and can stay in this condition for several years because parasites evade the immune response. When it becomes exacerbated it produces a cascade of immunological mechanisms. These cause parasite death, but also severe damage to the neighboring tissues in the host, especially to basal blood vessels, including a dense collagen wall around cysticerci, astrocitic gliosis, microglia and capillary vessel proliferation. Cysticerci can also develop in the eye, skeletal muscle and subcutaneous tissue, where they can remain asymptomatic or generate vision loss or muscle hypertrophy.

As in humans, *T. solium* cysticerci in swine lodge in the brain, the eye and the body musculature, but do not cause an apparent disease, although histological inflammatory changes surrounding cysticerci and electrophysiological alterations have been demonstrated. In humans, clinical features seemingly depend on the strain of the parasite. There are two genotypes of *T. solium*, one clustering African and Latin American parasite populations and the other grouping Asian populations. Interestingly, the gene flow within the different *T. solium* populations shows similar dispersion as the main maritime trade routes used between the 15th and 19th centuries: 'the middle passage' as the forcible route of African people to the New World was part of the Atlantic slave trade, and the total number of African deaths directly attributable to the Middle Passage Voyage is estimated to be up to two million. Another important maritime route during the last third of the 16th century was the 'Manila Galleon' that was inaugurated to sail the Pacific Ocean between Manila in the Philippines and Acapulco in Mexico in annual trips. Although the Manila Galleon carried mainly luxury products, it has been suggested that, through all the trips, approximately 35,000 people were exchanged, including slaves, convicts, government employees and missionaries. Interestingly, although the central nervous system is the most frequent lodging area of cysticerci, there are many more cases of muscle cysticercosis in people from Asia than from Latin America and Africa, supporting the existence of different genotypes having different virulence.

Intestinal taeniasis, caused by any one of the three species of tapeworms that lodge in humans, almost never induces significant symptoms; it is identified because proglottids can be released 2–3 times per week. In 1979, Thornton published a short and interesting report about the visit of a young overweight European woman to a slaughterhouse in a quest for a piece of measly beef to reduce weight because she was told that a sure method to reduce weight is 'to give myself a tapeworm'. This misapprehension has been common because there are no pathognomonic signs of *T. saginata* infection in man and even the most experienced clinician cannot be certain that a particular sign or symptom is due to a tapeworm infection. The most frequent symptom is the discharge of proglottids (93%) and this is a distinctive sign that can hardly go unnoticed by the patient, because of a sensation in the rectum followed by a crawling sensation in the perianal region and the thighs due to the discharge and movement of the proglottids. Remaining symptoms related to abdominal pain, nausea and weight loss occurs in one of each four tapeworm carriers. As a result of worm migration or due to mechanical effects, appendicitis, invasion of the pancreatic and bile ducts, intestinal obstruction and perforation, vomiting of proglottids can occur. The most important feature is to avoid *T. solium* adult infections because the tapeworm carrier is the main risk factor for acquiring human neurocysticercosis.

20.3 Characteristics of the parasite

The classification of human *Taenia* is as follows:

Kingdom: Animalia
Phylum: Platyhelminthes
Class: Cestoidea
Subclass: Eucestoda
Order: Cyclophyllidea
Family: Taeniidae
Genus: *Taenia*
Species: *Taenia solium* Linnaeus, 1758
Species: *Taenia saginata* Goeze, 1782
Species: *Taenia asiatica* Eom and Rim, 1993

The life cycle includes the adult, the egg and the metacestode, larva or, commonly named, cysticercus (Fig. 20.3). When a person ingests raw or semi-cooked pork or beef with cysticerci, the parasites remain alive and the scolex evaginates, adheres to the intestinal wall and in 3–4 months transforms into a fully-developed tapeworm. The adult worm measures several meters long and is constituted by a scolex or head (*T. solium* has a double crown of hooks, which are absent in *T. saginata* and rudimentary or sunken in *T. asiatica*), as well as four suckers, all used to attach to the intestinal mucosa. The scolex is followed by the neck, from which the strobila is formed, resembling a whitish ribbon formed by hundreds of immature, mature and gravid proglottids. The former are the closest to the neck and have not yet developed sexual organs, the latter are the farthest away and contain around 60,000 eggs each. Each mature proglottid has male and female sexual organs; therefore tapeworms are hermaphroditic organisms. Gravid proglottids are released with feces.

When humans, pigs or cows ingest microscopic eggs, gastrointestinal enzymes and bile induce the breakdown of the external cover of the egg, called embryophore, and the activation of the hexacanth embryo or oncosphere that liberates itself from the oncospheral membrane. Spherical newborn larvae (measuring 35–45 μm) cross the intestinal wall and, through circulation, arrive and transform into cysticerci in muscle, eyes and brain in *T. solium*; muscle, mainly cardiac, in *T. saginata*; and in the case of *T. asiatica*, parasites lodge in the liver. The cysticercus is a visible vesicle measuring up to 2 cm in diameter; it is whitish, transparent and contains an invaginated scolex. When humans ingest *T. solium* eggs, cysticerci lodge mainly in the brain causing neurocysticercosis, a disease with high morbidity and mortality.

The egg is the only life stage of the parasite that is found in the environment. Each egg contains one embryo that has six hooks (therefore is called hexacanth embryo) and it also releases enzymatic vacuoles, both of which facilitate invasion. It has been demonstrated that eggs survive in the environment for many months (except in hot dry summers or freezing winters). In the case of *T. saginata*, in whom the life cycle continues when cows graze, eggs are transported mainly by flies that normally stand on fresh feces; the eggs adhere to the wings or in the intestine while flying, and in this way, are disseminated in the environment and cows ingest them with the pasture. In contrast,

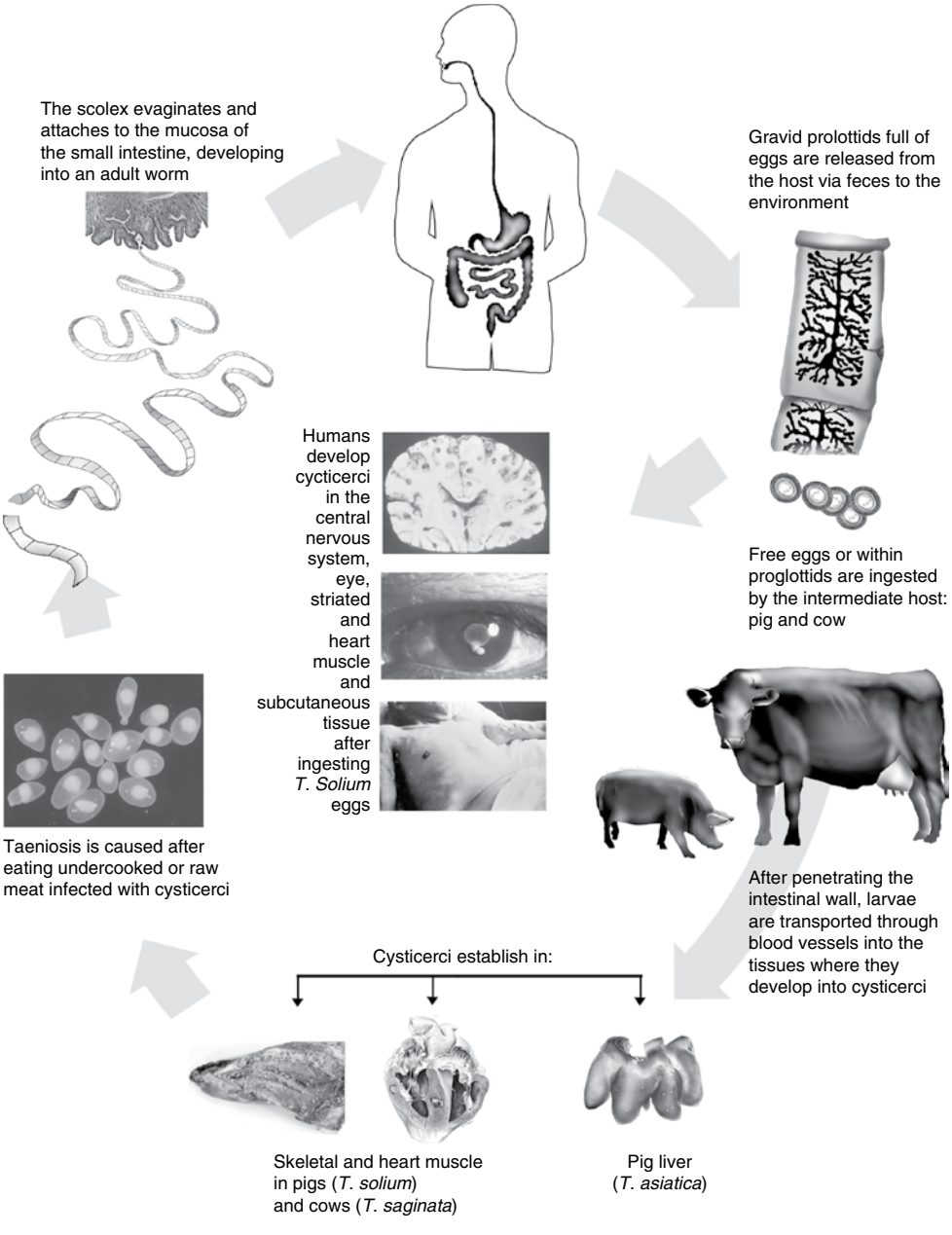

Figure 20.3 Diagram of the life cycle of the three human tapeworms (elaborated by Ana Viniegra). Reproduced from: Flisser A, Correa D, Avila G, Maravilla P. Biology of Taenia solium, Taenia saginata and Taenia saginata asiatica. In K.D. Murrell (ed.) Guidelines for the surveillance, prevention and control of taeniosis/cysticercosis. © WHO/FAO/OIE, 2005.

in *T. solium*, as Gemmell used to say: 'pigs are faster than flies' and ingest freshly discharged human feces. Therefore, the life cycle of *T. solium* depends on a tapeworm carrier in the close environment, and implies inadequate sanitary conditions and insufficient health education. Probably the same occurs in the transmission of *T. asiatica* eggs.

20.4 Epidemiology

Elimination of *T. saginata* taeniasis has been a methodological challenge. In many countries tartar meat is considered a delicacy and the parasite practically does not cause disease; furthermore, in Western countries beef carcasses, if infected, are frozen before being released for sale, while in autochthonous communities raw beef is part of ritual dishes. Therefore it is considered a cosmopolitan parasite.

Since its discovery, *T. asiatica* has been subject to many epidemiological studies in order to identify its frequency and obtain samples for molecular studies. Through this, its frequency has been importantly reduced. For example, in Korea 1.9% was found in 1969, 0.02% in 1997 and human taeniasis was not detected in the 2004 survey. In China during the period of 1998 to 2002, a total of 19,894 inhabitants of three ethnic minorities in Guangxi Province were surveyed for tapeworms; in total 927 (4.7%) persons discharged proglottids and were treated with arecoline nuts.

However, *T. solium*, that produces human cysticercosis, is found in countries of all continents that have inadequate sanitary infrastructure and insufficient health education (Fig. 20.4). This parasitic disease was identified during necropsy in 1–3% of brains in Latin American countries performed around the mid-20th century, and its importance became evident through the high frequency of neurocysticercosis in neurological hospitals, where up to 10% of new patients had the disease, and 50–70% of cases with late onset epilepsy were due to cysticerci lodged in the brain. Furthermore, the use of immunodiagnostic methods has shown that up to 15% of open populations, rural and urban, have anti-cysticercus antibodies. Presently, cysticercosis is still endemic in several countries of Latin America, Africa and Asia; furthermore, due to migration there are many neurological cases in developed countries, such as the USA, and recently tapeworm carriers in the USA and Muslim countries or regions have expanded interest in cysticercosis, proposing it as an emerging infectious disease.

Cysticercosis is a fascinating disease in its epidemiologic component. Most parasitological books show the *T. solium* life cycle including human beings as definitive hosts and pigs as the intermediate host. Nevertheless, it has always been considered that neurocysticercosis is acquired from vegetables and fruit – mainly strawberries, lettuces, coriander, etc. – that were watered with sewage which may have contained eggs and were eaten uncooked and unpeeled. In the last decade of the 20th century, after many field studies, the main risk factor was identified, which was the presence of a tapeworm carrier in the household or neighborhood. This finding changes the concept of the control of human neurocysticercosis, since it is easier to treat tapeworm carriers and provide minimal health education than to modify water handling and irrigation in developing countries. Nonetheless, it has been published that tapeworms are difficult to find, probably because they can be released only after mass treatment due to their low incidence and the lack of knowledge of being infected, or because diagnosis and/or treatment have low sensitivity. Most probably, tapeworms live in their human hosts for short periods of time (less than one year) before being expelled.

20.5 Detection of organisms

Traditionally, taeniasis has been diagnosed by detecting eggs in stools under microscopy or proglottids seen with the naked eye. These approaches are not very sensitive because they depend on the natural release of segments and on technical expertise. Coproantigens are

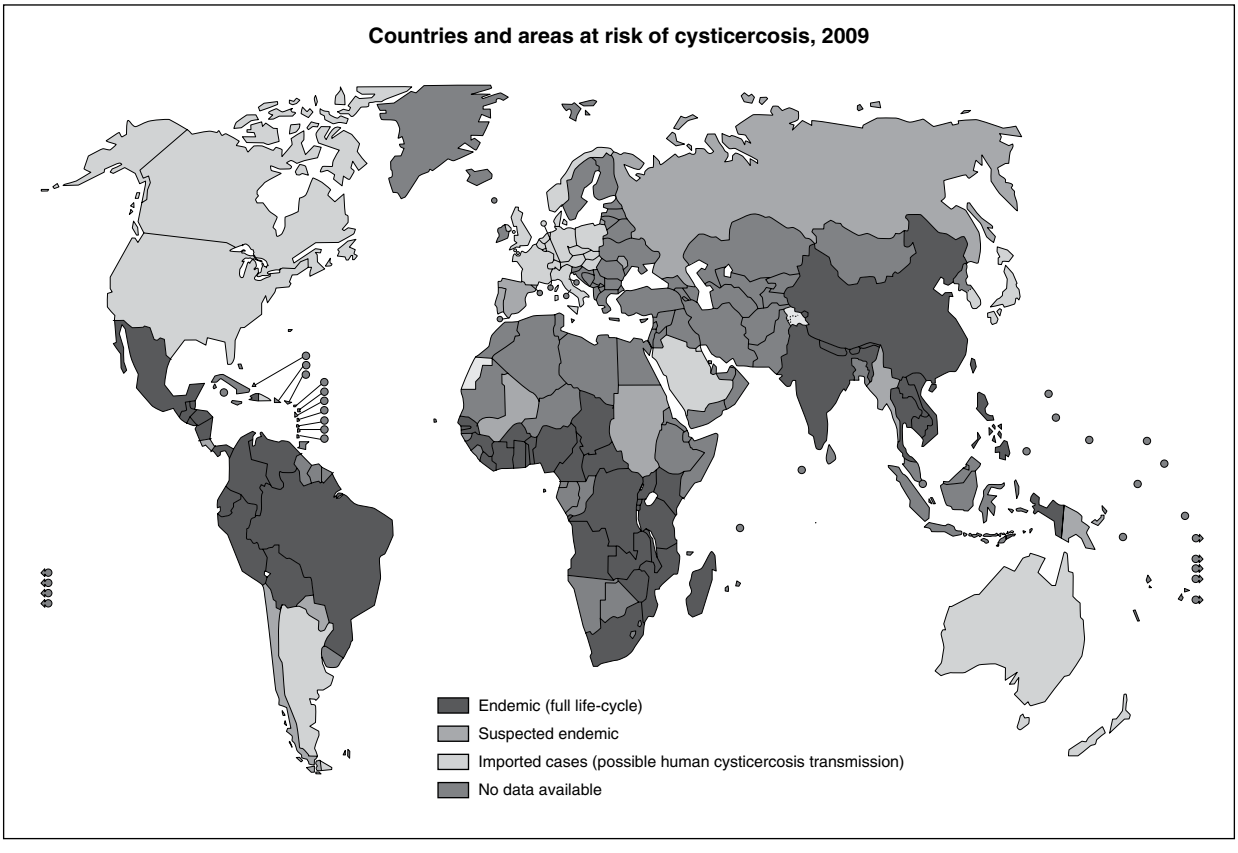

Figure 20.4 Map depicting the distribution and intensity of human cysticercosis. Reproduced from: First WHO report on neglected tropical diseases. WHO. Edited by DWT Crompton, 2010. © World Health Organization 2010.

parasite-specific products present in host feces that can be detected by immunologic techniques. Diagnosing human taeniasis by an enzyme-linked immunosorbent assay (ELISA), without necessarily observing eggs in feces, represents a significant advance. The assay can detect as little as 35 ng protein/ml of adult parasite antigens in stools. The sensitivity depends on the assay format employed and the quality of the immunized rabbit serum used to capture antigens. A high titer rabbit serum offers a higher sensitivity. Rabbit anti-*Taenia* antiserum is not commercially available at this time so that antibody titers and avidity may vary. Coproantigen detection by ELISA has already been applied for screening all three species of taeniasis in several field studies. Interestingly, parasite antigen can be found in prepatent infections and becomes undetectable shortly after removal of the adult worm, therefore it is very useful for epidemiological surveys and it indicates treatment success.

Since clinical manifestations of neurocysticercosis are nonspecific, most neuroimaging findings are not pathognomonic, and some serologic tests have low sensitivity and specificity. Thus a consensus was reached by a group of experts in the disease that provides diagnostic criteria for neurocysticercosis, based on objective clinical, imaging, immunologic, and epidemiologic data. These include four categories of criteria stratified on the basis of their diagnostic strength, as follows:

1. Absolute: histological demonstration of the parasite from biopsy of a brain or spinal cord lesion, cystic lesions showing the scolex on computed tomography or magnetic resonance, and direct visualization of subretinal parasites by funduscopic examination;
2. Major: lesions highly suggestive of neurocysticercosis on neuroimaging studies, positive serum enzyme-linked immunoelectrotransfer blot, commonly known as western blot, for the detection of anticysticercus antibodies, resolution of intracranial cystic lesions after therapy with albendazole or praziquantel, and spontaneous resolution of small single enhancing lesions;
3. Minor: lesions compatible with neurocysticercosis on neuroimaging studies, clinical manifestations suggestive of neurocysticercosis, positive cerebrospinal fluid ELISA for detection of anticysticercus antibodies or cysticercal antigens, and cysticercosis outside the central nervous system;
4. Epidemiologic: evidence of a household contact with *T. solium* infection, individuals coming from or living in an area where cysticercosis is endemic, and history of frequent travel to disease-endemic areas.

Interpretation of these criteria permits two degrees of diagnostic certainty: (a) definitive diagnosis, in patients who have one absolute criterion or in those who have two major plus one minor and one epidemiologic criterion; and (b) probable diagnosis, in patients who have one major plus two minor criteria, in those who have one major plus one minor and one epidemiologic criterion, and in those who have three minor plus one epidemiologic criterion.

20.6 Prevention and control

As knowledge of the global burden of cysticercosis/taeniasis due to *T. solium* is gained, the impact of the disease in health systems and animal husbandry becomes evident. Also, because the disease is related to poverty, any control strategy has to consider costs and

available resources. The most practical is the follow up by health institutions of cases of human cysticercosis (patients with brain cysticerci or subcutaneous nodules being notified to the National Health System) because this way tapeworm carriers can be identified and treated, since 'hotspots' (households with intestinal *Taenia*) have been associated to the disease.

Prevention can also be achieved if infected beef and pork are not sold; with adequate sanitary inspection at slaughter, meat has a higher commercial value. Meat has to be carefully checked, especially since the most frequently infected muscles may not harbor parasites, such as the heart in beef and cheeks in pigs, as well as lack of liver inspection in Asian countries. Pig breeding is a common practice among rural people, mainly because expenses are small and returns may be high. Pigs that freely roam complement their feeding with garbage and feces, in this way they also help to keep the community clean. Confining swine is costly, therefore if a control strategy is implemented it should offer economic incentives to small breeders to adopt adequate pig breeding practices.

Many people, including local populations in endemic areas, seldom understand the biological relation between a tapeworm and swine cysticercosis, and thus are unable to change practices in order to stop transmission. Health education must always be part of a control program because, if properly transmitted, it is long lasting and has shown high efficacy in generating consciousness regarding the importance of human and swine cysticercosis, and in understanding the feasibility of eliminating it. Health education was very well received by a rural population in Mexico because well-trained sociologists organized the intervention; this suggests that specialized personnel have to elaborate ad hoc programs for this purpose. Educational materials should be designed so that the reader finds them attractive and easy to understand. In the case of swine cysticercosis, the economic advantage of having healthy pigs should be emphasized, as was recently done in Tanzania, where the purpose of the information was 'breed healthy pigs, win more money', and in a book for children which explained the life cycle and indicted that if pigs are healthy, parents can pay tuition for their children for school. Krecek in South Africa prepared and distributed a poster in medical and veterinary offices, animal husbandry facilities, markets, schools, and even through the internet. It was prepared in such a way that, with minor illustrative adjustments, the text can be changed to any African language. Population-based cestocidal treatment intended to eliminate all possible tapeworm carriers is also a good alternative, but has been evaluated in only two population based studies: Ecuador and Mexico.

For over 20 years, research has been undertaken towards the evaluation of vaccination of pigs as an alternative measure for stopping *T. solium* transmission. Recently, a recombinant vaccine named TSOL18 has been demonstrated to induce close to 100% protection in swine in experimentally controlled studies and in field trials performed in Mexico, Peru and Cameroon. If prevalence of swine cysticercosis is reduced by efficient vaccination, the *T. solium* life cycle would be interrupted, as would transmission to humans. Furthermore, an efficient vaccine would reduce the low impact of insufficient chemotherapy for treatment of tapeworm carriers or their introduction to naïve areas. Nonetheless, since target populations for vaccination are those in rural and poor endemic areas of developing countries, the vaccine has to be cheap and accessible. TSOL18 seemingly has gained interest from the Global Alliance for Livestock Vaccines (GALV) that could arrange its production and distribution in Africa, where swine cysticercosis is found wherever it is being looked for.

Finally, neurocysticercosis may no longer be a public health problem in Mexico probably due to three factors:

1. The Mexican trends in publication over several decades: areas related to clinical aspects peaked from 1980–1990 and then declined; epidemiological studies began a decade later and increased modestly, whereas articles on basic biology have proliferated since 1991. These trends indicate a shift in scientific interest over the years from clinical and epidemiological aspects to basic research. This distribution parallels the decrease in cysticercosis in Mexico, because the information generated from immunological and imaging diagnosis, clinical case descriptions, evaluation of cestocidal treatment and epidemiological studies probably promoted publication of the Official Mexican Policy (NOM, from its name in Spanish) for Control and Prevention of Taeniasis/Cysticercosis in 1994 that was revised in 2004.
2. The aim of the NOM was to establish criteria, strategies and operational techniques for the implementation of preventive and control measures of taeniasis and human and porcine cysticercosis. This policy is mandatory throughout the country for all health staff providing care in public, social and private institutions as well as for professional and technical agricultural personnel, animal husbandry veterinarians engaged in private practice, in pig farms, producers, pig owners and anyone involved in the transport and commercialization of this species. The NOM also mandates that intestinal tapeworm carriers should be treated with a single oral dose (10 mg/kg) of praziquantel. When the NOM was published the Ministry of Health also designed and printed hundreds of thousands of leaflets with basic information aimed at different populations: pig farmers, butchers, cooks, food vendors and the general population; the pamphlets were distributed nationwide.
3. Social, economic and health conditions have improved substantially in recent decades in Mexico, including those related to neurocysticercosis, and this is also a relevant factor. It is important to note that this information indicates that cysticercosis in Mexico has been controlled but not eradicated (which from the biological standpoint is almost impossible), thus it is necessary to maintain active surveillance and health measures along with health education to maintain and even improve the current situation.

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21 Other foodborne helminthes

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21.1 Introduction

In most developed countries, the relationship between parasites and food safety has all but fallen off the radar with respect to consumer concern. The main drivers of complacency are modern inspection methods coupled with the understanding and acknowledgment that commercially-raised animals and properly-prepared foods offer products to the consumer that pose minimal risk. That being said, consumers incorrectly assume that organically-raised foods equate to pathogen-free foods. Clearly, these are not mutually exclusive and, indeed, may be inversely related. The increase in free-range and hunted animals as food sources has thus resurrected interest in zoonotic parasites as a source of human infection worldwide. Equally important is the escalation of anthropogenic effects resulting in the unwanted and uncontrolled movement of parasites to geographical areas where the pathogen was once considered nonendemic. Such movements have become far more prevalent in aquatic diseases, but the rapid rise in the feral swine population in the US and therefore transmission of associated diseases is one example of how this can affect the commercial swine industry.

Parasitic infections can be broadly divided into protozoan diseases such as cryptosporidiosis and toxoplasmosis and helminthic diseases such as taeniasis derived from beef or pork tapeworms. In this chapter, we would like to draw your attention to a few others that seem no longer to be given their due as human pathogens, but fall into one or more of the categories defined above and therefore warrant discussion.

In particular, this chapter reviews parasites of the genus *Trichinella*, which are gastrointestinal and tissue parasites once believed to be only contracted from swine but now known to be capable of infecting all mammals. In addition, we review three other helminths, all of which are derived from fish. These include the fish tapeworm *Diphyllobothrium* and two nematode parasites of the genera *Gnathostoma* and *Anisakis*.

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21.2 *Trichinella* sp.

21.2.1 Background

Trichinella spiralis was first described as a human pathogen in 1835 by Richard Owen (1835) with the help of a first-year medical student, James Paget. An autopsy performed on an Italian bricklayer who presumably died of tuberculosis turned up some very strange looking microscopic worms 'coiled up' in small nodules we now know to be enlarged muscle cells; hence the species designation 'spiralis' (Fig. 21.1). For many years following, the genus was thought to be monospecific; however, by the early 1970s, biological differences started to emerge among geographical isolates that led the scientific community to conclude that the genus was comprised of more than just one 'type' of *Trichinella*. Since that time, the number of different organisms encompassing this genus has grown dramatically; it is now believed to consist of eight species and at least four additional genotypic variants that have yet to be taxonomically defined. Other than for the presence of a collagen capsule that forms around the infected muscle cells of one group of *Trichinella* parasites, the organisms making up this genus are morphologically indistinguishable.

Today, two clades are recognized in the genus: one that becomes encapsulated following maturation in the muscle tissues and a second that does not. Those that comprise the encapsulated clade seem only to infect mammals. Among the three species that collectively make up the nonencapsulated clade, all can infect mammals as well; however, one species is also infectious for avian hosts and the other two can infect reptiles.

In recent years, infections of *T. spiralis* originating from pork meat – the most common source of human trichinellosis – have dwindled or become nonexistent in many developed countries throughout the world; however, infections originating from sylvatic hosts are on the rise. Among these outbreaks, infections derived from *T. spiralis* are less

Figure 21.1 *Trichinella spiralis* after tissue digestion presented in its typical coiled form. (Photograph courtesy of Dr. Dolores Hill, USDA, ARS, Animal Parasitic Diseases Laboratory USA).

common than infections resulting from one of the other *Trichinella* species or genotypes. A recent study showed that among the 4700 isolates of encapsulated *Trichinella* spp. thus far genotyped, approximately 45% were identified as *Trichinella britovi*, 40% as *T. spiralis*, and 10% as the freeze-resistant *Trichinella nativa*. All other encapsulated taxa combined account for only 5% of reported infections. Though the genus is believed to be quite ancient, originating during the Paleozoic, extant species are considered to be less than 20 million years old and, unlike the sylvatic species and genotypes, the genetics of *T. spiralis* is quite uniform worldwide except for a small group of isolates originating in Asia.

21.2.2 Life cycle

The life cycle of *Trichinella* is quite unique among parasitic nematodes. Whereas most nematodes have need for multiple hosts to complete their life cycles and often require a free-living stage to accomplish this task, *Trichinella* does not have a free-living stage and completes its entire life cycle within a single host. Furthermore, unlike most nematodes, *Trichinella* has both extracellular and intracellular stages.

If we begin with the encysted or intracellular stage, otherwise referred to as L1 or muscle larva, infected meat is first ingested by the unsuspecting host. The stomach enzymes (pepsin) in the presence of low pH (HCl) digest the muscle fibers surrounding the larvae and release them into the surrounding medium. Within hours, the larvae migrate to the jejunum and ileum where they burrow into the lamina propria of the villi. There they rapidly undergo four molts and by the third day become sexually mature. The sexually mature adults re-enter the lumen of the small intestine, where copulation takes place; the adult males die shortly thereafter. The females, on the other hand, re-enter the mucosa harboring fertilized eggs and by day 5–6 post infection, begin releasing newborn larvae (NBL) into the lacteals of the villi. This deposition of larvae can continue for weeks until each female has released 1000 or more NBL.

The NBL are carried first through the intestinal lymphatic system to the local lymph nodes, then on to the thoracic duct and the venous blood where they quickly enter peripheral circulation. The NBL have an absolute requirement for striated muscle cells and cannot live long outside. Studies have shown that most larvae find muscle cells within minutes to a few hours after entering the circulatory system. Approximately 40% of circulating larvae are removed each hour in circulation and by 3 hrs after release, 75% of NBL have disappeared. Little information is available as to the fate of all NBL. It is believed that, during migration, the larvae penetrate many different types of unaccepting tissues and either die there or migrate out and begin the search once again for a striated muscle cell. The parasite uses mechanical rather than biochemical methods to penetrate the sarcolemma of the muscle cell. Once inside, the larvae reprogram the muscle cell into a 'nurse cell' to support their growth and development without causing death to the cell. *Trichinella spiralis* becomes infective by the 14th day after infection. Over the next 30 days, depending on the species, the muscle larvae will grow to 1200 μm in length, coil within the cell possibly to conserve space and energy, and then await the process to begin anew. The fate of larvae that are not passed to a new host is the calcification of the nurse cell which in turn denies the parasite of host nutrients and eventually causes death. Clearly, humans are a 'dead end' host; however, the ability of *Trichinella* species to infect nearly all animals and the extended longevity of some species in tissues from sylvatic hosts provide an ideal system to perpetuate the species.

21.2.3 Epidemiology, prevention and control

The size of the genus, the ability of the various *Trichinella* species and genotypes to infect a broad range of hosts, and the cosmopolitan distribution of these organisms complicate the epidemiology. However, inasmuch as humans are a dead-end host, trichinellosis is best understood in the context of the epidemiology in animals. In general, one can partition transmission into two cycles, the domestic or synanthropic cycle, and the wildlife or sylvatic cycle. Clearly, humans are linked to both. Also, interactions can occur where these two cycles overlap.

Trichinella has been identified on all continents except Antarctica; however, there have yet to be any epidemiological studies performed in this region of the world. Sources of human infection include domestic livestock, i.e., pigs and to a much lesser extent horses, and hunted wild mammals such as bear, feral pigs, and foxes. In the United States, the rapid and unwanted expansion of the feral pig population in conjunction with the presence of *Trichinella* species including *T. spiralis* in wild animals has the potential to escalate the number of human infections in the coming years. In general, the most common hosts for harboring *Trichinella* infections are those belonging to the Suidae (52%) and Canidae (37%) families. Rodent and small farm animal infections are more highly linked to livestock infections rather than acting as an infiltrating reservoir from the surrounding ecosystem.

By definition, the domestic cycle must involve pigs. Surprisingly, the United States is among a select few developed countries that do not inspect pork products for the presence of *Trichinella*. Consumers are instructed to cook their pork to 145 °F/63 °C or freeze it for a minimum of 14 days at –10 °F/–24 °C to guard against unwanted transmission. It should be noted that ordinary curing and smoking does not kill *Trichinella*. It should also be noted that, while freezing works for *T. spiralis*, there are species of *Trichinella*, i.e., *T. nativa* and the *Trichinella* T6 genotype, which are quite resistant to freezing and can only be killed by adequate cooking. Surprisingly, the freeze-resistant genotypes comprise greater than 10% of genotypes isolated identified to date. However, given that most other types of *Trichinella* do not infect pigs well, the etiologic agent derived from domestic pigs is relegated to *T. spiralis.*

In developed countries, infections in the domestic food supply originating from pigs have been addressed by excluding uncooked food scraps from their feed and by raising pigs under confinement, thereby eliminating interaction with sylvatic sources of infection. However, it has not always been this way. A 1943 report from the National Institutes of Health estimated that approximately 17% of the US population was infected with *Trichinella*. By imposing controls over how pigs are managed, and the production and sale of processed pig meats which comprise 50% of commercially sold pork, reported infections have been reduced to less than 10 documented cases of trichinellosis per year in the US (Fig. 21.2). Among these cases, most now originate from wild game or from small, noncommercial farms that raise free-range pigs (Fig. 21.3). Initially, horses were never considered a viable source for human infection; however, several outbreaks in France resulting in thousands of infections and several deaths were eventually linked to *Trichinella murrelli* and horsemeat imported from the US. This dispelled theories that herbivores could not be sources of human trichinellosis. In certain regions of Eastern Europe, it was determined that horses are often fed and willingly consume animal products and garbage. It has since been documented that at least three species contribute to human trichinellosis derived

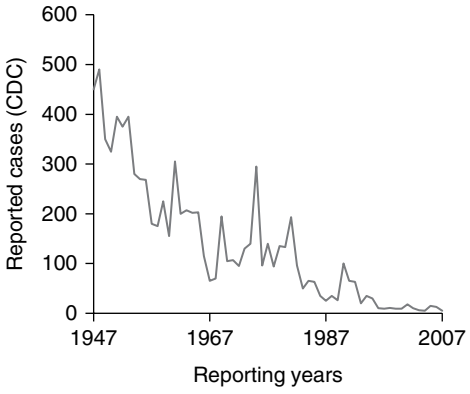

Figure 21.2 Reported cases of trichinellosis in the United States, 1947–2007. *Source*: Center for Disease Control and Prevention; www.cdc.gov

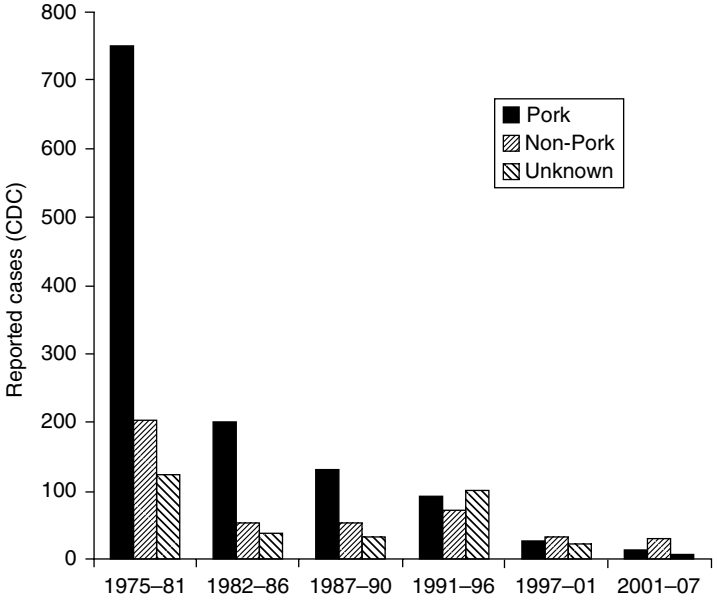

Figure 21.3 Sources of reported cases of trichinellosis in the United States, 1975–2007. *Source*: Center for Disease Control and Prevention; www.cdc.gov

from horsemeat, *T. spiralis*, *T. britovi*, and *T. murrelli*. In 2006, due to public outcry, the inspection and therefore slaughter of horses for human consumption was banned in the US; however, this ban was lifted in November 2011.

In contrast to developed countries, outbreaks of human trichinellosis persist in second and third world countries. Among those countries and regions showing high infection rates are China, Southeast Asia, Russia, Romania and Serbia. These are often the result of ethnic dishes being prepared from uncooked or improperly cooked meats. In other

cases, changes in pork production systems resulting from political and social turmoil have also been linked to the rise in infection rates; predominantly during the period ranging from the late 1980s through the early 1990s. Other notable areas are Argentina, and central and South America. In general, most these infections have been linked to pork products and *T. spiralis*. However, within these regions, infections can also involve more species than just *T. spiralis*; in Western Europe, for example, *T. britovi* predominates in wildlife and, unlike most sylvatic isolates, is capable of infecting boar and feral swine. Surprisingly, the geographical distribution of sylvatic *Trichinella* species is quite regional. Among the most common encapsulated species, *T. murrelli* is relegated to North America and in particular the US, *T. britovi* is predominantly found in Western and Central Europe, and the freeze-resistant genotypes *T. nativa* and *Trichinella* T6 are most exclusively observed in the Holarctic with rare incursions observed below the January –4-(–5)°C isotherm. Only *T. spiralis* has the most widespread distribution and these isolates exhibit a high level of genetic homogeneity throughout all regions of the world (except Asia) whereas Western Europe is considered the modern origins of *T. spiralis*. This species eventually was disseminated to North and South America as a result of modern man traveling westward.

21.2.4 Clinical manifestations

For the most part, trichinellosis goes either undiagnosed or incorrectly diagnosed because the clinical signs are not unlike those of the common flu, or because the low levels of infection that most people are commonly subjected to rarely generate meaningful clinical signs. In those cases where an individual is exposed to substantial numbers of larvae, different manifestations are exhibited in the enteral (intestinal) and parenteral (muscle) phases. During the enteral phase, abdominal discomfort, diarrhea, nausea and vomiting are most common and these occur as soon as one to two days after ingestion of infected meat. Occasional life-threatening manifestations include myocarditis, central nervous system involvement, and pneumonitis. Myocarditis and even cardiac failure have been linked to the migratory phase of the infection and the damage caused by the NBL as they try to find a suitable home in striated muscles.

The parenteral phase usually begins 2–3 weeks after infection and after the NBL have started developing within the muscle cells. Symptoms – which can continue for several weeks thereafter – are usually characterized by muscle aches, joint pain, fever, chills, periorbital and facial edema, itching, rashes, conjunctivitis, and peripheral eosinophilia among others. Neurologic involvement is not common (10–24%) but can result from migrating larvae invading the brain. Studies have been performed which show that, in severe cases, small lesions in the cerebral cortex and white matter can be identified using CT or MRI scans. However, diagnosis is generally difficult given that muscle biopsy is only suggestive of neurologic involvement provided other symptoms have presented themselves. Symptoms associated with acute illness generally diminish by 5–6 weeks after ingestion of infected meat.

Complicating the matter of clinical manifestations is the lack of uniformity in host responses to species other than *T. spiralis*, most notably, the freeze-resistant *T. nativa*, *T. britovi* and *T. murrelli. Trichinella nativa* has been characterized by excessive diarrhea; however, fewer and less severe symptoms normally associated with the enteral phase have been observed, and parenteral symptoms are virtually nonexistent. In addition, the onset of enteral phase symptoms is substantially delayed relative to *T. spiralis*. It was postulated that

these changes are linked to prior exposure to the parasite. This hypothesis was supported in a Canadian outbreak that occurred in 2000 where the etiologic agent was identified as *T. nativa*. In this case, the symptoms were not unlike those observed in classic infections derived from *T. spiralis*. Nonetheless, the symptoms did not manifest themselves until 30 days post-infection.

It has been reported that individuals infected with *T. britovi* experience milder disease, longer incubation periods, and reduced intestinal symptoms relative to individuals infected with *T. spiralis* infections. These observations were recently confirmed in an outbreak that occurred in Turkey involving 1098 individuals where no deaths were reported and the symptoms were considerably less severe than normally observed with *T. spiralis*. To date, there has been only one report of clinical signs resulting from a *T. murrelli* infection. Results from this one study suggest that encapsulation in humans occurs much later (10 weeks) than with *T. spiralis*, thereby resulting in differences in clinical manifestations. Delayed encapsulation was also observed in mice, albeit not to this extent. In this same study, viable muscle larvae were found in infected patients at least 6 yrs post-infection.

21.2.5 Diagnosis and treatment

As noted above, there is a plethora of visual signs leading one to a preliminary diagnosis of trichinellosis. In addition to visible signs, final confirmation is predicated upon additional biochemical studies as well as epidemiological information. High eosinophilia and increased serum creatine phosphokinase, transaminase and other muscle enzymes are the most frequently-observed features. The increased levels of muscle enzymes may occur in concert with muscle atrophy and contractions; however, one cannot draw conclusions regarding the level of infection from these data. Other common attributes of infected patients are arthralgia, leukocytosis (>10,000/mm^3) and eosinophilia (>1000/mm^3). The detection of *Trichinella*-specific circulating antibodies and, when available, the examination of muscle biopsies will confirm the visible signs of trichinellosis. Dupouy-Camet et al. (2002) developed an algorithm for using available information to arrive at a meaningful diagnosis of trichinellosis absent a muscle biopsy. In addition to diagnosis of the disease, DNA-based tests exist to inform the clinician as to the species or genotype of *Trichinella*. This information can be very helpful in explaining not only the patient's symptoms, but also the origins of the infection. Of course, positive muscle biopsy is required to perform these DNA-based tests.

Usually, by the time an adequate diagnosis of infection can be made, the infection is already several weeks old. Nonetheless, medical treatment is generally directed at the enteral phase of the infection and, for the most part, the symptoms. Data showing elimination of worms from the muscles are equivocal and dependent upon the time, duration, dosage and choice of treatment. To address the parasitemia, nonpregnant patients and those greater than 2 years of age are treated with anthelmintics such as mebendazole (5–10 mg/kg/day) or albendazole (15 mg/kg/day) for 10–15 days. These drugs have been shown capable of ameliorating symptoms of the muscle stage. Pyrantel (10 mg/kg) for 4 days and levamisole (2.5 mg/kg/day) have been used as well but target only the intestinal stages of the infection. Anthelmintics are also accompanied by glucocorticosteroids such as prednisolone (30–60 mg/day for 10–15 days) to help alleviate inflammation and other symptoms of the disease.

The cosmopolitan nature of *Trichinella* species in conjunction with the overlap among the domestic and sylvatic cycles suggest that elimination of infection will

continue to be a challenge where confinement facilities for domestic pigs and well-developed inspection procedures for consumed pork products are lacking. Those individuals with a long-standing heritage in the preparation of ethnic foods will always be at higher risk. Attempts have been to develop vaccines against the parasite, but these attempts have thus far been unsuccessful. Natural antigens derived from homogenized parasites, larval secretory products, synthetically-produced tyvelose side chains, which are highly immunodominant in the ML stage, and a plethora of recombinant proteins, many of which focus on secreted products, have all met with limited success. For these reasons, cooking and freezing meats along with continued education about the risks of infection from non-commercial sources of meat remain the best avenues to limit human disease going forward.

21.3 *Diphyllobothrium* spp.

21.3.1 Background

Diphyllobothriids represent a group of pseudophyllidean tapeworms comprising at least 13 species where the adults reside in the intestine of fish-eating birds and marine or terrestrial mammals. In some areas of the world, a few species have been reported with relative frequency in people who consume marine or anadromous fish containing infective larvae (plerocercoids). At least eight species of *Diphyllobothrium* are known from marine mammals; however, other terrestrial mammals may acquire the infection, such as dogs which are especially receptive if they are fed on raw fish. In contrast to the anisakid nematodes which generally infect man as the larval stage only, diphyllobothriids can grow and mature in the human intestine and produce a zoonotic disease known as diphyllobothriasis. For years, most human cases in the old and new worlds were thought to be caused by a single species, the broad fish tapeworm *D. latum*; however, it is now known that other congeners such as *D. pacificum*, *D. nihonkaiense*, and *D. klevanobskii* are also found in humans in South America, Japan, and the Russian Far East, respectively. In Alaska, six species of the genus have been recorded in humans, i.e., *D. latum*, *D. dendriticum*, *D. lanceolatum*, *D. urse*, *D. dalliae*, and *D. alascense*, of which *D. latum* is the most common. At least 13 congeners have been implicated in human cases (Table 21.1). Anadromous fishes such as salmon (*Onchorhynchus* spp.) may become infected during both the freshwater and marine phases of their life cycles. As such, it is possible that humans may become infected by eating fish from marine or freshwater environments. In the marine habitats, salmon acquire plerocercoids of diphyllobothriids for which marine mammals are supposed to be the definitive host. However, the commercial distribution of salmon contributes to the infection of people in localities widely separated from the coast. The current distribution of species of *Diphyllobothrium* is confusing, as *D. latum* is still diagnosed in areas where the main sources of infection are marine salmonids and where there is no history of freshwater diphyllobothriasis. In recent years, molecular methods have proven to be very useful in identifying the species causing human diphyllobothriasis. For instance, some authors were able to identify *D. nihonkaiense* from a human in France; however, this person was infected by eating Pacific salmon from Canada.

In order to identify potential trends in the way researchers have focused on describing different aspects of *Diphyllobothrium* spp. and the zoonotic disease (diphyllobothriasis) caused by some of the other species, we surveyed literature referenced in ISI Web

Table 21.1 Species of *Diphyllobthrium* Reported from Humans and their Geographic Distribution

Species	Geographic distribution
Diphyllobothrium alascense	North America: Kuskokwim Delta, Alaska
Diphyllobothrium cameroni	Japan
Diphyllobothrium cordatum	Northern Seas, Greenland, Iceland
Diphyllobothrium dalliae	North America: Alaska. North Asia: Siberia
Diphyllobothrium dendriticum	Circumpolar; introduced elsewhere
Diphyllobothrium hians	North Atlantic; Probably in the North Sea
Diphyllobothrium klebanovski	Eastern Eurasia: Sea of Japan, Sea of Okhotsk; Probably in Alaska
Diphyllobothrium lanceolatum	North Pacific, Bearing Sea
Diphyllobothrium latum	Fennoscandia, western Russia, North and South America; reported from Cuba, Korea
Diphyllobothrium nihonkaiense	Japan
Diphyllobothrium pacificum	Peru, Chile, Japan
Diphyllobothrium ursi	North America: Alaska, British Columbia
Diphyllobothrium yonagoensis	Japan, eastern Siberia

of Science (http://scientific.thomson. com/products/wos/) containing the phrases '*Diphyllobothrium*' or 'Diphyllobothriasis' in the title, abstract or keywords, and found >493 and 64 references, respectively. In the last decade, 125 papers out of the 493 were published referencing some aspect of the biology of *Diphyllobothrium* species, including the taxonomy, evolutionary biology, biogeography, as well as isolated reports on the presence of some of the species as human parasites. This clearly indicates a renewed interest in this tapeworm. Needless to say, there are many other bibliographical sources not considered by the ISI Web of Science, considered as grey literature, that deal with diphyllobothriasis from an informational point of view, where the main highlights of the disease are mentioned.

21.3.2 Life cycle

Humans and fish-eating mammals, either marine (such as seals and sea lions) or terrestrial (such as bears and foxes), and a few fish-eating birds such as the Herring gull are considered definitive hosts. Marine or freshwater copepods and fish are the intermediate hosts. There are two ways in which the life cycle of diphyllobothriids may be completed: either through a human-to-human infection via fish or through a sylvatic life cycle that involves wild animals and fish, where humans become infected through the consumption of infected tissues. Numerous fish species are known to be infected with the plerocercoid, e.g., the Arctic char (*Salvelinus alpinus*), the Rainbow trout (*Onchorhynchus mykiss*), Northern pike (*Esox lucius*), Yellow perch (*Perca flavescens* and *P. fluviatilis*), Burbot (*Lota lota*), Sockeye salmon (*Onchorhynchus nerka*), etc. The plerocercoid is present in the fish musculature and infects humans following the consumption of raw, inadequately cooked or minimally processed fish. Adult tapeworms are found in the intestine of their definitive hosts. These cestodes are among the largest

parasites of humans since they can reach between 2 and 15 m in length. Pieces of the strobila or just operculated eggs are passed in feces. Once they reach the water, they develop after a 14-day period and hatch into the free-living larvae (coracidium). Coracidium must be eaten during the next 12 hrs by the first intermediate host, a copepod (*Cyclops* spp. or *Diaptomus* spp.) for the life cycle to continue. Once ingested, the larvae penetrate the gut and enter the hemocoel and develop into the next larval stage, the plerocercoid, in a 10–21-day period. The copepod is then ingested by a suitable second intermediate host and, once in the intestine, penetrates the gut wall to enter the body cavity where it encysts and matures into the plerocercoid larvae. In the case of *D. latum*, the procercoid enters the fish muscle and matures into a plerocercoid. The definitive hosts – i.e., birds, wild mammals, or humans – become infected when they feed upon fish with the plerocercoid which is located either in the body cavity or the flesh. The life cycle of this tapeworm may involve a third intermediate host represented by a large piscivorous fish. If that fish becomes infected, the plerocercoid reinvades the muscle of that fish and, if eaten raw or uncooked, enters the intestine of the definitive host and matures within a 5–6 week period.

21.3.3 Epidemiology of human diphyllobothriasis

As in the case of anisakids, the transmission of diphyllobothriids to humans is via the foodborne route through fish dishes that are common in specific parts of the world. This implies the consumption of raw or undercooked fish, either marine or freshwater. Diphyllobothriasis is commonly associated with cold waters, i.e., it results from a circumpolar and boreal parasite, in North America, Eurasia, Japan and South America; however, sporadic reports have surfaced from places such as Cuba, the Middle East and Malaysia. Several human case reports indicate that the disease has declined in some countries but increased in others such as Chile, Peru and Argentina. Apparently in this region of the world, diphyllobothriids were thought to be introduced by fish stocking and human immigrants. Now, however, infections derived from native fish species and Pacific salmonids are being reported. Some hypotheses indicate that tapeworms were there long before human immigration.

The most common diphyllobothriid, *D. latum*, is considered as an anthropogenic cestode, especially in the holarctic region, even though at least seven species of pinnipeds including walrus, harbor seal, ringed seal, hooded seal, Mediterranean monk seal, and harbor porpoise may become infected. The number of human cases of diphyllobothriasis is uncertain because many infections are undiagnosed as symptoms are nonspecific and it is not generally considered to be a serious disease. In addition, since diphyllobothriasis is not a severe disease it is usually not reported to health authorities. A recent estimation indicates that the number of cases of this disease worldwide is around 20 million. However, the global distribution of the disease is only partially known because some reports are based on human cases, but most papers report infections occurring in the fish intermediate hosts, or even in marine mammals or fish-eating birds as definitive hosts. Based on the widespread distribution of *Diphyllobothrium* spp. in the world and the way this tapeworm is transmitted among their hosts, it is possible to find the parasite in aquaculture systems resulting from fish becoming infected by feeding on either the first intermediate host or other infected fish. Infection levels in aquaculture systems are expected to be low if fish are maintained on artificial feeds. Needless to say, this zoonosis occurs most frequently in areas of the world that have food preferences for

wild-caught fish consumed raw or undercooked. Additionally, the life cycle of species of *Diphyllobothrium* is maintained primarily in the wild, independent of humans. Thus, the tapeworm life cycle will not be affected if eliminated from the human population. This parasite can be easily disseminated considering the host preferences for marine and freshwater fish, piscivorous birds, and mammals such as bears, cetaceans, pinnipeds, and even humans.

Human settlements are another risk factor that has caused the introduction and maintenance of the zoonosis in human communities due to the contamination of local aquatic environments with feces. Domestic animals, and particularly dogs, are another source of infection of this zoonosis, and those fed on raw fish help to maintain the parasite life cycle. As pointed out by several authors, diphyllobothriasis exists as a zoonotic disease within a continuum that links animal (wild and domestic) and human populations, and this equilibrium may be altered by anthropogenic disturbances which include the intensification of fish production, translocation of human and animal populations, and cultural changes in eating habits.

21.3.4 Clinical signs, diagnosis, and treatment

Most people who are infected with *Diphyllobothrium* have no clinical signs. If symptoms occur, these may include abdominal discomfort or pain, diarrhea, weakness, weight loss and anemia (caused by vitamin B12 deficiency). In some severe infections, obstruction of the intestine may occur, and sometimes pieces of the worm are vomited. Rarely, migrating proglottids can cause inflammation of bile ducts or the gall bladder. Tapeworms may remain active living in the human intestine, releasing eggs or even proglottids within the feces, causing no apparent damage to infected individuals. Researchers have actually been infected experimentally with *D. latum* where the symptoms of infection were nonspecific and mild.

Diagnosis of this disease is very simple and requires the identification of the causative agent by examining a stool sample to find either the eggs or proglottids. Identification is usually accomplished to genus level; however this is irrelevant when treating the disease since all *Diphyllobothrium* species respond to the same drugs. Molecular probes will be particularly useful to help identify the sources of infections of human cases, and can be used on purified eggs, or in proglottids. On the other hand, identification of the plerocercoids of diphyllobothrids from fish by morphological criteria remains troublesome. As such, DNA technology is becoming more important in diagnosing potential diphyllobothrids in the fish flesh that may cause the disease after human consumption.

In the past, diphyllobothriasis has been treated with praziquantel or niclosamide, but apparently these drugs are not reliable in cutting the scolex loose. If the scolex is left in the intestine, the tapeworm will eventually grow again. The best treatment is a diatrizoic acid injection into the duodenal wall. The use of this acid guarantees that the entire worm, including the scolex, is detached leaving nothing behind.

21.3.5 Prevention and control

As with all other fishborne diseases such as anisakiasis, diphyllobothriasis might be controlled by using drug therapy in the human population. However, since the life cycle of this tapeworm can be maintained in the wild, several prophylactic measures have to be considered to avoid the risk of infection. For instance, fish need to be properly cooked or, if

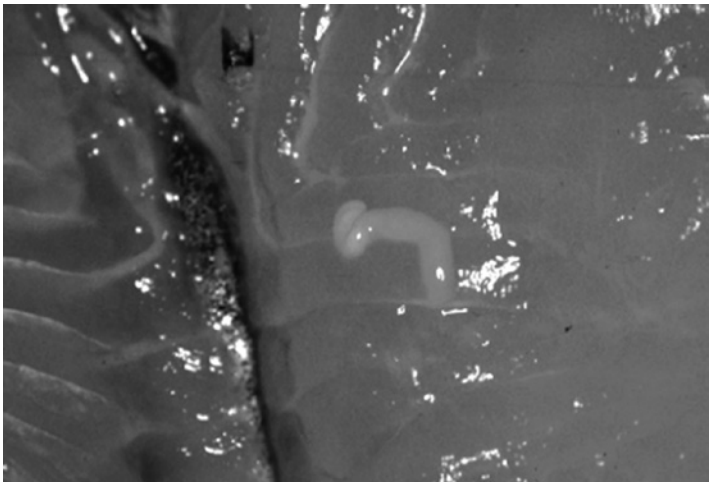

Figure 21.4 A plerocercoid larva of *D. nihonkaiense* in masu salmon, *Oncorhynchus masou masou* in Japan (*Source*: http:/fishparasite.fs.a.u-tokyo.ac.jp, with permission from the author)

eaten raw, it should be frozen at –10 °C (or below) for two days to kill the tapeworm larvae. Since the consumption of raw fish is the major problem, a careful inspection of raw fillets is highly recommended. Secondly, more strict regulations in the commercialization of the potentially infected fish species need to be established. A third measure that may help to mitigate the disease, particularly in endemic areas, is educating the locals to not defecating in water because, if the tapeworm larvae cannot reach the intermediate hosts, the life cycle is interrupted. In addition to that, pets such as cats and dogs need to be treated with anti-parasitic drugs on regular basis because they represent a source of infection to humans, especially in areas where they are fed on raw fish.

As previously mentioned, diphyllobothriasis is commonly associated with cold waters, and even though people who are infected with *Diphyllobothrium* are asymptomatic, infection with the tapeworm may cause problems in human populations in these endemic areas. This foodborne disease has persisted in endemic areas because the life cycle of diphyllobothriids may be completed through a human-to-human infection via fish, or through a sylvatic life cycle involving wild animals, fish and eventually humans. It is now known that numerous fish species can become infected with the *Diphyllobothrium* plerocercoid (Fig. 21.4). At least 60 reports and publications of human infections caused by *D. latum* and other diphyllobothriids have been listed in a 30-yr period (1975–2006) where areas not endemic to this zoonotic disease, such as Brazil, in recent years showed an increase in the number of human cases. This might be related to an increase in the commercial distribution of some fish species as salmonids that can contribute to the infection of people in localities where the life cycle of the parasite is not normally completed. As for Brazil, any other place in the world may become an area of the distribution of this tapeworm since it has the potential to emerge as a zoonotic disease in these new areas.

A clarification of species infective to humans should be a priority as a research program on Diphyllobothriasis in order to establish the source of infection so that public health measures can be taken. The use of molecular tools to identify the species of human

diphyllobothriasis in different areas of the world is necessary. It has also been suggested the need for a thorough study of the molecular biology of *D. latum* from the boreal region of North America, as well as an investigation into the origin of plerocercoids as the source of infection to humans. This is suggested in relation to commercial practices to avoid disseminating this zoonosis to areas currently free of infection. There is always a concern that the presence of this parasite may become established and entrenched in nonendemic areas if the transmission dynamics can be sustained by the local ecosystems.

21.4 *Gnathostoma* spp.

21.4.1 Background

Gnathostomiasis is a foodborne parasitic zoonosis and is a systemic infection caused by migrating nematode larvae of several species of the genus *Gnathostoma* (Nematoda). A total of 12 *Gnathostoma* spp. have been described; among these *G. spingerum* is the species that causes disease in humans although other species such as *G. hispidum*, *G. doloresi*, *G. nipponicum* and *G. binucleatum* can also infect humans. It is endemic in areas where people eat raw freshwater fish or shellfish, such as Thailand, Japan and other parts of Southeast Asia. An increase in the number of infections caused by this parasite has been reported in Latin America, particularly in Mexico. In nonendemic areas, an increase in the number of cases has been reported probably due to the fact that migration has increased substantially over the past few decades and people from the tropics and subtropics have settled in Europe and other Western countries. In many cases, they harbor the parasites and may develop the disease.

21.4.2 Life cycle

All species of the genus *Gnathostoma* have similar life cycles except for the second paratenic and definitive hosts. During this cycle, crustaceans and copepods act as first intermediate hosts while freshwater fishes and snakes serve as second intermediate hosts. Dogs, various felines, and wild mammals are the definitive hosts of this parasite. These animals harbor the adult parasites in a tumor-like tissue in the stomach or in the esophageal wall and eggs are passed into the feces to the environment and hatch in the water where they release the first-stage larvae. These larvae develop into second stage larvae when these are ingested by small copepods (*Cyclops* spp.). Third-stage larvae (Figs 21.5 and 21.6) develop and migrate through the tissues and encyst in the muscles of their transport hosts, where they remain as infectious larvae. These larvae are passed into a wide spectrum of paratenic hosts. The life cycle is completed when a definitive host ingests a second intermediate host infected with mature third-stage larvae (L3). Humans are paratenic hosts and they are infected by eating third-stage larvae encapsulated in raw or partially cooked freshwater fish, especially *Monopterus alba* (swamp eel), *Fluta alba* (eel), *Charias batrachus* (catfish), and *Channa striatus* (snake-headed fish). Once in the human host, the larvae migrate through various tissues causing symptoms related to the 'larva migrans.' Third-stage larvae from infected meat could also penetrate the skin of food handlers rather than being ingested. In any event, humans are the dead-end host since the female gnathostome fails to mature to the adult stage.

Figure 21.5 Advanced third-stage larvae of *Gnathostoma binuclaetum* from the muscle of the Pacific fat sleeper *Dormitator latifrons*. (Photograph courtesy of Sylvia Paz Díaz-Camacho Universidad Autónoma de Sinaloa, México)

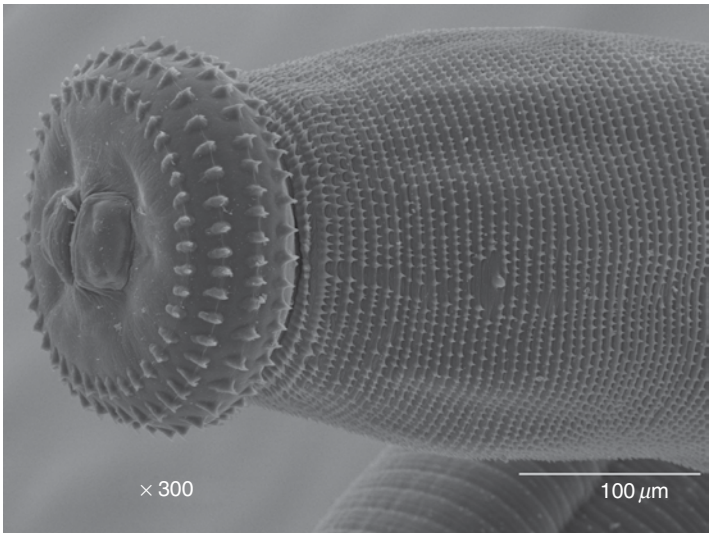

Figure 21.6 Scanning electron microscope micrograph of the advanced third-stage larvae of *Gnathostoma* sp. from the muscle of the Pacific fat sleeper *Dormitator latifrons* (Photograph courtesy of Berenit Mendoza-Garfias, Universidad Nacional Autónoma de México, México)

21.4.3 Epidemiology

The endemic areas of *Gnathostoma* spp. have been reported predominantly in Japan and Southeast Asia, particularly Thailand. Also, several reports have indicated that gnathostomiasis is present in Cambodia, Laos, Myanmar, Indonesia, the Philippines and some parts of China, Sri Lanka, and India. More recently, this disease has been considered a problem in Mexico (due to consumption of ceviche) and in Guatemala and South America (Peru and Ecuador). *Gnathostoma spinigerum* is widely distributed in Japan, Thailand, and Vietnam, and to a lesser extent in Bangladesh, Sri Lanka, Indonesia, Laos, Myanmar, and China. In America, the main agent causing gnathosomiasis is *G. binucleatum*, which is distributed in Mexico and Ecuador. Cases of gnathostomiasis have also been reported in Zambia and most recently in Botswana. Interestingly, the first proven endemically-acquired cases of gnathostomiasis in Australia was recently reported in Calder River in northern Western Australia. Dietary habits are the main cause for the transmission and expansion of the disease. Also, transmission is related to the consumption of food in local restaurants where almost no regulations are considered in the storage and fish consumption.

21.4.4 Clinical manifestations

Gnathostomiasis presents a wide spectrum of clinical manifestations which are related to the number of larvae ingested, the *Gnathostoma* species and the organs and tissues that are affected by parasite migration. Thus clinical signs depend of the external or internal forms of the disease. In the external form, worms migrate within the cutaneous and subcutaneous tissues and symptoms are characterized by intermittent migratory swellings of different sizes, sometimes reflecting the larval migratory tracks that occur, most commonly in the torso rather than in the limbs or face. The intermittent migratory swellings occur as indurate erythematous plaques (Fig. 21.7), itching, generally with little pain. The systemic symptoms present in the visceral form include fever, arthralgias, myalgias, malaise, anorexia, nausea, vomiting, diarrhea, and epigastric pain. These may be present as soon as 24 to 48 hours after the ingestion of the larvae. These manifestations are related to affected organs. Some patients may suffer from vision impairment caused by larva invading the eye and symptoms such as swelling of the eyelid (Fig. 21.8), pain and itching of the eyelid, photophobia, headache, nausea, and vomiting can be present. In cases where brain infection is present symptoms include pain, fever, meningitis, nausea, vomiting, an impairment of sensation of pain, temperature, weakness of limbs, paralysis, and unconsciousness.

21.4.5 Diagnosis

Cutaneous gnathostomiasis involves a migratory swelling, which is the primary physical sign in establishing the diagnosis but this is not practical for visceral disease. Thus, other characteristics need to be considered, such as the presence of eosinophilia, and obvious exposure risk. The latter is related to residence in or travel to an endemic area and consumption of food that potentially contains the larval form of the parasite. Definitive diagnosis of gnathostomiasis requires isolation of the larvae of the helminth from the lesions they cause; however, when this is not possible other tests need to be carried out to help in the diagnosis of gnathostomiasis. In this context, serological tests have been developed to detect this disease. Among these, Western blot has been reported to have a high sensitivity and 100% specificity.

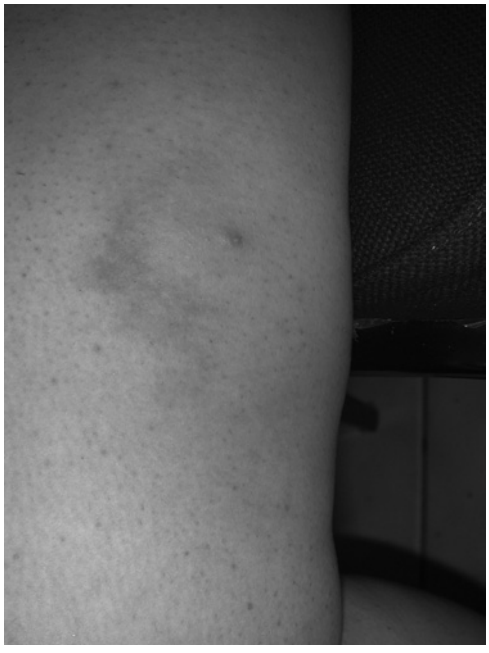

Figure 21.7 A cutaneous manifestation of gnathostomiasis caused by the third-stage larvae of *Gnathostoma binucleatum*. A case of a female with an indurated erythematous edema in her thigh (Photograph courtesy of Sylvia Paz Diaz-Camacho Universidad Autónoma de Sinaloa México).

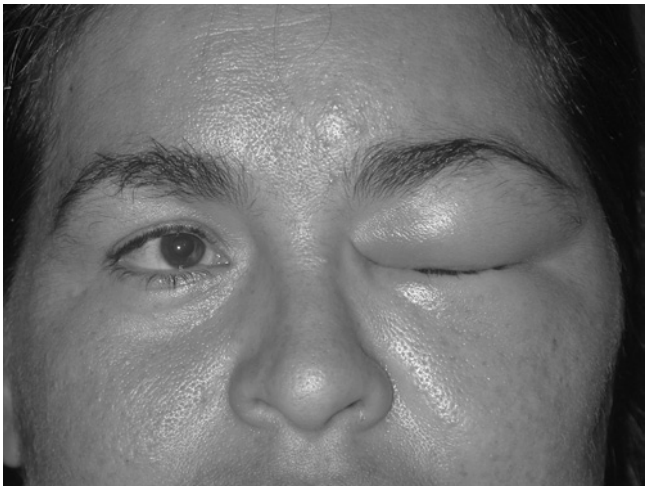

Figure 21.8 A deforming erythematous edema without plaque caused by *Gnathosthoma* frequently present in the face. (Photograph courtesy of Sylvia Paz Díaz-Camacho Universidad Autónoma de Sinaloa, México).

Currently, the diagnostic tool is the skin test and ELISA for IgG antibody against the third-stage larvae, but cross-reactivity is a common occurrence. This may be reduced if, in the ELISA assay, detection of IgG1 antibody is used as a screening test for humans with suspected gnathostomiasis and detection of IgG2 antibody is used to confirm the diagnosis.

21.4.6 Treatment

Ivermectin has been used to treat gnathostomiasis and this drug is effective either as start dose of 0.2 mg/kg or in doses of 0.2 mg/kg on two consecutive days. Although it is considered safe and effective, some concern exists about its use in children younger than 5 years of age or weighing less than 15 kg. The studies involving ivermectin have had fairly small sample sizes ($n = 17$ to 21), and therefore this drug needs to be tested in a larger prospective study. Albendazole also has been used to treat gnathostomiasis at a dose of 400 mg twice a day for 21 days and has been found to have minimal side effects. In one study, cure was achieved in 93.9 or 94.1% of patients receiving 400 mg twice daily or 400 mg once daily, respectively. Reductions in eosinophil counts and in IgG antibody also were noted. Future trials would be useful to investigate the use of combined treatment with both albendazole and ivermectin and to determine whether relapse rates are lower with combination drugs than when one single drug is used.

21.4.7 Prevention

Due to the global distribution of gnathostomiasis, the large variety and wide distribution of animals that are intermediate hosts, and the common dietary practices in endemic areas, eradication of the parasite is difficult to achieve. Therefore, public health education is essential to change the eating habits in areas with high levels of endemicity, and travelers need to be aware of the potential consequences of eating local delicacies. Adequate cooking is the best way to ensure that the larvae are killed, although freezing infected meat to –20 °C for 3–5 days is also effective. Marinating fresh fish in lime juice is ineffective since the parasite is still viable even after five days in lime juice. However, whilst travelers continue to choose the exotic and remote areas of the world, gnathostomiasis will be seen with increasing frequency in the West and other regions where it is not endemic and it should be considered by physicians as another emerging imported disease.

Gnathostomiasis was once fairly localized, but now is much wider spread, the incidence is increasing, and numerous areas are becoming endemic for the helminth. Therefore, to prevent transmitting gnathostomes to nonendemic regions, prevention and control strategies must include the collaboration of public health authorities, physicians and researchers. This, together with the establishment of national surveillance systems, will allow the detection, diagnosis, and treatment of gnathostomiasis.

21.5 *Anisakis* spp.

21.5.1 Background

Anisakids, as broadly defined, constitute a group of around 20 genera of ascaroid nematodes that inhabit, as adults, the gastrointestinal tract (mostly the stomach) of fish, aquatic mammals and reptiles, or fish-eating birds as definitive hosts. Humans are not natural definitive hosts of this worm, but they may become infected as accidental hosts by consuming uncooked fish or cephalopods (squids) harboring third stage larvae ((L3), Fig. 21.9). In humans, larvae do not mature to provoke the zoonotic disease known as Anisakiasis or Anisakidosis. This disease is frequently reported in areas of the world where seafood is consumed raw, lightly pickled or salted in dishes such as sushi, sashimi, ceviche, and pickled herring (Spanish boquerones). Historically, only two species of anisakids have been

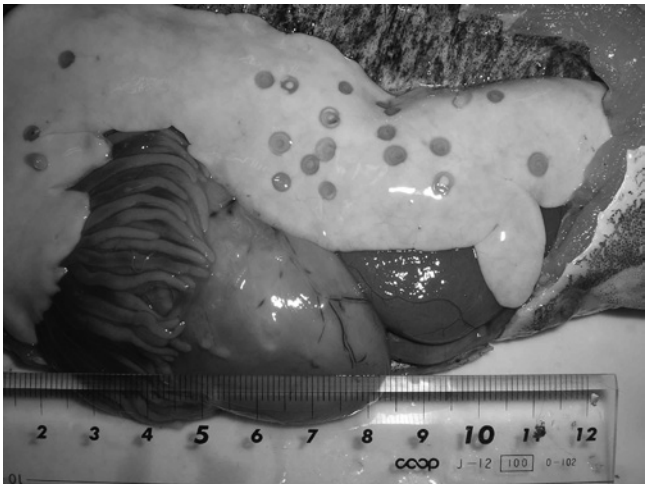

Figure 21.9 Third-stage larvae of *Anisakis* sp. from the body cavity of a clupeid fish (arenque). (*Source*: http://en.wikipedia.org/wiki/Image:4-S05A_010.jpg. Image of public domain)

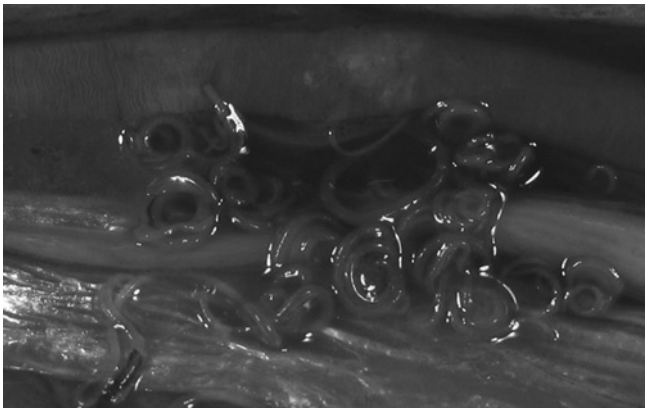

Figure 21.10 *Anisakis simplex* on the liver of Alaska pollack (*Theragra chalcogramma*) in Japan (Source: http:/fishparasite.fs.a.u-tokyo.ac.jp, with permission from the author)

considered to cause anisakiasis in humans with an apparently cosmopolitan distribution, the herring worm or whale worm *Anisakis simplex* (Fig. 21.10) and the cod worm or seal worm *Pseudoterranova decipiens*. However, recent findings through molecular data have demonstrated that both species may actually represent a complex of cryptic species, with at least three different species comprising the *A. simplex* complex, and six different species comprising the *P. decipiens* complex. Even though the detection and identification of human infections is difficult, very recently another species, *Anisakis pegreffi*, was recognized by molecular techniques based on mitochondrial DNA sequencing as the cause of human intestinal anisakiasis in the Mediterranean Sea.

We surveyed literature referenced in the ISI Web of Science (http://scientific.thomson.com/products/wos/) containing the phrases '*Anisakis*' or 'Anisakiasis/Anisakiosis/Anisakidosis' in the title, abstract or keywords and found >965 and 410 references, respectively. A good

deal of information has accumulated on this zoonotic disease, and the bibliographical records under the term '*Anisakis*' deal with almost every aspect of this nematode species, from taxonomic records of the presence of larvae in a wide array of marine fishes to some aspects of their phylogenetic relationships, ecology and biogeography. However, considering just the disease, it is worth pointing out that 214 of the 410 records, i.e., 53%, were published in the last decade. Clearly, this is an indication of a renewed interest in the zoonosis but may also coincide with an increase in the prevalence of the organism. The interest in this disease derives from the fact that some food habits have increased human exposure to fishborne parasitic infections. Also, food availability, in combination with human and society behavior related to customs and cultural beliefs, has impacted the way food is prepared. The second half of the last century and the first decade of the current century witnessed a great variation in human diet. Profound increases in world trade have facilitated larger changes in the way food is processed and in the diets of local human populations.

21.5.2 Life cycle

Anisakids utilize marine vertebrates as definitive hosts. Marine mammals such as dolphins, sea lions, and whales are common natural hosts of anisakids. In particular, the life cycle of *Anisakis simplex*, the best-known species, includes whales as definitive hosts. A generalized life cycle starts with the adult male and female worms living in the stomachs of their hosts where they mate and produce eggs that are passed into the seawater to embryonate within the host´s feces. Eggs require an incubation period before hatching into free-living larvae. These larvae are then ingested by the first intermediate hosts, particularly crustaceans, where they continue to develop while living in the hemocoel; eggs can be eaten directly by the crustacean. Crustaceans containing the L3 larvae are then eaten by fish or squid where the larvae penetrate the intestine to invade the tissues where they usually encapsulate. The life cycle is rather complex since small fish and squid may be eaten by other larger predatory fish or cephalopod. The cycle is complete when the definitive host feeds upon one of these intermediate hosts infected with L3 larvae, the larvae break free in the stomach and develop into adults. The whole definitive and intermediate host range of anisakids has not yet been fully determined, and there seems to be some level of host-specificity in the definitive hosts; however, only minimal specificity exists for their intermediate hosts. Considering such a complex life cycle within the context of marine food webs, it is not uncommon that accidental hosts, typically fish or cephalopods, can become infected with anisakids by eating intermediate hosts.

21.5.3 Epidemiology of human anisakiasis

Humans are accidental hosts who usually become infected by eating raw or undercooked fish or cephalopods. Transmission of anisakids to humans is associated with the foodborne route through fish dishes that are common in different parts of the world where the consumption of raw or undercooked fish or squids is common. The epidemiology of such infections is sometimes difficult to characterize since usually no attempts are made to distinguish between the infecting anisakid species. A wide variety of marine fish species have been found as intermediate hosts for *A. simplex*. Some examples include the spotted chub mackerel in Japan, herring in Western Europe, and farm reared salmon, Pacific herring, and Atlantic cod in North America. Anisakiasis occurs all over the world, with most

cases reported in North Asia and Western Europe. Of the more than 20,000 cases reported thus far, over 90% are from Japan, with a record of almost 2000–3000 cases diagnosed annually. The number of records of anisakiasis around the world has increased during the last three decades, and cases have been found in areas of the world such as the United States, Mexico, Canada, the United Kingdom, Belgium, Egypt, Korea, the Phillipines, Chile, and New Zealand. Recently, the first report of human anisakiasis in Australia was presented, along with several reports of the presence of anisakid larvae in marine fishes. This is the result of an increasing demand for seafood and a growing preference for raw fish among consumers that in turn increases the risk of exposure to anisakid larvae, among other fishborne parasitic diseases. It seems likely the increase in reported cases of anisakiasis is largely due to the use of new and more efficient diagnostic techniques but also due to an increased awareness of this zoonotic disease. As a consequence, reports in the literature have shown a marked increase in the prevalence of anisakiasis in the last 30 years throughout the world.

21.5.4 Clinical signs, diagnosis, and treatment

Human anisakiasis can present several symptoms which depend mainly on the location of the larvae in the human body. The most common form of the disease is the gastrointestinal anisakiasis, with symptoms that include sudden abdominal pain, nausea, diarrhea, and frequently urticaria, most of which result from allergic reactions in the digestive tract. There are, however, two manifestations of the disease; noninvasive and invasive. Noninvasive anisakiasis might be asymptomatic since larvae remain in the alimentary canal, without penetrating the mucosal wall and worms are detected when they are expelled by coughing, vomiting or defecating. Instead, invasive anisakiasis results from larvae penetrating the digestive tract and producing lower abdominal pain, nausea and diarrhea. Chronic anisakiasis due to larval invasion may produce abscess or eosinophilic granulomas. This occurs occasionally when larvae penetrate completely the wall of the stomach or intestine and enter into the body cavity and form tumor-like structures. The disease is commonly misdiagnosed because symptoms are often vague. Common diagnoses are appendicitis, acute abdomen, stomach ulcers, or ileitis. Also, symptoms of anisakiasis have been known to mimic other diseases such as stomach tumors, and inflammatory bowel disease.

The definitive diagnosis of anisakiasis requires identification of the causative agent. In this case, stool examination is unhelpful and diagnosis is usually made by upper endoscopy which allows for the identification of the worm morphologically and/or genetically. Radiologic films and immunological assays such as the complement fixation test, enzyme-linked immunosorbent assay (ELISA) and Western blot, among others, have also been used successfully. However, serologic tests need to be taken with caution because these can yield false positive reactions due to cross-reactivity with other ascarids and even other parasite antigens. Endoscopy is one of the most useful tools in both the diagnosis and treatment. Removal of worms using a fiberoptic endoscope, especially during the early course of the infection, minimizes the chance of allergic responsiveness and leads to immediate improvement of symptoms. In the case of intestinal anisakiasis, surgical intervention to remove the affected tissue might be required. No standard chemotherapeutic treatments have been described for anisakiasis; however, the anthelmintic albendazole has been used occasionally.

21.5.5 Prevention and control

Considering the increase in popularity of culinary practices in countries (other than Japan) that have a strong tradition of eating raw or lightly-cooked fish and shellfish, the number of cases of anisakiasis is expected to increase in the coming years. As such, preventive and control measures for anisakiasis need to focus on handling, storage and cooking procedures for fish and shellfish. Three main actions are needed to decrease the risk of infection by anisakids. First, commercially-important fish, particularly those already proven to be suitable hosts for L3 larvae of anisakid species, should be immediately eviscerated. This action would prevent the migration of worms from the viscera to the flesh. In addition, examination of fillets on a light table is highly recommended even though it might be inefficient in detecting all L3 larvae. Second, fish products should be frozen according to the regulations imposed by Federal Agencies, particularly if fish are used for raw or semi-raw consumption. For instance, it has been recommended that fish should be frozen at –20 °C or below for a minimum of seven days. Finally, it is highly recommended that if neither of the first two recommendations are put in force, fish should be thoroughly cooked prior to eating. Since anisakid larvae seem to be resistant to alternative food processing such as salting or smoke-curing, it is highly recommended to cook fish and shellfish to reach a temperature of 60 °C.

Since anisakiasis is very common in North Asia and Western Europe, but also occurs throughout the world, further research is needed to fully understand the epidemiology of this zoonotic disease specifically involving transmission to humans via the foodborne route through fish dishes. Even though only two species are implicated as the cause of human disease (the herring worm or whale worm *Anisakis simplex* and the cod worm or seal worm *Pseudoterranova decipiens*), future research is needed to better understand the life cycle and transmission dynamics of all anisakid species since they show potential to become human parasites, i.e., some species represent possible cases of emerging disease pathogens.

It has been pointed out the need to conduct research on anisakiasis in three different areas. The first area needing attention is parasite biology involving not only transmission dynamics, but also the extent of geographic distribution, host range, and prevalence in both definitive and intermediate hosts. This information will be very useful not only to endemic countries, but also to those with a higher than normal risk of exposure. For example, in countries like Mexico, although human anisakiasis has not yet been reported, larvae of anisakid nematodes have been found in marine fishes. Research on the parasite biology should also include proper identification of the etiological agent, and the discrimination among anisakid species using molecular techniques. For a complete review of anisakid nematode biology, the reader should refer to a recent paper published by Mattiucci and Nascetti (2008). In that review, the authors examined the current knowledge and advances in the taxonomy, epidemiology, geographical distribution, population genetics and phylogenetic relationships of species belonging to the genera *Anisakis*, *Pseudoterranova* and *Contracaecum* since the introduction of molecular markers, highlighting ecological implications and co-evolutionary processes.

A second suggested area of research is the epidemiology of anisakiasis, asking why this disease is basically only associated with certain species of anisakid larvae (*A. simplex* and *P. decipiens*). Determining the potential risk of infection by other species of anisakids will be crucial to evaluating the impact that this disease may have in the near future in nonendemic areas, and helping to establish more strict control

measures to avoid the dissemination of the disease. It will also assist in refining the key diagnostic methods to properly determine the causative agent of anisakiasis in humans. Finally, some authors have suggested that one of the most important issues needing future study is the discovery of hypersensitivity reactions to anisakid allergens, because these may produce different pathological reactions in people handling and ingesting fish products.

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22 Foodborne viruses

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22.1 Introduction

Viruses transmitted by the fecal–oral route, sometimes referred to as enteric viruses, are now recognized as the leading cause of foodborne illness in the United States and are the second most-reported cause of foodborne disease outbreaks in Europe. These illnesses occur in individuals of all ages, genders, and professions but tend to have greater severity in children and the elderly. They are also responsible for substantial health care-associated costs and economic losses among food producers, processors, handlers, and consumers.

Many virus types can be transmitted by foodborne routes, but from an epidemiological perspective, human noroviruses (NoV) and hepatitis A virus (HAV) are the two most important. NoV is most significant by virtue of the sheer numbers of cases; HAV because it causes a more severe disease. For all enteric viruses, however, their biology and pathology contribute to the ease with which they are transmitted. Viruses are not free-living organisms and require a live host cell in which to replicate. Enteric viruses, then, cannot replicate outside of the human body, but when present in the environment (or foods) they tend to be persistent and resistant. They are primarily transmitted by the fecal–oral route, and when shed in the feces of infected humans, their numbers can be high: millions or even billions of virus particles per gram of fecal material. Enteric viruses are also highly infectious, causing disease at very low doses. Unfortunately, because infected individuals may or may not show symptoms it is sometimes difficult to know whether an individual is infected.

In addition to NoV and HAV, there are other enteric viruses that can be transmitted by foodborne routes. In fact, the vast majority of foodborne disease cannot be attributed to known pathogenic agents. For example, while there are an estimated 9.4 million annual cases of foodborne illness each year in the US caused by known pathogens, four times this number of annual cases (38.4 million) cannot be attributed to known pathogens; viruses are undoubtedly responsible for many of these cases. Further, for a number of reasons, nearly half of the principal foodborne pathogens (responsible for high US incidence of gastrointestinal cases), including human NoV, were identified in the past 25 years suggesting that

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additional principal foodborne pathogens are likely to be identified in the near future. Rotaviruses and astroviruses are well recognized but their transmission by foodborne routes has only recently been documented. Viruses of zoonotic origin can also be transmitted by contaminated foods. Examples of these viruses include the avian influenza viruses (H5N1), hepatitis E virus (HEV), and swine influenza virus, all of which can be transmitted enterically by animal fecal material, with varying documentation of cross-species transmission to humans by consumption of contaminated animal products.

For many reasons, the food industry is ill equipped to manage foodborne viruses. Because foodborne virus infection may be asymptomatic, it is difficult to identify infected food handlers and separate them from food. Food handlers who appear healthy (never exhibited symptoms or resolved symptoms) may be reservoirs of disease due to the long duration of asymptomatic shedding. The high amount of virus shed by infected individuals combined with the low dose needed to infect consumers increase the likelihood of transmission of foodborne viruses to food. Once in the environment or on foods, enteric viruses are generally stable for weeks (on foods or surfaces) or months (in water), and relatively resistant to heat, disinfection and pH extremes. Because only a small fraction of virus is required for infection, food handlers will have difficulty in ensuring complete environmental disinfection and ensuring effective handwashing. This also means that, once a food source is contaminated, it will be difficult to completely eliminate or inactivate the virus needed to ensure prevention of infection. Finally, contaminated foods will not show any signs of spoilage, as they might with bacterial contamination.

The food industry also lacks technology to sample, survey, and detect foodborne viruses on foods. For example, in the US, there are no standardized assays for the routine detection of viral contamination in foods and environmental samples. In addition, most food quality control systems still rely on bacterial indicators as proxies for the presence of pathogenic viruses and bacteria. Unfortunately, bacterial and viral indicators are poor indicators for viral pathogens.

This review will focus on viruses from the food-safety perspective by describing the epidemiology of the most common agents, our ability (or lack thereof) to detect viruses in foods and environmental samples, the food types most susceptible to contamination, and what can be done to prevent enteric virus contamination and/or remove it if present in foods. We will focus on human NoV and HAV because of their significant epidemiological link to foodborne disease, but point out that there are many other viral agents that may be transmitted by foodborne routes (Table 22.1). Other, as yet unrecognized, agents are likely to emerge over time.

22.2 Health and economic impact of foodborne viral outbreaks

The U.S. Centers for Disease Control and Prevention (CDC) estimate that, each year, there are 47.8 million episodes of foodborne illness and, of those caused by known pathogens, 59% are caused by viruses. Annually, foodborne viruses account for over 15,000 hospitalizations (0.03% of cases) and over 150 fatalities in the US (<0.1% of cases). In the European Union (EU), approximately 700 viral foodborne disease outbreaks were reported in 2008, a figure which has increased 3% annually since 2006. In developing nations, human NoV alone may cause up to 1.1 million hospitalizations (197 per 100,000 children) and 218,000 deaths (39 per 100,000 children) in children every year. In 2005, HAV infected an

Table 22.1 Foodborne Viruses

Virus	Family	Shedding	Transmission routes	Environmental stability	Infectious dose	Attack rate	Method of detection*
Astrovirus (HAstV)	Astroviridae	Unknown, thought to coincide with diarrhea	Foodborne, possibly waterborne, person-to-person	Unknown	Unknown	Unknown	*Clinical*: RT-PCR, ELISA *Foods*: RT-PCR
Norovirus (previously 'Norwalk-like viruses') (NoV)	Caliciviridae	One mo; up to a yr. in immuno-compromised patients	Foodborne, waterborne, person-to-person, fomites, vomitous	Highly stable	18 virons	20–53%, depending on transmission and setting	*Clinical*: RT-PCR, ELISA *Foods*: RT-PCR
Sapovirus (previously 'Sapporo-like viruses') (SaV)	Caliciviridae	2 wks, up to 300 days in immuno-compromised patients	Mainly person-to-person, rarely foodborne (e.g., shellfish)	Unknown	Unknown	Unknown	*Clinical and Foods*: RT- PCR
Hepatitis E (HEV)	Hepeviridae	1–2 wks before and 2–4 wks after symptoms	Zoonotic foodborne, waterborne, parenteral, person-to-person	Unknown	Unknown	Unknown	*Clinical*: RT-PCR, ELISA, *Foods*: RT-PCR
Echovirus	Picornaviridae	Unknown	Waterborne, person-to-person	Unknown	Thought to be <20 PFU	Unknown	*Clinical*: RT-PCR *Water*: cell culture
Hepatitis A (HAV)	Picornaviridae	10–14 days before symptoms through 12 wks after infection	Foodborne, waterborne, shellfish, person-to-person, parenteral	Highly stable	Thought to be 10–100 virons	Unknown	*Clinical*: RT-PCR, ELISA *Foods*: RT-PCR
Rotavirus (RV)	Reoviridae	>10 days in adults, up to 2 weeks in infants	Rarely foodborne, waterborne, person-to-person	Moderately stable	1 cell culture infective unit, unknown in children	Unknown	*Clinical*: RT-PCR, ELISA *Foods*: RT-PCR

*ELISA (enzyme-linked immunosorbent assay); RT-PCR (reverse transcription polymerase chain reaction)

estimated 119 million people globally, with about one-quarter of these infections resulting in symptomatic illness with as many as 34,000 deaths.

The public health burden of foodborne viruses, which includes the cost of illnesses, outbreaks, hospitalizations, and deaths, is serious. Individuals who contract viral illness may incur direct costs associated with medical care or hospitalizations, or indirect costs such as those associated with unanticipated transportation or childcare, or the loss of income from missed work time. Companies may experience loss of productivity due to ill or absent employees, and when an outbreak is directly linked to a specific facility or company, losses due to temporary closures, decontamination costs, and loss of customer base or brand reputation can be severe. Companies that produce and distribute contaminated foods also incur costs associated with product recalls, poor sales or changes in trade, and legal liability. For example, Scharff et al. (2012) found that foodborne viral illnesses (mainly HAV) are estimated to cost the US $3.79 billion (95% confidence interval: $1.45 to $7.16 billion each year) every year in medical care, lost productivity and quality of life costs.

22.3 Epidemiology and clinical characteristics of foodborne viruses

22.3.1 Human noroviruses

Human NoV are small-round-structured viruses in the family *Caliciviridae* having a diameter ranging from 27–40 nm and containing a single stranded, positive, polyadenylated RNA genome about 7.6 kilobase pairs (kb) in length. The genome is divided into three open reading frames (ORFs) – ORF1 codes for six nonstructural proteins, ORF2 and ORF3 encode the major (VP1) and minor (VP2) capsid proteins, respectively.

Norovirus is divided into genogroups, and within genogroups, into gentotypes. Each genogroup is defined as a cluster of virus strains that reproducibly group together on one branch of a phylogenetic tree and are separate from other clusters. The phylogenetic tree must be constructed from complete capsid amino acid sequences. There is 46% nucleotide divergence between the five genogroups. Three genogroups (GI, GII, and GIV) are known to infect humans. Within genogroups, the virus is classified into genotypes. There has been an evolution in the definition of genogroups but an international working group has recently proposed a unified nomenclature. From 2007 onwards, new genotypes will be assigned if: (1) at least two geographically diverse complete capsid sequences are detected; (2) 'clustering should be robust using more than one accepted phylogenetic method, including a branch support test'; and (3) 'using the inferred distances from these phylogenetic methods, the average distance between sequences of the new Genotype cluster and sequences of the nearest genotype cluster, should not overlap within two standard deviations of each other'. As of 2006, there were eight recognized GI genotypes (GI.1–GI.8), 17 recognized GII genotypes (GII.1–GII.17), and one GIV genotype (GIV.1). The most prevalent genotype worldwide is GII.4, which is the only strain to have caused global epidemic NoV outbreaks.

Noroviruses are the leading cause of nonbacterial gastroenteritis worldwide and are estimated to cause over 58% of all foodborne illness of known etiology in the US, equivalent to over 5.4 million estimated cases per year. In 2008, NoV caused 30 reported outbreaks in Europe, with 1020 confirmed outbreak cases. It is important to keep in mind, however, that these numbers are underestimates because many cases and outbreaks are not recognized.

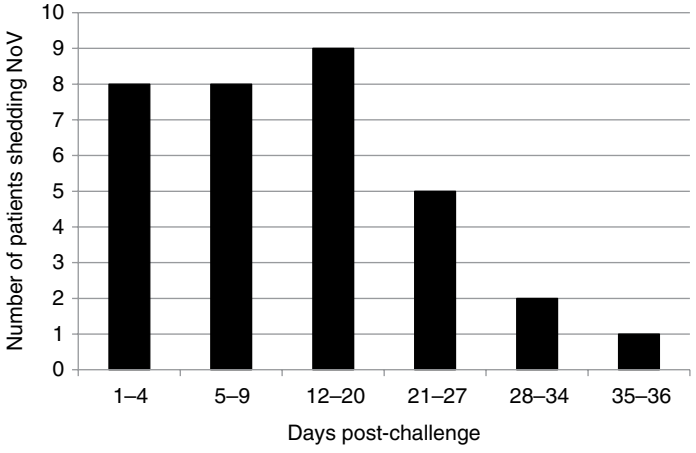

Figure 22.1 Norwalk virus can be shed in volunteers for up to 1 month. Thirteen volunteers were dosed with groundwater seeded with safety tested Norwalk virus inoculum on day 0. Stool or vomitus samples were collected on the indicated days and assayed for Norwalk virus by RT-PCR. Stool samples were not collected beyond 36 days post challenge.

From a foodborne disease perspective, human NoV are most often transmitted by fresh produce, molluscan shellfish, and ready-to-eat (RTE) foods.

Human NoV are highly transmissible for many reasons. Transmission of NoV can occur through multiple routes including the fecal–oral route through person-to-person contact, fecal contamination of food, water, fomites, or contact with aerosolized vomitus from an infected person. Once infected, individuals may or may not present with symptoms. After a typical incubation period of 1–3 days, symptoms may include fever, headache, acute diarrhea, and vomiting which also serve to facilitate the transmission of virus. Symptoms usually last 1-3 days, but may last longer in those patients needing hospital care. After symptoms subside, a person may continue to shed NoV particles for 2–8 weeks; immunocompromised patients have been documented to shed the virus for several months up to two years (see Fig. 22.1). Asymptomatic shedding presents challenges for the prevention of NoV transmission, particularly in the food industry when asymptomatic food handlers are unaware that they are contagious. Data from our human challenge studies with healthy volunteers suggest that one-fifth to one-third of infected individuals, depending on challenge study, shed virus asymptomatically. The virus is shed at a high titer and has a low infectious dose (ID_{50} may be as low as 18 virons). Norovirus is stable in the environment – its RNA can be detected on surfaces for weeks and in water for months. Its RNA can be detected after pH extremes (<4), high temperatures (>90 °C), and disinfection with hand-sanitizers or ethanol. Sodium hypochlorite is relatively effective against NoV if used properly.

Seroprevalence studies indicate that practically all adults in the world have been infected with at least one NoV strain, but prior infection does not necessarily lead to immunity. While short-term immunity (<6 months) has been documented in individuals challenged multiple times with the same virus, long-term immunity (>2 years) has not been documented. Currently, there are no commercially-available vaccines for NoV, although this is an active area of research. For example, Atmar et al. (2011) conducted a randomized, placebo-controlled trial of a Norwalk virus vaccine using a virus-like particle (VLP) as the immunogen. They found that vaccination significantly reduced the frequency of Norwalk

virus gastroenteritis (69% of placebo recipients vs. 37% of vaccine recipients, P=0.006) and infection (82% of placebo recipients vs. 61% of vaccine recipients, P=0.05).

22.3.2 Hepatitis A virus

Hepatitis A virus (HAV) is a small, nonenveloped, spherical virus in the family Picornoviridae that is 27 to 32 nm in diameter and contains a single positive strand of RNA which is 7.5 kb long. The genome includes two noncoding regions (NCR) at the 5' and 3' ends, and a single open reading frame (ORF) consisting of the P1 region, which encodes the structural four capsid proteins (VP1, VP2, VP3 and a putative VP4), and the P2 and P3 regions encoding several nonstructural proteins. Hepatitis A virus has a high degree of antigenic (amino acid) and genetic (nucleotide) conservation. There is only one major serotype, but several genotypes and subgenotypes. Sequence variation within the VP1/P2A junction is used to define genotypes: genotypes have >15% nucleotide variations between isolates and subgenotypes have 7.0–7.5% nucleotide variation. Hepatitis A virus strains are categorized into six genotypes (I–VI), three of which (I, II, III) are of human origin. Genotypes I and III, compared to other genotypes, are the most prevalent genotypes isolated from humans, and subtype IA is responsible for the majority of cases worldwide.

HAV can be transmitted by consumption of contaminated water, by person-to-person contact, by parenteral routes or by foodborne routes. It is estimated that, in the US, foodborne HAV causes an estimated 1500 illnesses per year, 100 hospitalizations (30% of lab-confirmed cases) and nearly 10 deaths (2.5% of lab-confirmed cases). However, the proportion of HAV cases caused by foodborne transmission is most likely underestimated because of the prolonged disease incubation period, making it difficult to confirm whether food has been implicated in infection and, if so, which specific vehicle was responsible. Several high-profile foodborne HAV outbreaks have occurred over the years, including those associated with molluscan shellfish, ready-to-eat (RTE) foods, and fresh produce items like lettuce, berries, and green onions.

The infectious dose of HAV is unknown but assumed to be low. Similar to NoV, prolonged periods of virus shedding, a high degree of environmental stability and resistance to inactivation all contribute to the transmissibility of HAV. For example, HAV is resistant to low pH (<4) for weeks and persists under low temperature conditions. The virus is considered relatively heat resistant. Likewise, HAV is relatively resistant to free chlorine, requiring a sodium hypochlorite treatment.

The illness begins with a prodrome of nonspecific symptoms that may include fever, headache, fatigue, nausea, and abdominal discomfort. These nonspecific symptoms are followed by classic hepatitis symptoms such as jaundice and inflammation of the liver. There is no specific treatment for infection with HAV; the disease is self-limiting and normally lasts 4–6 weeks, but can last several months. In children under 6 years of age, HAV infection is usually asymptomatic or only mild, without jaundice. Adult infection is generally symptomatic with severity increasing with advanced age. Shedding of the virus in stools can begin 10–14 days before the onset of symptoms and can continue for up to 12 weeks after infection. For instance, HAV-infected individuals have been shown to be viremic for as long as 30 days before and 60–80 days after the onset of symptoms. Similar to NoV, the long shedding time of HAV-infected individuals increases the risk of HAV transmission.

Unlike human NoV, infection with HAV induces protective immunity. In areas with poor hygiene conditions, HAV is often endemic and the majority of infections occur during childhood, providing protective immunity to the adult population. As hygiene conditions

improve, the number of childhood cases declines, and adults no longer acquire protective immunity or are protected through herd immunity, and therefore a greater proportion of the adult population is susceptible to infection.

Prior to 1995, passive immunization with immunoglobulins (IG) was the only preventive option, and is still used in cases of possible exposure or if the vaccine is unavailable and only short-term immunity (3–5 months) is needed. The US CDC recommends IG for post-exposure prophylaxis for all food handlers if a co-worker is diagnosed with HAV. The CDC does not recommend IG administration to patrons because they are at lower risk of infection than the food workers, and they are unlikely to be identified and administered IG within the recommended two weeks post-exposure.

An inactivated HAV vaccine was first licensed in 1995. There are four HAV vaccines currently available internationally. All are from inactivated HAV and are administered via intramuscular injection to the deltoid. Two licensed in the United States are HAVRIX® (GlaxoSmithKline, Belgium) and VAQTA® (Merck & Co., New Jersey) – these vaccines are each licensed in more than 40 other countries. Two other vaccines licensed internationally are: Epaxal® (also sold as HAVpur® and VIROHEP-A, Crucell, the Netherlands), licensed in 37 countries, and Avaxim® (Sanofi Pasteur SA, France), licensed in more than 100 countries worldwide. HAVRIX® and VAQTA® are recommended for anyone 12 months or older – two doses given 6–18 months apart are 94% and 100% effective for the vaccines respectively, and either vaccine will give protective immunity for >25 years in adults and >14 years in children. Avaxim® is generally used for short-term immunity in adults, or to boost other HAV vaccines – it is recommended only for people 16 years and older, one dose is 96% effective (compared to 87% efficacy after one dose of HAVRIX) and will give protection for 36 months, and a second dose 6–12 months later will give 10 years protective immunity. Two doses of Epaxal® given 12 months apart have been shown to be 100% effective at preventing HAV for a duration of >25 years. Recently, several emerging antigenic variants of the HAV were isolated and are thought to be resistant to the vaccines.

The US recommends HAV vaccination for all children at 1 yr, and for persons at increased risk for HAV infection including: people traveling to HAV-endemic countries, men who have sex with men (MSM), people with clotting-factor disorders or chronic liver disease, and people at occupational risk. The vaccine is not recommended for children under 1 yr or pregnant women because there has not been enough study of the vaccine effects in these populations. Mandatory vaccination of all food handlers against HAV has been proposed to prevent contamination of foods with HAV by food handlers, but mandatory vaccination has not been advocated universally, due its high cost and logistical difficulties. Some US counties and states, as well as countries (i.e., Austria) now require mandatory vaccination of their food handlers against HAV.

22.4 Detection of enteric (foodborne) viruses

22.4.1 Clinical samples

Viral infections are usually diagnosed using immunologically-based methods, or methods based on nucleic acid amplification. For example, diagnosis of HAV infection is usually done by enzyme immunoassay (EIA). This EIA detects immunoglobulin antibodies to HAV in the serum of patients. Hepatitis A infection can also be diagnosed by extraction of HAV RNA from stool and, infrequently, serum (using methods for HCV extraction from serum)

followed by amplification by reverse transcription PCR. In the absence of laboratory diagnostic reagents, acute hepatitis A infection can also be diagnosed by assessment of liver function, including testing urine and serum for bilirubin.

Clinical diagnosis of NoV infection, compared to HAV, is more complicated. In the 1970s and 1980s, electron microscopy and/or immune electron microscopy was occasionally used as a diagnostic tool, but this was mostly limited to research settings. Efforts to develop EIA methods have met with limited success due to poor assay sensitivity and specificity, largely as a function of high antigenic diversity within the NoV genus and the lack of broadly reactive antibodies. Two relatively recent EIAs, IDEIA and RIDASCREEN, and one enzyme-linked immunosorbent assay (ELISA), Denka NV-AD, are commercially available in some parts of the world for detection of NoV in clinical (stool) samples. However, RIDASCREEN is the only method approved in the US. Because of the aforementioned poor sensitivity and specificity, commercially-available ELISAs for NoV detection cannot be used for routine clinical diagnostics; rather, they should only be used in conjunction with the diagnosis of patients suspected to be involved in a NoV outbreak. In the future, salivary antibody assays for detection of NoV infection may be promising, as collection of saliva samples is more attractive than stool samples, but these will still be subject to the sensitivity and specificity issues associated with using antibodies. In public health laboratories, NoV infection is diagnosed by detection of NoV RNA in stool samples using reverse transcription-PCR, and this method is generally considered to be more sensitive and specific than immunological assays. However, at the time of this writing, there were no PCR-based clinical diagnostic kits available for routine use in patient diagnosis in the US.

22.4.2 Food and environmental samples

Detection of enteric viruses in food and environmental samples is much more difficult relative to clinical samples because of (i) the low levels of virus contamination present in naturally-contaminated samples; (ii) the need to test fairly large samples sizes (≥25 g); and (iii) the presence of matrix-associated organic compounds that can inhibit downstream molecular-based detection methods. Because enteric viruses are either difficult or impossible to culture in vitro, there are no universal or rapid culture-based methods available. Instead, it is necessary to concentrate and purify the viruses from the sample matrix prior to the application of molecular amplification methods for detection. After nucleic acid amplification, sequencing or molecular probes can be used to identify and/or confirm virus contamination. It is important to note that amplification of genetic material cannot distinguish between infectious virus and noninfectious viruses and this remains an important limitation of current methods. In summary, the major steps required for detection of viral contamination in food and environmental sample matrices are as follows: (1) virus concentration and purification; (2) nucleic acid extraction; (3) detection; and (4) confirmation. Each step is discussed in greater detail below.

The goal of the concentration and purification step is to separate the viruses from the sample matrix and to reduce the total sample volume while maximizing virus recovery. There is no universal method for concentration and purification of viruses from foods, but most methods rely on the ability of viruses to behave as proteins in suspension, their tenacious attachment to matrix particulates, their ability to withstand exposure to organic solvents without loss of infectivity, and the combined use of standard separation methods such as centrifugation and filtration. In almost all instances, two or more methods must be used sequentially, and the choice of those methods is matrix-specific. For example, enzymatic pretreatment, such as with proteinases, can be used to digest matrix-associated

organic matter prior to further extraction steps. Organic solvents extraction (like Vertrel or chloroform) can be used to remove lipids from the sample matrix prior to virus concentration. For surface-contaminated products, manipulation of pH and/or ionic conditions can facilitate disassociation of viruses from organic matter. Viruses can be co-precipitated with residual matrix components using organic flocculants or polyethylene glycol (PEG). Once precipitated, simple centrifugation can be used to recover viruses from the matrix. For water or relatively 'clean' liquid samples, filtration, ultracentrifugation or ultrafiltration can be used for virus concentration. New methods for recovery of HAV and NoV from produce have included the use of a desorption buffer to facilitate removal of surface-associated virus, followed by anion exchange filtration to concentrate any virus present in the buffer, and finally elution of viruses off the filter membrane. A commonly-used method for virus concentration from molluscan shellfish includes dissection of the virus-rich digestive diverticula, tissue digestion using proteinase K to release the virus from the matrix, and centrifugation to pellet the sample homogenate containing the RNA.

Even though viruses are resistant to these viral concentrations and purification treatments, there is usually virus loss with each sample manipulation. Therefore, minimizing the number of steps of sample concentration and purification is an important component to optimizing methods so that they result in the most efficient virus recovery. The optimum method for concentration and purification of viruses will vary by virus and matrix type.

In the field of food microbiology, methods like immunomagnetic separation have gained favor for target concentration and separation. Unfortunately, it is difficult to obtain purified HAV antibody, and there are no widely reactive antibodies available for human NoV capture. In fact, for some strains, there are no commercially-available antibodies at all. As an alternative, other ligand-bound magnetic separation methods have been developed for NoV. The most important to date has been the use of histo-blood group antigens (HBGAs), which can be purchased with biotin labels to facilitate their conjugation to streptavidin-coated magnetic beads. Specific HBGAs are the putative host cell binding sites for certain human NoV strains, but not all strains bind to HBGAs and so these methods still lack broad reactivity. Some investigators have used porcine gastric mucin in place of HBGAs as a less expensive alternative but, unfortunately, these mucins are still not broadly reactive with all NoV strains.

After the viruses have been concentrated and purified from the sample, the next step is to obtain purified viral RNA. This entails the steps of capsid lysis and additional sample clean-up to remove any remaining matrix compounds that could inhibit RNA amplification. A good extraction method is able to remove most inhibitory substances while efficiently recovering the viral RNA. Many methods have been studied over the years, but most investigators use guanidinum thiocyanate (GTC)-based methods as they effectively lyse the virus capsid and protect the integrity of the viral RNA by preventing residual nuclease activity. When followed by a secondary clean-up step using silica particles, this method usually results in high yields and quality of viral RNA that is amenable to nucleic acid amplification without the need for extensive dilution concentrate.

The most commonly-used method for detection of viruses in foods and environmental samples is molecular amplification using reverse transcription PCR (RT-PCR). In RT-PCR, the RNA is reverse-transcribed first to complementary DNA (cDNA), and then the cDNA is amplified by a thermostable DNA polymerase. The specificity of the RT-PCR reaction is determined by the primers, which are engineered to be complementary to a DNA sequence specific for the organism of interest. The assay sensitivity is affected by many different factors, including but not limited to buffer composition, annealing temperature, enzyme efficiency, and degree of matrix-associated inhibition.

Early efforts in nucleic acid amplification resulted in amplicons that had to be resolved using gel electrophoresis and, later, their sequences were confirmed by Southern hybridization. Because of these considerations, this 'conventional' RT-PCR assay was labor- and time-intensive, providing only presence or absence (nonquantitative) results. Over the last decade, these methods have been replaced by quantitative real-time RT-PCR (RT-qPCR). Most qPCR methods combine nucleic acid amplification with hybridization by incorporation of fluorescently labeled DNA probes specific to internal amplicon sequences in the reaction. Hence, the amplicon identity is confirmed while the reaction is progressing. It is also possible to perform qPCR using a nonspecific dye that detects the production of double stranded DNA that occurs during amplification. With either method, the amount of fluorescence increases as DNA amplification progresses. By using an RNA standard to create a standard curve, and comparing the sample fluorescence to the curve, one can estimate the initial amount of virus present in a sample. Theoretically, RT-qPCR amplification reactions are considered more sensitive and faster than traditional RT-PCR but, in practice, sensitivity is variable and determined by the same factors as in traditional RT-PCR. Primer design for NoV detection is critical, and several primer sets have been developed to detect a broad range of strains within one genotype, although primers that can detect all known strains of NoV have not yet been developed. The ORF1-ORF2 junction is the most highly conserved region and is usually used for genogroup-specific detection. Because the major capsid protein (VP1) sequence is used to define NoV genotypes, primers based on part of the VP1 sequence (e.g., region D) are used for genotyping NoV within a particular genogroup. Depending on the virus load and inhibitory substances present in different foods, different primers and different probes may perform differently in PCR reactions. To address this, multiple primer and probe sets are sometimes used in one PCR reaction for the detection of NoV in foods. For HAV detection, the primers most often used target either the VP1/P2A junction or the 5' untranslated region (UTR) of the viral genome, and genotypes can be distinguished using the VP1/P2A junction.

A sample that tests positive by RT-qPCR should be considered as a presumptive positive, with further steps to confirm that the amplicon sequence is indeed consistent with the target virus. This usually requires that the sample be re-screened using conventional RT-PCR, the amplicon cloned, and then submitted for sequencing. Unfortunately, this is frequently more difficult than anticipated, especially if the RT-qPCR signal intensity is low which suggests a small number of amplicon targets. As the amplicon target concentration decreases, so does the likelihood that the investigator will be able to obtain a usable clone for sequencing. Recently, a real-time PCR protocol has been developed for simultaneous amplification and sequencing of viruses, which would simplify the process for detecting viruses in foods or other samples.

22.4.3 Other detection considerations

Besides traditional negative and positive PCR amplification controls, most investigators also include sample extraction controls and internal amplification controls. The purpose of the sample extraction control is to monitor the efficiency of the virus concentration, purification and the extraction steps. The extraction control consists of a sample of the same food matrix that is seeded with a high concentration of a similar, usually nonpathogenic virus (e.g., feline calicivirus, mengovirus, and canine calicivirus). This control sample is processed in the same manner as the experimental samples. An internal amplification control (IAC) is a non-target nucleic acid sequence added to the sample amplification tube that is co-amplified with the

target sequence. IACs are meant to identify whether inhibitors from the sample matrix are impeding the amplification of the target nucleic acid (reviewed in Hoorfar et al. 2004). In a negative sample (no target RNA) with an IAC added, the IAC will amplify and the sample will not. In a positive sample (with target RNA), if amplification of the IAC RNA is inhibited, then this suggests that amplification of the target RNA is also inhibited. Homologous (competitive) IACs are constructed with non-target RNA flanked by the primer binding sites. They are amplified in the same tube and using the same primers as the sample, but are identified using a different probe. Homologous IAC and RNA compete for amplification. This competition can lower the amplification efficiency and therefore lower the assay limit of target RNA detection. To avoid this competition and to avoid a false negative result, it is recommended that the size of the IAC be larger than the target sequence and that the lowest IAC concentration that can be reproducibly amplified be used. Heterologous (non-competitive) IACs are designed to be amplified in the same tube and in a separate reaction than the target using a primer and probe different from the target. The primer and probe is usually that of synthetic nucleic acid or a gene present in any microorganism at a level higher than the target. The IAC and target do not compete for primers, and the IAC amplification must be limited by a minimal concentration of IAC primer. The disadvantages of heterologous IACs include that the amplification of the heterologous IAC does not necessarily reflect the amplification of the primary target, and that two PCRs (target and IAC) reactions must be developed to work under the same conditions, which may not be optimal for either reactions. The main advantage of heterologous (non-competitive) IACs is that the same heterologous IAC can be used in assays of different target RNAs (provided the assays can work under the same conditions). The homologous (competitive) IACs, although more time consuming to develop, are generally recommended because they avoid the risk of multiples primer interaction and allow both PCR reactions to run under identical conditions with the same primer.

As previously mentioned, successful amplification of the viral genome does not necessarily equate to the detection of infectious virus. To discriminate between infectious and noninfectious virus, methods are being developed to capture viruses having intact capsid antigens, evaluate viral capsid integrity, or assure that viral RNA is complete. Such methods are reviewed by others. Recently, porcine mucin has been proposed as a method for excluding noninfectious NoV from detection by RT-PCR. In these experiments, NoV that was exposed to disinfection treatments could no longer effectively bind to porcine gastric mucin-conjugated magnetic beads. Additional research and validation is necessary before these methods can be integrated into standard protocols for virus detection in foods.

As methods for detection of viruses in different food matrices have developed over the past decade, standard methods have begun to be accepted across the field. There are now validated methods for the detection of viruses in shellfish and berries. The European Committee on Normalization has a working group (CEN/TC 275/WG6/TAG4 'Detection of viruses in food') focused on the development of a European Union standard method for the detection of NoV and HAV in key foods and bottled water.

22.5 Transmission of foodborne viruses, outbreaks, and their prevention in high-risk commodities

The relative importance of food amongst enteric virus transmission routes (person-to-person contact, consumption or bathing in contaminated water, and contact with contaminated inanimate objects [fomites]) is unknown, but because enteric viruses are transmitted readily

between persons, a single foodborne case has the potential to result in a large propagated outbreak. Food remains an important vehicle of enteric virus transmission because it is easily and regularly introduced into the body and it has widespread global distribution. Foods differ in their likelihood and mechanisms of contamination by foodborne viruses. Contact with fecal material (or vomitus) is the ultimate source of virus contamination, and this can occur directly (for instance, by using human fecal matter as a fertilizer for crop production) or more often, indirectly by contact with fecally-contaminated materials or objects (e.g., hands, surfaces, waters, etc.). The contamination event can be focal or diffuse and can occur at any stage of the farm-to-fork continuum (production, harvest, processing, preparation, and consumption). Though contamination of large amounts of food, such as an entire field of strawberries or a large batch of frosting, results in dilution of the initial virus concentration, because foodborne viruses are infectious at low doses, even a small amount of final contamination, after environmental dilution, has the potential to result in disease.

There are two primary ways to control foodborne transmission of enteric viruses: (i) prevent the initial contamination event; or (ii) process the food so as to inactivate viruses when and if they are present. General approaches to prevent the initial contamination event include (i) policies to prevent disposal of raw (untreated) human fecal material; (ii) strict sanitation and hygiene for individuals having direct contact with foods; and (iii) installation of barriers (gloves, preventing symptomatic workers from having contact with foods) to prevent contamination. Example of challenges to these approaches include (i) severe weather events on farms (produce) or growing waters (shellfish) that lead to contamination by untreated human fecal material; (ii) inability of hand sanitizers to inactivate certain viruses on hands; and (iii) the difficulties in identifying asymptomatic and infected food handlers. General approaches to process the food so as to inactivate viruses include (i) the use of compounds with improved antiviral activities (produce washes, chicken rinsates); and (ii) food processing technologies that inactivate viruses (hydrostatic high pressure processing, cooking, irradiation). Examples of challenges to these approaches include (i) the inability of anti-viral compounds or food processing technologies to achieve inactivation levels sufficient to inactivate all viruses on foods without unacceptable effects on sensory qualities or product shelf life. The global importance of prevention and control of foodborne viruses has recently come to the forefront in efforts put forth by the Codex Alimentarius Commission, which recently published the *Guidelines on the Application of General Principles of Food Hygiene to the Control of Viruses in Food*.

Three types of commodities are usually associated with foodborne viral outbreaks, each with distinct transmission pathways: fresh produce, molluscan shellfish, and ready-to-eat (RTE) foods. Each of these is discussed in greater detail below, with a focus on contamination routes, notable outbreaks of viral foodborne disease, and strategies for prevention and control.

22.5.1 Fresh produce

Fresh produce refers to fruits and vegetables that are likely to be sold to consumers unprocessed or minimally processed (raw), and can be intact (e.g., whole carrots, strawberries), cut during harvesting (e.g., broccoli, celery), or 'fresh-cut', meaning the produce is processed to be pre-cut, packaged, and ready to use (e.g., ready-to-eat salad mixes). Fresh produce items are more prone to virus contamination because they are frequently consumed raw or minimally processed, there is no virus 'kill' step, and there are many opportunities for contamination from farm-to-fork such as during production (via contaminated irrigation

water, use of human sewage for fertilization, by soil), harvesting (via the hands of pickers), in processing/packing (via water or hands), and/or during preparation (via hands of food preparers).

Enteric viruses, like NoV, may be found on produce. In a recent study, evidence of human NoV contamination was identified in 33%, 50% and 28% of lettuce samples purchased at markets and catering companies in Belgium, France, and Canada, respectively. In the US, foodborne disease outbreaks involving produce have generally had more illnesses per outbreak than those traced to other foods.

22.5.1.1 *Viral contamination of and transmission via produce*

Unfortunately, the relative importance of the various routes of virus contamination of fresh produce is unknown. Clearly, soil and irrigation water are important potential sources of virus contamination to fresh produce. In this case, the product becomes contaminated by exposure to untreated or inadequately treated sewage effluents, or by direct defecation of workers (or their children) in the fields. In developing countries, irrigation with untreated or insufficiently treated wastewater is relatively common and the incidence of foodborne pathogens on fruits and vegetables has been shown to increase with the use of insufficiently treated wastewater for irrigation. Wastewater treatment plants, while effective at reducing bacterial contamination of water, have been shown to have detectable levels of enteric viruses even in their 'clean' effluent waters (after secondary treatment). Guidelines for the microbial quality of irrigation waters vary from country to country and by water type but most use fecal indicator bacteria like E. coli as criteria. As mentioned previously, elevated fecal indicator counts do not predict the presence of viral pathogens. For example, in a study of farms in South Korea, enteric viruses, including enteroviruses and NoV, were found in groundwater and on raw produce, even when groundwater concentrations of bacterial indicators were below recommended levels. Even if poorly treated wastewater is not used as a source of irrigation water, other sources like groundwater or surface water may be contaminated. Groundwater can become contaminated from nearby solid waste or sewage disposal, and surface waters are susceptible to contamination from sewage discharge.

Once soil or water is contaminated with enteric viruses, the viruses can survive for weeks or months, depending upon factors like soil composition, temperature and moisture, resident microorganisms, and virus type. For example, both murine NoV and HAV have been shown to maintain their infectivity in manure and biosolids after months of storage. Human NoV have been documented to persist and remain infectious in water for months.

Contamination of fresh produce during harvesting and packing can occur as a consequence of inappropriate hygiene behaviors of workers handling the product. A survey of nearly 3000 reports from American farms suggested that 94% of all fruit acres and 87% of all vegetable acres surveyed were harvested by hand. Leon-Felix et al. (2010) found that the hands of between one-quarter to one-third of Northern Mexican green bell pepper classifiers and packers showed evidence of human NoV contamination, and 30–45% of the peppers processed also showed evidence of human NoV contamination. Many countries import fresh produce in order to provide a year-round supply. If the exporting countries have hygiene and agricultural practices below the standards of the importing countries, the consumers are at elevated risk.

Despite these potential contamination routes, it is often difficult to identify the exact source of contamination during fresh produce production. For example, in a 1999 outbreak of GII NoV in Finland that was traced to frozen raspberries imported from Eastern European countries, contaminated irrigation water was suspected to be the source, but traceback was complicated

and this source was never definitively proven. The source of a 2006 large GII.1 NoV outbreak in Finland associated with raw vegetables was likewise never identified. Blueberries originating from a single orchard were responsible for an HAV outbreak in New Zealand, and the associated sanitary audit identified multiple opportunities for contamination by pickers. The largest U.S. foodborne outbreak of HAV was linked to green onions produced in Northern Mexico, perhaps contaminated by young children defecating in the fields during harvest.

22.5.1.2 *Virus outbreaks associated with produce*

Contaminated produce has the potential to result in virus outbreaks occurring on a multinational scale. For example, semi-dried tomatoes were implicated in HAV outbreaks in Australia (two in 2009), France (2010), and the Netherlands (2009–2010), and all four outbreaks were caused by highly similar genotype IB strains of the virus. Contamination was thought to have occurred during production, harvesting, or shipping, but the raw product could not be definitely traced back to its originating source. Because the processing methods were highly variable, the outbreaks were of long duration (7 months), and there was no evidence of HAV infection in the manufacturing plant workers. Investigators concluded that it was unlikely that an ill person involved in processing could have caused the outbreak. The ultimate source was thought to be tomatoes contaminated during production in a country with endemic HAV, but a complicated international supply chain prevented product trace-back to a specific farm.

Once pathogens are deposited onto the surface of fresh produce items, many factors influence their attachment and subsequent survival. Attachment of viruses to produce can be affected by surface characteristics, such as the irregular leaves of leafy greens or the rough ridges of cantaloupes. Moisture, pH, and the amount of organic matter on the plant surface are other important factors. Nonetheless, although there is evidence of pre-harvest internalization of NoV and hepatitis A in leafy vegetables, most investigators believe that this is of minimal importance relative to surface contamination. It has been noted that some fruits and vegetables produce natural antiviral compounds that may accelerate loss of viral infectivity.

In closing, it should be mentioned that there are limited surveys, and therefore a need, to quantify the magnitude and prevalence of enteric virus contamination of produce on farms and packing sheds.

22.5.1.3 *Prevention of viral outbreaks associated with produce*

Because fresh produce is usually consumed raw or with minimal processing, control strategies have focused on preventing virus contamination before it can occur. Guidelines for produce safety at the production (farm) phase place a strong emphasis on Good Agricultural Practices (GAPs) – the policies and practices farms should follow to prevent contamination of fresh produce with enteric pathogens during growing, harvest, and packing. In general, these guidelines help the producers identify and manage potential sources of contamination: in the case of viruses, these sources are usually fecally-contaminated soil amendments or irrigation water, and food handlers who are ill or shedding viruses. Many organizations in developed nations have put GAPs policies in place, and a full accounting of all the programs available is beyond the scope of this work. In fact, to address the duplicity of efforts, US federal food safety agencies, in cooperation with trade organizations, have begun GAPs harmonization efforts. On a more international scale, GLOBALG.A.P., a nongovernmental organization, functions as a global partnership of over 100 European, North American, and other food service retailers, producers, suppliers, and certification bodies, with the goal

of creating one unified set of international GAP standards. They currently have a set of six standards and an annual certification process based on the WHO Codex Alimentarius Commission HACCP guidelines. As more members recognize the GLOBALG.A.P. certification process, there is likely to be pressure on countries interested in trading produce internationally to develop a national GAP system to comply with these standards. For example, GLOBALG.A.P. has been working with the People's Republic of China since 2005 to establish a national certification system, ChinaGAP, so Chinese producers can be certified in international recognition of their produce safety.

Despite national and international GAPs efforts, there remain challenges in implementing guidelines, including issues with training, monitoring and evaluation. For example, despite several training programs, farm workers are frequently itinerant and poorly compensated. Communication between farm managers and workers may be complicated by language and cultural barriers and workers may lack the understanding or motivation to apply training into action. Monitoring and evaluation of GAP compliance presents its own set of financial and logistic challenges, as these GAP compliance programs are almost always voluntary and do not carry the force of law. Smaller producers sometimes believe they are at an economic disadvantage as they cannot afford large, expensive GAPs initiatives (and associated third party audits) that are required by large retailers as a prerequisite to the purchase of their products. In the case of developing countries, GAP compliance may even be viewed as a potential trade barrier.

22.5.2 Molluscan shellfish

22.5.2.1 *Viral contamination of and transmission via molluscan shellfish*

Molluscan shellfish are exoskeleton-bearing aquatic invertebrates that obtain their food from the water column by filter feeding activities. They include species such as clams, mussels, oysters and scallops. The ultimate source of enteric virus contamination in shellfish is marine growing waters that are contaminated with human fecal matter. This can occur from various sources: discharges of untreated or undertreated wastewater and sludge, failing septic systems, or illegal dumping of human waste, especially from marine vessels. Infrequently, molluscan shellfish may also become contaminated with enteric viruses during preparation for consumption, usually due to poor hygiene practices of infected food.

In the process of filter feeding, molluscan shellfish process large volumes of water and not only collect microbial contaminants, but also concentrate them. Based on molluscan biology, if shellfish are grown in human sewage-contaminated waters, they can harbor high concentrations of enteric viruses. Rates of bioaccumulation are affected by environmental factors like water temperature or salinity, as well as the type of mollusc and its size. In addition to bioaccumulation, there is evidence that human NoV actually bind with some degree of specificity to shellfish digestive tissues using carbohydrate structures similar to their human ligands. A recent study by Provost et al. (2011) suggested that enteric viruses may persist in the phagocytic blood cells (hemocytes) of shellfish, allowing them to be sequestered. Consequently, there are a number of mechanisms that promote enteric virus accumulation and persistence in contaminated molluscs.

The prevalence of enteric viruses in molluscan shellfish is variable, but can be quite high. Assays of oysters and mussels around the world have found NoV prevalence to vary from 4.5–37%. Mesquita et al. (2011) tested 15 batches of shellfish from class 'A' growing and harvesting waters in Portugal and found two (13.3%) NoV-positive batches and three (20%) HAV-positive batches of shellfish.

22.5.2.2 *Virus outbreaks associated with shellfish*

NoV, HAV, sapovirus, astrovirus, enterovirus, rotavirus Aichi virus, among various enteric viruses, have been implicated in foodborne disease outbreaks linked to molluscan shellfish. Outbreaks are often linked to point-source contamination of harvest waters with raw human sewage. For example, in the largest HAV outbreak in history, clams impacted by the disposal of untreated residential effluent into rivers in Shanghai, China sickened at least 292,000 people. Contamination of shellfish by sewage effluents may result in outbreaks caused by multiple virus types or strains, see Case Study 1.

Point-source contamination of shellfish harvesting waters can also occur on a smaller scale, when individual boaters discharge waste materials. Since fecal matter from an infected individual may contain millions to billions of virus particles per gram, and the human NoV infectious dose is low, the stool from one single infected individual can be enough to contaminate a shellfish bed that is 25 m deep and approximately the area of a football (soccer) field (200,000 m^3), and cause an outbreak. Harvesting oysters from prohibited waters has also been associated with enteric virus outbreaks.

Despite the importance of point-source contamination, there have also been instances of more widespread contamination events, as was the case for a 2004 British Columbian oyster-associated NoV outbreak traced to 45 groceries and restaurants, 18 suppliers and producers, and 14 geographically-distinct harvest sites, for which the source of contamination was never identified. Outbreaks have even been associated with product originating from approved waters. An outbreak of human NoV in Oregon, US, was traced to oysters grown by US-approved growers in South Korea and legally imported to the US. Not only was this outbreak unexpected, it also demonstrates the impact of contaminated food on a global scale.

Case Study 1 An International Outbreak Due to Viral Contamination of Shellfish with Sewage

In December 2002, the French public health authority received reports of outbreaks of acute gastroenteritis from several areas across the country. Questionnaires were sent to all persons affected to assess the source of the outbreak and 12 fecal samples were collected from patients from various regions to determine the etiological agent. That same December, Italy's public health unit became aware of over 200 gastroenteritis cases. Similarly, questionnaires with questions on time, place, and symptoms were distributed to ill persons and fecal samples were collected for pathogen identification.

The questionnaires from both countries revealed a strong correlation between the consumption of shellfish and the development of illness. Investigators collected oyster samples from leftovers at private homes, and all samples were found to be positive for norovirus (NoV). Because multiple strains were found in both the oyster samples as well as the fecal samples, it was initially difficult to link these two separate national outbreaks. Investigators soon discovered that all samples contained the same rare strains of NoV (i.e., GII.8), indicating the possibility that all cases had a common source. Interestingly, these oysters had undergone the mandatory two-day depuration several days before the outbreak was reported.

Contamination of food with multiple strains of NoV is often associated with sewage contamination of water. Further investigation found that all of the oysters consumed in France and Italy were produced from the same harvesting area south of France. Heavy rain and wind earlier in December caused water runoff and sewage treatment failures that likely contaminated the oyster beds. Following this flooding, the surveillance network in the area did in fact detect increased coliform counts and *E. coli* contamination in shellfish, although levels remained within the European regulation of 230 *E. coli* per 100 g. Consequently, harvesters were mandated to subject shellfish to depuration for two days before bringing them to market and before the outbreak was identified. While depuration has been shown to effectively eliminate *E. coli*, it is a poor method for eliminating viruses. Because this outbreak occurred with depurated oysters, it illustrates the limitations of current water surveillance methods and recommended post-harvest shellfish treatments in preventing viral outbreaks (LeGuyader et al. 2006).

22.5.2.3 *Prevention of viral outbreaks associated with shellfish*

Harvest areas in the US, Europe, and other countries have regulations in place to prevent dumping of human waste near shellfish beds, as well as to monitor growing waters for potential human fecal contamination. For example, the U.S. Food and Drug Administration provides guidelines for the safe growing, monitoring, processing, and distribution of shellfish in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish In the US, growing waters are classified as approved, restricted, or prohibited. Waters may be 'conditionally approved' to be open during periods when waters meet the 'approved' standards, or similarly 'conditionally restricted' and open during periods when waters meet the 'restricted' standards. The National Shellfish Sanitation Program (NSSP) determines the classifications for growing waters based on a sanitary survey, levels of pathogens, marine toxins, and either total or fecal coliform concentrations. Unfortunately, there is little correlation between the levels of fecal indicator bacteria and the presence of enteric viruses in molluscan shellfish or their growing waters. Table 22.2 gives a description of the standards and consequences of each classification.

The European Union (EU) Shellfish Growing Waters Directive (2006/113/EC) provides member states with guidelines and imperative standards for the quality of shellfish waters, and the frequency of monitoring. However, there is no microbial standard for water quality in the EU – rather, such parameters are limited to pH, suspended solids, metals, and others. Instead, shellfish are tested directly and monitored for acceptable levels of fecal coliforms (300/100 g mollusc) and *E. coli* (230/100 g mollusc) in their tissues. As is the case for indicator levels in harvesting waters, the levels of fecal indicator bacteria in shellfish meat are relatively poor predictors of virus contamination. This lack of correlation is explained by a number of phenomenon, the most important of which are the extended environmental persistence of viruses relative to Gram-negative bacteria, the fact that virus concentrations in shellfish often exceed those of the fecal indicator bacteria and the poor removal efficiency for viruses relative to bacteria.

Once contaminated with viruses, the risk of infection from shellfish consumption is high because this product is normally eaten raw or only lightly cooked. Common cooking treatments like steaming, grilling, stewing, and frying will not completely inactivate human enteric viruses, and outbreaks caused by viruses have been associated with cooked shellfish. Cooking must be thorough to inactivate viruses in shellfish, and results are variable.

Depuration – a common purification method that involves allowing shellfish to filter pure water– adequately eliminates bacteria but has been shown to have limited success with virus elimination For example, it has been reported that depuration of oysters for 48 hours reduced *E. coli* levels by 95%, whereas NoV levels were only reduced by 7%. There are several other novel strategies for the treatment of shellfish once they are contaminated. High pressure processing (HPP) is highly effective at eliminating pathogenic bacteria (e.g., *Vibrio* spp.) in oysters and also facilitates shucking. Unfortunately, at pressure-time conditions commonly used by the industry, HPP is not effective at eliminating NoV and HAV. In short, there are currently no commercially-effective methods to eliminate viral pathogens from shellfish that successfully maintain the organoleptic properties of the 'raw' product.

Table 22.2 US standards for Classifications of Shellfish Growing Waters from the U.S. Food and Drug Administration National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish, 2009

	Standards						
			Total coliforms		Fecal coliforms		
Classification	**Sanitary survey**	**Bacterial pathogens*, marine poisons****	**Median or geometric mean of 15 samples (MPN/100 mL)**	**<10% samples exceeding (MPN/100 mL)**	**Median or geometric mean of 15 samples (MPN/100 mL)**	**<10% samples exceeding (MPN or CFU/100 mL)**	**Consequence**
Approved	No public health hazard from fecal contamination sources	None found	70	140–330†	14	28–43†	Growing waters open, no post-harvest requirements
Restricted	Limited fecal contamination sources identified	None found	700	1386–3300†	88	163–300†	Growing waters open, shellfish must undergo post-harvest relaying or depuration
Prohibited	Point or unpredictable fecal contamination sources identified	Any found	Doesn't meet approved or restricted standards		Doesn't meet approved or restricted standards		Growing waters closed

* *Vibrio vulnificus* and *Vibrio parahaemolyticus*
** Paralytic Shellfish Poison (PSP) and Neurotoxic Shellfish Poison (NSP)
† Depending on which assay is used

22.5.3 Ready-to-eat (RTE) and prepared foods

22.5.3.1 *Viral contamination of and transmission via RTE foods*

Ready-to-eat (RTE) foods are those foods that have been completely processed or prepared and do not require a terminal heating step prior to consumption. Examples include many store-bought foods, like potato salad, sandwiches, and baked goods, or pre-packaged foods, like fruit cups. Poor hygiene practices of infected food handlers are almost always the source of virus contamination of these products. Because the seroprevalence of virus infection in the general population is high, particularly for human NoV, there is a high probability that food handlers will be infected with enteric viruses. Symptomatic food handlers are at highest risk for contaminating RTE foods because they are shedding virus in high numbers in their feces (or vomitus) and they are frankly ill. However, food handlers, like any normal individual, may still shed virus pre-symptomatically, post-symptomatically, or asymptomatically. As described previously, enteric viruses can be shed fecally for weeks after symptoms have resolved. Viruses can be even be transmitted by healthy individuals who have simply had contact with infected people. Virus transfer from fingers to foods (e.g., ham, lettuce) and preparation surfaces (e.g., stainless steel) has been documented, as well as the movement of virus from gloved hands to lettuce.

22.5.3.2 *Virus outbreaks associated with RTE foods*

Many outbreaks of both HAV and human NoV have been traced to RTE foods. The U.S. CDC has estimated that almost 50% of all NoV outbreaks are due to contamination from an ill food handler, and an international review of human NoV outbreaks cited that a food handler was implicated in 17 of 40 outbreaks (43%) occurring between 2000 and 2007. One, or few, infected food handlers can cause outbreaks of great magnitude as described in the following examples.

22.5.3.3 *Prevention of viral outbreaks associated with RTE foods*

There are several strategies to prevent transmission of viruses during preparation of RTE foods, both in commercial settings and in homes or institutions. First, if food handlers are symptomatic, the U.S. CDC and other public health agencies recommend they be removed

Case Study 2 Outbreak in Austria caused by Ill Foodhandler

In Austria, in January 2006, nearly 200 of 500 total employees at a telephone company fell ill with gastroenteritis. Stool samples from patients revealed that the cause of illness was norovirus (NoV), and questionnaires distributed to ill patients confirmed that all of the cases ate at the company cafeteria. The investigators gathered information on foods consumed by the ill individuals and found that the greatest risk factor for contracting illness was eating a salad prepared and served on a specific day.

On January 18th, five days before the outbreak was reported to the Austrian Agency for Health and Food Safety, a young kitchen assistant fell ill with diarrhea and decided to remain at work for fear that a prolonged absence would cause her to lose her job. On-site inspection revealed that the kitchen lacked several basic hygienic requirements, such as functional hand-washing facilities. After the kitchen was closed for inspection and cleaning on January 23rd, the number of new cases rapidly declined. Before the kitchen was re-opened, all kitchen staff received training in food hygiene. This outbreak reflects the importance of enforcing hygiene practices in preventing outbreaks as well as the impact of sick-leave policies in the food industry (Schmid et al. 2007).

from food preparation tasks until 2–3 days after symptoms resolve. While local health departments may recommend or require ill workers to take time off, unpaid sick leave may incentivize workers to avoid reporting illness to supervisors. Even if these recommendations are followed, demonstrated virus shedding for weeks or months after symptoms resolve means that food handlers who return to work may still transmit the virus if they are not practicing ideal hand hygiene. In the home, sick individuals should avoid preparing food and be excluded from food preparation areas.

Because asymptomatic food handlers may still transmit virus, constant and proper attention to hand hygiene is imperative in preventing the contamination of foods with enteric viruses. There is compelling evidence that alcohol-based topical agents and hand gels cannot be relied upon to inactivate human NoV on hands. Handwashing with soap is the primary recommended hand hygiene behavior, and alcohol-based sanitizers are only recommended as a supplement to proper handwashing.

The U.S. FDA Food Code (Section 2.301) details exact requirements for handwashing procedures and when hands need to be washed. To clean their hands, food employees must use a sink used exclusively for handwashing and vigorously wash their hands and any exposed part of their arms for 20 seconds using soap. They must also clean under all fingernails before rinsing under running water and drying with an approved drying device (e.g., paper towel, air dryer). If necessary, they must shut the water off with a paper towel or other barrier to avoid recontaminating their hands. This procedure must be followed each time a food employee: touches any part of their or another's body other than the hand or arm; uses the toilet; cares for animals; coughs; sneezes; uses a handkerchief or tissue; eats; drinks; smokes; uses soiled equipment or utensils; changes tasks during food preparation; switches from preparing raw to ready-to-eat foods; prepares to put on gloves; and engages in any activity that may contaminate their hands. While these recommendations are necessarily rigorous food handlers may find them difficult to enforce given their multitude of responsibilities and the speed and efficiency that a food service position demands. In fact, a 2004 FDA study found that 73% of restaurants were out of compliance with these regulations.

The U.S. FDA does not allow employees to contact RTE foods with their bare hands except in certain circumstances (e.g., washing produce), and instead requires them to use 'barriers' such as single-use gloves and other utensils like spatulas, tongs or deli tissue. When working with any food, gloves must be single-use and discarded when damaged, soiled, or when the worker changes tasks. Slash-resistant or cloth gloves are generally only permitted for use with foods that will be cooked afterwards (e.g., shucking oysters or cutting meat).

Teaching and enforcing proper hygiene practices is an important public health measure in preventing outbreaks, but hand hygiene compliance and behavior change are complex issues. While many food safety training programs focus on knowledge gain, there are many other determinants of behavior. There are many conceptual frameworks developed to define the determinants of handwashing behavior – one that is used globally is the Water and Sanitation Program's FOAM framework. The FOAM framework identifies the opportunities, abilities, and motivations of people needed to improve handwashing behavior. Opportunity determinants are external factors that affect the chance of good handwashing being performed, such as easy access to appropriate handwashing facilities and the social norms of the workplace. Ability determinants reflect each person's perceived capacity to wash their hands, and include their knowledge of disease transmission or proper handwashing procedures. Motivation determines whether an individual wants to wash their hands, and includes employees' perceived risks, competing priorities, and other emotional or social drivers.

Comprehensive handwashing behavior change programs in the food service industry need to address the main barriers to individual opportunities, abilities, and motivation. Opportunity barriers include inaccessible or inconvenient hand-hygiene facilities or lack of supplies (soap, warm water), positive reinforcement for compliers and negative reinforcement (e.g., penalties) for non-compliers, and lack of time to wash hands appropriately (understaffing). Ability barriers include a lack of handwashing guidelines and a lack of knowledge about germ theory or disease transmission. Motivational barriers include the perceived negative effects of handwashing (skin irritation), interference with other job priorities, perception that handwashing is not prioritized by superiors or peers, lack of a food safety climate at work, lack of role models, and lack of a culture of compliance.

Characteristics of successful hand-hygiene programs are reviewed in Pittet (2000). Successful programs address opportunity barriers by making hand hygiene easy and convenient. Successful programs also address ability barriers through education and training of proper handwashing practices. Successful programs address motivation barriers by improving workplace social norms through enforcing employee sanctions and rewards. Motivation is also achieved through routine observation with feedback, placing reminders in the workplace, providing for skin care (e.g., lotion), involving all levels of business (individual, management, and institution), and avoiding understaffing and excessive workloads so that food handlers do not need to choose between washing their hands and their other work responsibilities.

In addition to proper sick-leave policies and hand hygiene, to prevent contamination of foods by enteric viruses, the CDC recommends disinfection of food preparation surfaces with 1000–5000 ppm of chlorine (1:50 to 1:10 dilution of household bleach, equivalent to 5–25 tablespoons per gallon of water) and washing linens with detergent and hot water followed by machine drying. These rigorous recommendations are based on the absence of other reliable disinfectants for enteric viruses. Detergents have been shown to be ineffective at reducing NoV concentrations, and washing surfaces with detergents can spread NoV via the cleaning tools and hands. Low (<2) and high (>12) pH, UV light disinfection and 70% ethanol also have been ineffective at completely eliminating NoV. The U.S. Environmental Protection Agency (EPA) provides a list of over 50 commercial products demonstrated to be effective against Norovirus – many list sodium hypochlorite as the active ingredient. Even 5000 ppm available chlorine has been shown to be ineffective at completely eliminating NoV on surfaces. In addition, such high concentrations of chlorine may not be practical or acceptable in the food service industry because chlorine can be corrosive, toxic, irritate the eye, skin, mucous membrane, and upper respiratory tract, as well as being a possible carcinogen.

Food handlers and the retail food sector are concerned about NoV-associated vomiting events. NoV-associated vomiting events have led to outbreaks on airplanes, cruise ships, hotels, and restaurants. These vomiting events by food handlers or customers, despite decontamination activities, led to cases of NoV infection several days to weeks after the initial contamination event. Vomitus, from NoV-infected individuals, has not yet been assayed to quantify ranges of concentrations of norovirus but sufficient NoV is transmitted from vomitus samples to persist in the environment and cause infection in individuals either through direct contact or aerosols. There are no guaranteed decontamination guidelines for NoV-associated vomiting events but suggested guidelines by the CDC, based on laboratory-based NoV disinfection studies (discussed in the preceding paragraph) recommend: (i) protection of clean-up personnel with barrier protection like gloves; (ii) minimizing aerosolizing of vomitus during clean-up; (iii) transportation of soiled surfaces or laundry, or

disposal; (iv) chlorine bleach solution for disinfection of surfaces; (v) practicing frequent handwashing; and (vi) washing soiled laundry at the highest temperature and cycle length and then air-drying. Other state and county department of health have more specific guidelines for vomitus removal and disposal, use of non-chlorine bleach disinfectants, for disinfection of surfaces in which chlorine bleach concentrations may vary from 1:10 to 1:250 diluted in water depending on surfaces to be disinfected, and for washing of laundry. Though these guidelines are inconsistent with each other or have not been demonstrated to be effective at norovirus inactivation under the specified condition, they generally follow CDC guidelines based on laboratory studies and therefore may be used following a vomiting event. It is important to note that though the Environmental Protection Agency has approved several disinfectants for use against NoV, these disinfectants have been tested using NoV surrogates that are weaker than human NoV and therefore may not be as effective as chlorine bleach (which has been tested against human NoV) to inactivate human NoV. In summary, following a NoV-associated vomiting event it is best to use chlorine bleach for disinfection of surfaces and complement, but do not replace, CDC's guidelines with other guidelines for NoV-associated vomiting event disinfection.

22.6 Conclusions

In conclusion, existing and emerging foodborne viruses will continue to cause morbidity and mortality among consumers because of common transmission routes, industry behaviors and practices and consumer behaviors and practices. Therefore, it is important to improve existing strategies to address known foodborne viruses and simultaneously proactively anticipate novel emerging foodborne viruses. As described at the beginning of this chapter, it is likely that in the next decade novel emerging foodborne viruses will be identified that can be associated with proportions of currently unknown foodborne illness cases. Therefore, four general recommendations are proposed including discovery, prevention, confirmation, and inactivation of foodborne viruses.

1. **Discovery**. Research efforts should focus on identifying novel viruses that may explain the unknown foodborne illness cases through a combination of epidemiologic and laboratory partnerships. Re-emerging viruses may also be responsible for a proportion of foodborne cases and therefore existing viruses should also be considered.
2. **Prevention**. Food industry groups, including produce, shellfish, and RTE industries have worked together with each other, government, and the research community to identify specific prevention behaviors and practices with existing data. These specific behaviors and practices need to be evaluated by the research community to assess their effectiveness at foodborne virus prevention so that they can be enforced or discarded. In the meantime, industry can work with behavioral researchers to identify effective strategies for training and compliance of these human behaviors and practices. It is important to emphasize that in RTE foods hygiene and regulations are in place in many developed countries, but a poorly-paid, unskilled workforce with high turnover will present continual challenges to proper training and hygiene behavior. Two recurring needs from industry are (i) the identification of acceptable levels of risk on foods by viruses (i.e., sterility in foods are usually impossible) and (ii) identification of cost-effective strategies to survey pathogenic viruses on foods or, instead, to identify strategies that will survey effective surrogates of pathogenic viruses. A possible

surrogate for existing and emerging foodborne viruses could be the detection of fecal matter on foods by chemical (or microbial) technologies.

3. **Confirmation**. As described in this chapter, currently foodborne viruses are difficult to detect and classify on environmental and food samples. In addition to new detection, confirmation, and classification technologies, harmonization efforts of these effective technologies among government and industry would assure that viruses can be reproducible detected, confirmed, and classified.
4. **Inactivation**. Ideally, inactivation of foodborne viruses would be the last resort but in reality these efforts must parallel prevention efforts. The largest obstacle to research on technologies to inactivate foodborne viruses is that regulatory agencies require ineffective surrogate viruses in the approval of these technologies and do not require the use of human enteric viruses. Researchers should also be cognizant of consumer and industry needs, and adapt their research to address these needs (e.g., effective technologies to inactive viruses from a vomiting event).

Partnerships between industry, government, and researchers were responsible for the tremendous strides in the past two decades to identify new emerging viruses and prevent and control known existing viruses. Similar and stronger partnerships will ensure that the next few decades yield improved protection for global consumers.

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23 Seafood toxins

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23.1 Introduction

All of the major seafood toxins have microbial origins. Most are produced by microscopic phytoplankton while a few are the products of marine bacteria and cyanobacteria. Among seafood products potentially contaminated by these natural toxins, molluscan shellfish pose a different management problem than fish due to their filter feeding; Clams, mussels, oysters, cockles, and other bivalves can very rapidly accumulate the toxins, necessitating that harvest be curtailed. Most of the seafood toxins found in fish and shellfish are produced as secondary metabolites in dinoflagellates and diatoms, and are often metabolized further prior to harvest. The most threatening of these naturally-occurring toxins in fish and shellfish are potent neurotoxins and a few of them can be fatal to humans. Nevertheless, seafood continues to increase in popularity, and commercial seafood products are rarely implicated in illnesses due to seafood toxins. This success in ensuring a safe supply of seafood has resulted from the considerable efforts of regulators, the seafood industry, and academic researchers.

Rapid onset of symptoms is one of the first indications of seafood intoxication. Compared with microbial infections, symptoms due to seafood toxins take effect much more rapidly, in minutes to a few hours. When outbreaks do occur, doctors and other health professionals must know how to recognize symptoms and quickly choose treatments. Detailed symptomology and also etiology are described below for each type of seafood intoxication, together with descriptions of how the impacted seafood is monitored and associated analytical methodology.

23.2 Shellfish toxins

In addressing the 'shellfish toxins' to be discussed here, emphasis will be placed on those which are known to cause human illness. The five major shellfish poisoning syndromes include: paralytic shellfish poisoning (PSP); amnesic shellfish poisoning (ASP); diarrhetic

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

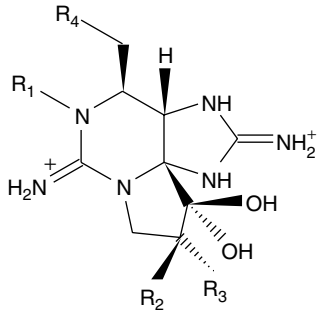
shellfish poisoning (DSP); azaspiracid shellfish poisoning (AZP); and neurotoxic shellfish poisoning (NSP). Although the associated toxins are primarily considered molluscan shellfish toxins, fish as well as other invertebrates feeding on plankton can become toxic also, and this kind of transfer of the toxins via food chains can also produce toxic crustacean shellfish, primarily in their viscera. Finally, there are other 'shellfish toxins' that often occur in combination with these toxins but which have no demonstrated public health impact nor oral toxicity in laboratory animals, and are mentioned only briefly. This latter group of toxins, which include the yessotoxins, pectenotoxins, and cyclic imines among others, is mentioned only because of their historical importance and interference in mouse bioassays. Although still included in some areas of the world in regulatory legislation, mainly as an artifact of initial use of intraperitoneal (IP) injection mouse bioassays, these toxins are generally de-emphasized in health protection efforts, or are slated for deregulation due to lack of oral toxicity. In the interest of brevity, the reader is referred to other sources such as toxicology reviews for additional information.

23.2.1 Paralytic shellfish poisoning (PSP)

Paralytic shellfish poisoning is often first noticed as a tingling or burning sensation in the lips. At high doses paralysis of the extremities and loss of motor coordination occur and without treatment death by respiratory paralysis can occur. Since the toxic effect is temporary (due to reversible toxin binding), a respirator is often all that is needed to save the life of a victim.

The potent neurotoxins responsible for paralytic shellfish poisoning are known as the saxitoxins. It was first believed that a single compound (the parent compound saxitoxin) caused PSP. At this time, over 20 different saxitoxins have been discovered. This is perhaps the best-understood suite of toxins, since most of the toxin structures were elucidated years ago and most are now commercially available as certified reference materials. The most common saxitoxins are shown in Fig. 23.1. Many make a significant contribution to the total toxicity, although their toxicities vary widely, with the more positively-charged congeners the most potent. Stereochemistry also plays a central role in toxin potency, and epimerization and other interconversions among the various forms modulate their overall toxicity. Many species of dinoflagellates are now associated with the saxitoxins. At northern latitudes these include *Alexandrium catenella*, *A. excavatum*, *A. fundyense* and *A. tamarensis. Gymnodinium catenatum*, *Pyrodinium bahamense* and *Gonyaulax polyedra* are found at more southerly latitudes. Dinoflagellates exist in free-swimming and cyst forms, and although classic 'red tide' blooms are caused by the free-swimming forms, cysts (deposited in bottom sediments) can remain toxic for several months. Toxin profiles produced by a particular dinoflagellate strain in a fixed location are well defined for a given area but vary depending on the shellfish species ingesting them. Overall, the most remarkable differences in toxin profile are observed across the different dinoflagellate species.

Although the mouse bioassay remains the predominant method of detection in the management of PSP in shellfish, this situation is rapidly changing because as of 2012 three additional official methods of analysis (OMA) have been approved by AOAC Int. Those US states with most active monitoring programs for preventing PSP analyze thousands of samples over the course of a year and must return results rapidly enough to allow closure of toxic harvest areas and prevention of illness. Thus, high sample throughput is critical for success, and refinements to the official methods such as automation and more rapid separations



	R_1	R_2	R_3	R_4
Carbamoyl saxitoxins				
STX	H	H	H	$OCONH_2$
GTX II	H	OSO_3^-	H	″
GTX III	H	H	OSO_3^-	″
NEO	OH	H	H	″
GTX I	OH	OSO_3^-	H	″
GTX IV	OH	H	OSO_3^-	″
Decarbamoyl saxitoxins				
dc-STX	H	H	H	OH
dc-GTX II	H	OSO_3^-	H	″
dc-GTX III	H	H	OSO_3^-	″
dc-NEO	OH	H	H	″
Sulfamate saxitoxins				
B_1	H	H	H	$OCONHOSO_3^-$
C_1	H	OSO_3^-	H	″
C_2	H	H	OSO_3^-	″
B_2	OH	H	H	″
C_3	OH	OSO_3^-	H	″

Figure 23.1 The saxitoxins.

emphasize improved method efficiency. Two liquid chromatography methods and a receptor binding assay for detecting the saxitoxins have now been validated and approved as official methods of analysis. Beginning in Canada with the Canadian Food Inspection Agency (CFIA) and Alaska in the United States, North America is beginning to slowly phase out use of mouse bioassays as monitoring authorities embrace the higher sensitivity of post-column oxidation (PCOX, or AOAC 2011.02) HPLC-based monitoring for the saxitoxins versus the traditional mouse bioassay. Approved HPLC methods for the saxitoxins, including the pre-column oxidation HPLC method (AOAC 2005.06) are also being implemented in other areas of the world such as in Europe and Australia.

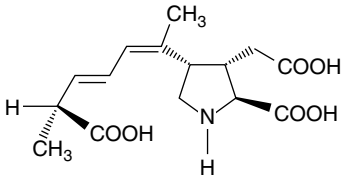


Figure 23.2 Domoic acid.

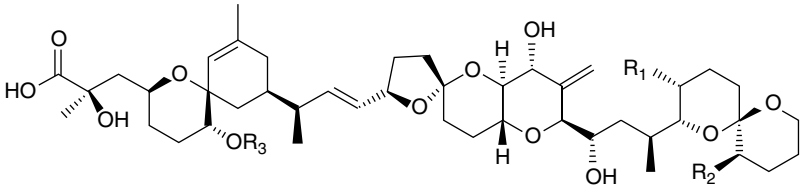
23.2.2 Amnesic shellfish poisoning (ASP)

Domoic acid is a neurotoxic amino acid with the structure shown in (Fig. 23.2). It binds to glutamate receptors in the brain, thereby causing continuous stimulation of nerve cells and eventually lesions are formed. Victims experience headache, loss of balance, disorientation, and the usual gastrointestinal symptoms typical of most food poisoning episodes. The most characteristic symptom, however, is persistent and apparently permanent loss of memory, thus the term amnesic shellfish poisoning. In December 1987, 156 individuals became ill after ingesting blue mussels (*Mytilus edulis*) cultivated and harvested at Prince Edward Island, Canada. Four elderly people died. Domoic acid (up to 900 ppm) was found in the implicated mussels. It is believed that the domoic acid was produced by the diato, *Nitzschia pungens f. multiseries* and then retained by the mussels. This incident introduced amnesic shellfish poisoning, and domoic acid has now been found in many different areas of the world. In addition to causing human illnesses, domoic acid also has significant impact on the environment. In 1993, massive mortalities of pelicans in California were traced to domoic acid, and since then it has been implicated in many marine mammal deaths. Shortly after the pelican deaths were observed, domoic acid was also found in the Pacific Northwest area of the US and may have been the cause of human illnesses in Washington State. The relatively low toxicity of domoic acid [LD_{50} (i.p.) of 3.6 mg domoic acid/kg mouse] and lethality at 4 mg/kg in the monkey *M. fascicularis*, has led to the establishment of an action level of 20 ppm.

Injection of domoic acid into mice does not provide sufficient sensitivity and so no official methods for domoic acid detection use mouse bioassay. The presence of two conjugated double bonds in the molecule results in a strong absorbance at 242 nm, which allows for detection by monitoring absorbance at this wavelength. Domoic acid is readily determined in mussels using conventional reversed-phase HPLC. Two official AOAC procedures for detecting domoic acid in shellfish exist, one based on acid extraction and HPLC-UV detection and another is a commercial ELISA. Careful timing of the acid-extraction step in AOAC HPLC method is crucial since domoate is acid-labile, and another HPLC-UV procedure using neutral aqueous methanol has gained favor and is now listed by Codex Alimentarius as reference method. Domoic acid is also readily detected by LC-MS/MS, and in New Zealand this approach is used in monitoring domoic acid as well as several lipophilic toxins.

23.2.3 Diarrhetic shellfish poisoning (DSP) and azaspiracid shellfish poisoning (AZP)

Both diarrhetic shellfish poisoning (DSP) and azaspiracid shellfish poisoning (AZP) are caused by lipophilic toxins and diarrhea and other gastrointestinal symptoms are the predominant symptoms in both. DSP is however more prevalent than AZP and is caused by a suite of lipophilic acids (Fig. 23.3) versus the lipophilic alkaloids (Fig. 23.4) causing AZP.



	R_1	R_2	R_3
OA	CH_3	H	H
DTX-1	CH_3	CH_3	H
DTX-2	H	CH_3	H
DTX-3	H or CH_3	H or CH_3	Acyl

Figure 23.3 The okadaic acids.

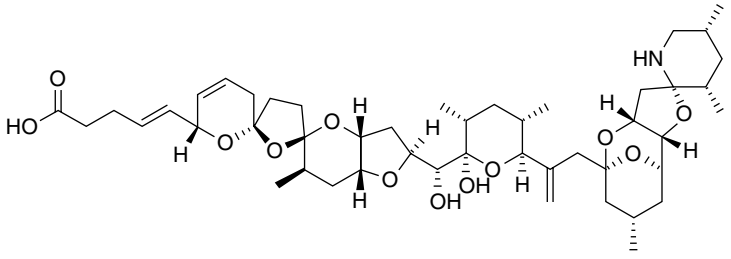


Figure 23.4 The azaspiracids.

Pharmacologically, DSP also occurs through a different mechanism and is much better understood than AZP, as is discussed in more detail below.

DSP was first reported in Japan,in 1978. In Europe, DSP accounts for numerous human illnesses including a 2010 outbreak in Italy impacting 300 victims. Canada has had DSP outbreaks in both its Atlantic and Pacific waters, and in general it appears that DSP has become an emerging problem in North America. The first documented outbreak of DSP in the US occurred due to a recreational harvest and impacted three victims in August 2011 in Washington State, with a simultaneous outbreak involving commercial shellfish and 60 individuals just north in British Columbia, Canada. Prior instances of shellfish contamination by the toxins implicated in DSP have also occurred in the Gulf of Mexico and also the east cost of the US although without any outbreaks.

Predominantly self-limiting, DSP nonetheless causes severe gastroenteritis and is recognized as a worldwide threat to human health. Many thousands of consumers of molluscan shellfish are affected and considerable economic hardship resuls in the shellfish industry. Human symptoms include diarrhea (92%), nausea (80%), vomiting (79%), and abdominal pain (53%). Because these symptoms are not unique to DSP it is thought that this illness is very likely one of the most under-reported maladies. The absence of unique symptoms, especially those indicating neurotoxicity (paralysis, etc.) often implicates DSP rather than other marine intoxications. DSP is distinguished from bacterial infection by its rapid onset time and heat stability. The first symptoms occur a few hours after ingestion. The onset time

is as short as 30 minutes in severe cases. After three days victims recover with or without medical treatment. Although DSP-implicated shellfish harvested in Japan have been reported to contain many different toxins (Fig. 23.4, only OA derivatives are shown) in most areas of the world the predominant toxins are okadaic acid (OA), and its derivatives dinophysistoxin-1 (DTX-1), and dinophysistoxin-3 (DTX-3) the latter referring not to a specific toxin but to a mixture of fatty acid esters of the other toxins. These are also the only forms of the group that cause severe diarrhea. Although DSP generally refers to poisoning from contaminated bivalves, crabs have also been implicated; in 2002 in Norway several hundred people became ill from recreational harvests. The other notable aspect of the incident was that over 90% of the okadaic acid congeners present in the crabs were a mixture of fatty acid esters of okadaic acid. Since the DTX-3 forms have low toxicity in mouse bioassays and pharmacological assays, it is believed that the illnesses in Norway involved gastric hydrolysis of these toxins to release their more active forms, in this case okadaic acid. Further, the toxins appear to have accumulated in the crabs via the food chain since DSP-toxic mussels were found in the area. Similarly, in the 2011 Pacific NW outbreak a high percentage of the toxins implicated were esterified (DTX-3) toxins.

Okadaic acid and derivatives may cause diarrhea by stimulating the phosphorylation of a protein that controls sodium secretion by intestinal cells. This is similar to that caused by cholera toxin. One form, DTX-1, in addition to diarrhea, causes severe injuries to intestinal mucosa. Okadaic acid and DTX-1 have also been found to be tumor promoters. Okadaic acid and derivatives are potent inhibitors of protein phosphatases-1 and 2A. Tumor promotion may stem from increased phosphorylation proteins that are substrates for protein kinase C and dephosphorylation of these enzymes. Mussels collected in Sweden, The Netherlands, France and Spain contain OA as the major toxin, and in Norway mussels collected from one area were found to contain OA as the main toxic constituent, while DTX-1 was predominant in another area.

The toxins are produced by dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*. Difficulties in culturing *Dinophysis* sp. under laboratory conditions and their low population densities in the sea make the assignment of a species to a particular DSP outbreak challenging and somewhat controversial. Okadaic acid or DTX-1, and in some cases both toxins, have been found by HPLC in *Dinophysis acuminata*, *D. acuta*, *D. fortii*, *D. mitra*, *D. norvegica*, *D. rotundata*, *D. tripos*, and *Prorocentrum lima*. DSP toxins have also been associated with *D. sacculus*. Cell densities of *D. fortii* as low as 200 cells/liter have been associated with shellfish toxic to humans.

The first analytical method developed for DSP was a mouse bioassay. An official mouse bioassay scheme published by the Ministry of Health and Welfare, Japan (1981) is cited by many investigators and this method, or a variation of it, was first used for monitoring shellfish toxicity in many of the areas impacted by DSP. The first HPLC methods used in monitoring used pre-separation labeling of the toxins with a fluorescent tag to allow detection. Due to the high cost of LC-MS/MS there remains interest in alternatives, so a column-switching clean-up version of this approach was recently developed. At the present time, LC-MS/MS is used extensively for detecting the okadaic acids and the azaspiracids, as well as yessotoxins, pectenotoxins and other lipophilic toxins. These methods were first developed in Canada and New Zealand and modified versions of these procedures, able to quantify many toxins in a single run, are now used around the world. In 2011, EU Reference Laboratory for Marine Biotoxins and several of the marine biotoxins National Reference Labs of Europe validated a (subsequently) EU-approved LC-MS/MS procedure for these and other lipophilic marine toxins.

Azaspiracid shellfish poisoning first appeared in the Netherlands in 1995 and mussels implicated in the outbreak were traced back to Irish waters. Since only trace levels of the okadaic acids were found, this inconsistency with the strong DSP-like symptoms spurred detailed investigations. The structures of the toxins responsible, named azaspiracids (AZAs) for their carboxylic acid, alkaloid, and spiro configurations (Fig. 23.4) were then determined. Although the mechanism of azaspiracid poisoning has not been proven conclusively, studies have shown that the gastrointestinal toxicity and diarrhetic symptoms could result from AZA-induced F-actin, cytoskeletal, and tight junction protein changes. Another remarkable difference belies a very different mechanism of intoxication in AZP versus DSP: although both produce diarrhea when orally ingested by humans, it is notable that the azaspiracids do not induce diarrhea in mice when injected into the peritoneum, in contrast to the okadaic acids. Instead, prior to death, AZA-injected mice show progressive paralysis, dyspnea, and convulsions.

Some methods developed for the detection or quantification of DSP toxins detect the pharmacological activity (PP2A inhibition) of the OA toxin group. An advantage of the PP2A assay is that assay response reflects the toxin mechanism and thus correlates well with the (mouse bioassay) toxicities of the various okadaic acid congeners. On the other hand, reliance on detection methods for DSP toxins specific for only OA and its derivatives, although better than no methods at all, may not be adequate if resident phytoplankton in shellfish growing areas are capable of producing the azaspiracids. Presently, the only analytical methods that can detect both the OAs and the AZAs are based on LC-MS/MS. LC-MS/MS methodology satisfying EU requirements for analysis of lipophilic toxins has recently been validated by the EU Reference Laboratory on Marine Biotoxins in Vigo, Spain, working with the National Reference Labs of Europe.

23.2.4 Neurotoxic shellfish poisoning (NSP)

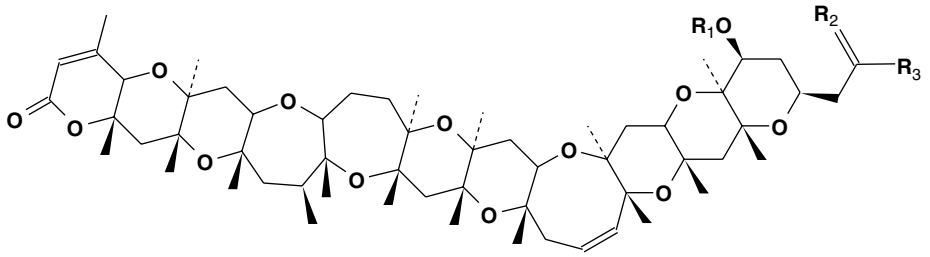
Striking in their appearance, the classic 'red tides' most often associated with toxic plankton blooms are caused by the marine dinoflagellate *Karenia brevis* in the Gulf of Mexico and particularly Florida. Shellfish impacted by an intense *K. brevis* bloom can be toxic to humans consuming them. The illness is termed neurotoxic shellfish poisoning (NSP). Some of the symptoms of NSP are similar to ciguatera although there is no documented case of a human fatality due to NSP (compounds causing NSP are much less toxic to mammals than ciguatoxin). Reversal of hot and cold sensation, a noteworthy paresthesia caused by ciguatera poisoning, can also occur with NSP. Fortunately, NSP is also of much shorter duration than ciguatera at 1 to 72 hours (17 hours duration is often observed). Another characteristic is the occurrence of massive fish kills during a bloom of *K. brevis*. This occurs when the cell densities reach about 5×10^5 cells/l. The fish die so rapidly that there is no chance for fish exposed to *K. brevis* to become toxic to humans.

It is now known that although the brevetoxins are introduced into the environment by *K. brevis* as a group of polyether lactones known as the brevetoxins (Fig. 23.5) much of the toxicity of *K. brevis* is caused by metabolites rapidly produced in shellfish. Of the algal toxins, six of the brevetoxins (Type 1) are based on a single structural polyether backbone and an additional three (Type 2) are based on a different polyether backbone (Fig. 23.5). The metabolites consist of a diverse group of compounds considerably more polar than their algal precursors (Fig. 23.6)

Although the entire Gulf Coast is impacted, within the United States most blooms of *K. brevis* occur in Florida, and this state has historically had the greatest impact from brevetoxins. A 1987 red tide reached North Carolina waters as a result of transport by currents. Control measures for the brevetoxins are somewhat different than the other toxins

Type 1
Skeleton

		R_1	R_2	R_3	
Brevetoxin	2	H	CH_2	CHO	
	3	H	CH_2	CH_2OH	
	5	$COCH_3$	CH_2	CHO	
	6	H	CH_2	CHO	(27, 28-β-epoxide)
	8	H	O	CH_2Cl	



Type 2
Skeleton

		R_1	R_2	R_3
Brevetoxin	1	H	CH_2	CHO
	7	H	CH_2	CH_2OH

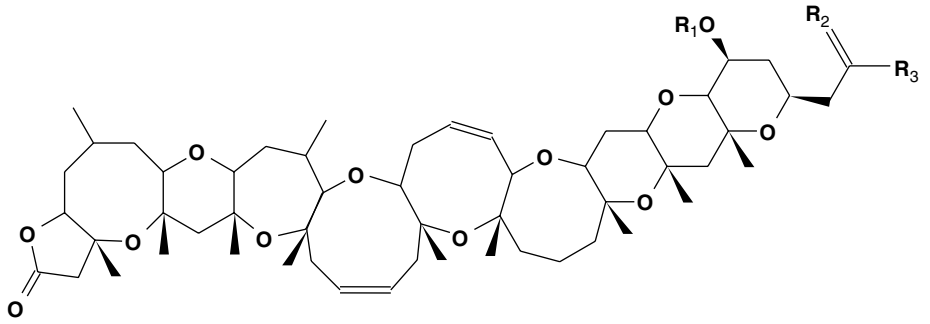


Figure 23.5 Brevetoxins produced by K. brevis.

in that plankton densities are used in harvest closures, while analysis of the toxin levels determines when the harvest can be reopened. When *K. brevis* cells reach 5000/L, the Florida Department of Natural Resources (FDNR) closes waters to shellfish harvesting. Mouse bioassay is used to reopen waters to harvesting following the bloom. Alternative assays are being explored. New Zealand has also been impacted by brevetoxins.

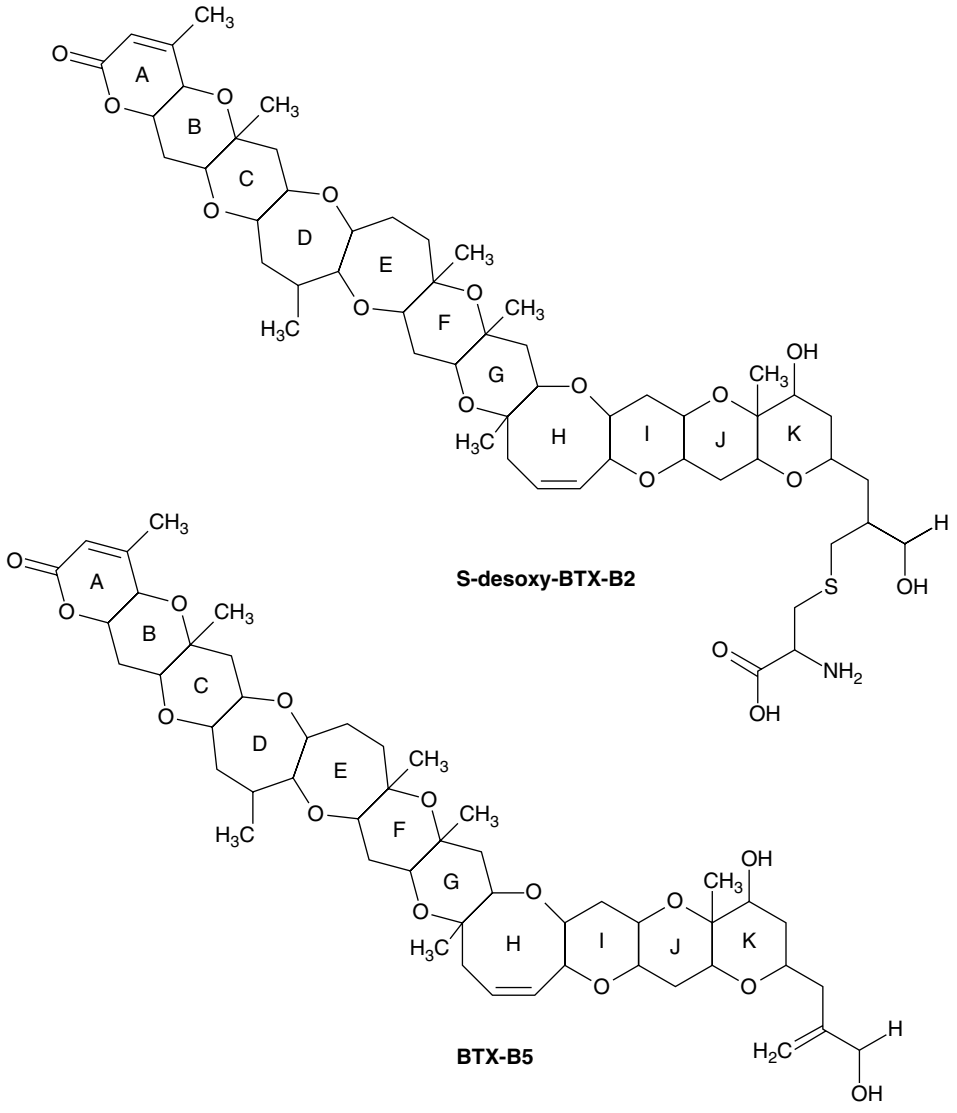


Figure 23.6 Brevetoxin metabolites found in shellfish.

Several different methods for the detection or quantitation of brevetoxins are available or are under development including: (a) animal bioassays (mouse, fish); (b) molecular pharmacological assays using voltage dependent sodium channel preparations; (c) immunoassays such as radioimmunoassays (RIA); enzyme-linked immunosorbent assay (ELISA); and d) high pressure liquid chromatography (HPLC). Many of these methods have potential for application to shellfish monitoring in the management of NSP. At this time, only the mouse bioassay has been applied to the detection of the brevetoxins in shellfish.

Brevetoxins are detected in shellfish meats by a standardized mouse bioassay. Toxicity is determined by using the relationship of dose to death time of mice injected (intraperitoneally) with toxic residues extracted from shellfish with diethyl ether.

23.2.5 Control measures for shellfish toxins

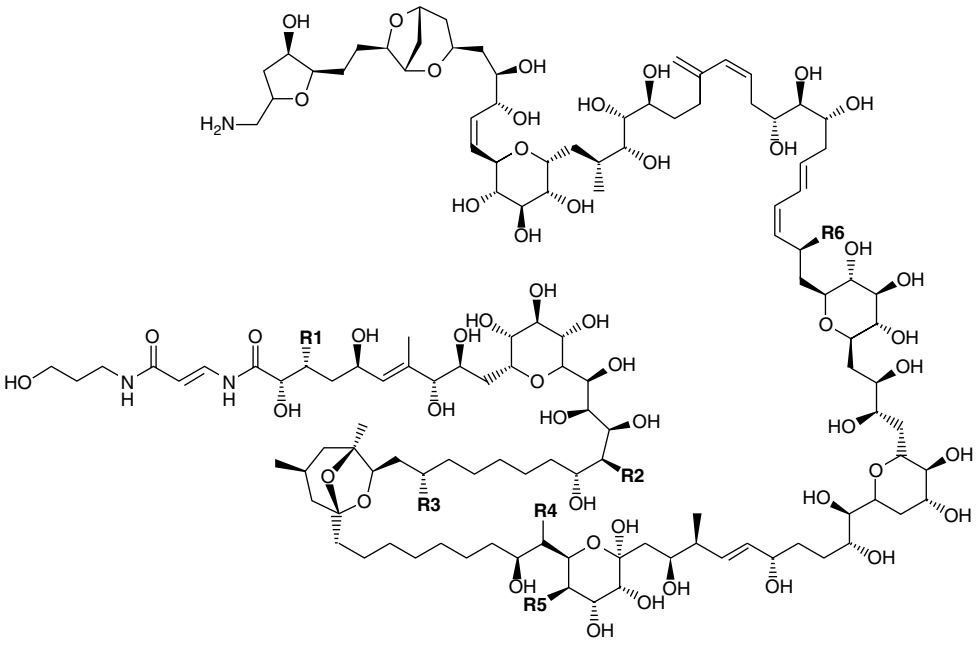
Generally, monitoring shellfish for most toxins is performed at the harvest level. Growing areas, natural or by aquaculture, are monitored and closed or opened for harvest following testing. In the US, shellfish monitoring for biotoxins is the responsibility of the individual state health and natural resources agencies participating in the Interstate Shellfish Sanitation Conference (ISSC). Federal oversight of ISSC is by the National Shellfish Sanitation Program. Historical data have been accumulated over many years of monitoring, and generally the seasonal nature of the plankton blooms allows down-scaling of shellfish sampling during cold months and increased sampling during warm weather. Yearly ISSC meetings are held at the regional level to review data, make changes in requirements, and so on. Some international trade partners participate also in biennial meetings. Shellfish-producing countries of the European Union must follow EU Directives and the European Union Reference Laboratory on Marine Biotoxins coordinates validation studies of detection methods, proficiency studies, and detection methodology training. In recent years, EU directives have required member countries to adopt newly validated chromatographic methods for many of the shellfish toxins. On an international level, Codex Alimentarius establishes detection methodology guidelines, with an emphasis on reference methods to be used if trade disputes arise.

23.3 Palytoxins

Palytoxin (PlTx) is among the most potent of the nonprotein toxins. The structures of palytoxin and analogs, referred here collectively as the PlTxs (Fig. 23.7) are also highly complex and have much higher molecular weights than most of the other marine toxins. Palytoxins were originally isolated from the soft coral *Palythoa*, and some illnesses and at least one dog death have resulted from its mishandling in home aquariums. In seafood safet,y palytoxins are regarded as an emerging problem in Europe, since congeners of this predominantly warm-water toxin are also produced by dinoflagellates of the genus *Ostreopsis* which are now found in increasing numbers in the semi-tropical and temperate waters of the Mediterranean. Although no documented cases of molluscan shellfish poisonings due to the palytoxins have been recorded as of November 2012, shellfish collected in the Aegean Sea in Northern Greece were found to be contaminated with palytoxins in studies conducted between 2004 and 2006 and harvest waters are closed whenever the organisms or toxins are detected. Human illnesses associated with *Ostreopsis* so far are due to the inhalation of sea spray. The toxins are readily released from these dinoflagellates by wave action since they are unarmored and easily ruptured. In these outbreaks due to inhalation, symptoms such as rhinorrhea, cough, fever, bronchoconstriction and wheezing are observed. Not all species of *Ostreopsis* produce the toxins, and so far the organisms most often implicated in the Mediterranean are strains of *Ostreopsis ovata*.

Historically, palytoxins have appeared throughout tropical marine food webs, and so as seafood toxins they are not exclusively molluscan shellfish toxins. Illnesses and deaths associated with consumption of palytoxin-contaminated crabs and fish in tropical subtropical environments have been reported over the years. Unfortunately, data from these outbreaks are scarce, often preventing confirmation of a direct relation between the illness and the PlTXs.

Considerable progress has been made in the development of LC-MS/MS methods for the PlTXs, leading to the discovery of new congeners. Mouse bioassays have been used since the discovery of the palytoxins, and a hemolysis assay that also includes use of a specific



	R1	R2	R3	R4	R5	R6
Palytoxin	CH_3	OH	CH_3	H	OH	OH
42-hydroxypalytoxin	CH_3	OH	CH_3	OH	OH	OH
Ostreocin-d	H	H	H	OH	H	OH

Figure 23.7 Palytoxin and congeners (ovatoxins not shown).

antibody has provided a useful screening method. Cytotoxicity assays and ELISAs have also been developed but no official methods for the detection and control of the palytoxins have been validated, primarily due to the scarcity of purified PlTxs to use as toxin standards.

23.4 Fish toxins

The toxins more often associated with fish are Fugu poisoning, scombroid poisoning, and ciguatera. In comparison with the shellfish toxins they pose unique problems in their management.

23.4.1 Ciguatera

Ciguatera is a term designating an illness caused by eating a variety of reef fishes and the carnivorous fish that feed on them. It is most prevalent in the Caribbean and South Pacific. Symptoms include gastrointestinal disturbances and (more diagnostic) neurological problems (e.g., paresthesia and dysesthesia-temperature reversal), and cardiovascular disorders. The toxic effects can last from several days to several months with resurgence of some symptoms after several years. These effects are also cumulative. It is estimated that Ciguatera

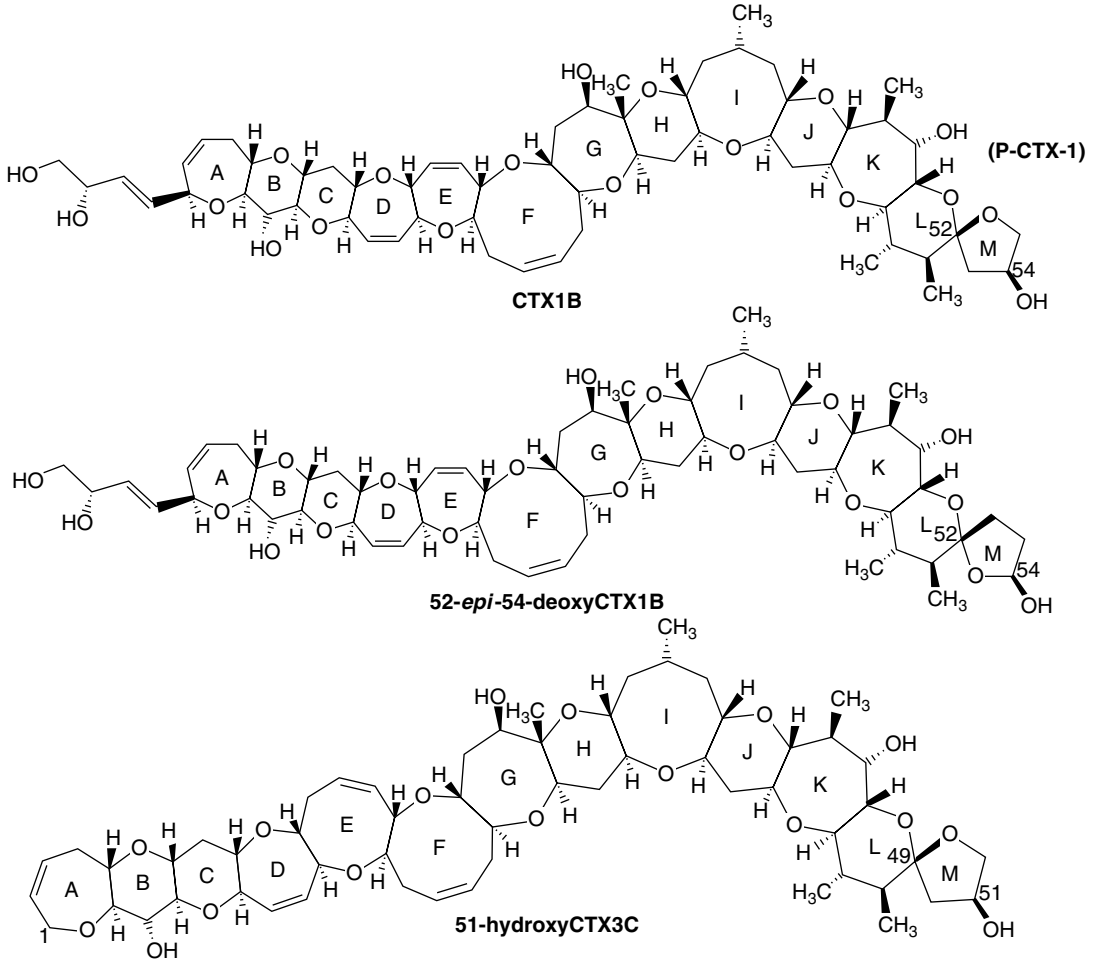


Figure 23.8 Pacific ciguatoxins implicated in illnesses.

causes annually over 20,000 illnesses and in the US more than one-third of all finfish-borne illness outbreaks. It is now recognized that levels as low as 1 ug/kg of Caribbean ciguatoxins and in Pacific Ocean forms, as low as 0.1 ug/kg, can cause intoxication in adults. The vast majority of these illnesses are caused by recreational harvesting, but climate change may be responsible for the appearance of ciguatoxins in commercial species, such as amberjack harvested in the Gulf of Mexico, not previously implicated.

As with most of the seafood toxins, originally the term ciguatera was defined more by the epidemiological aspects and symptomology of the illness than by the chemistry or structure of the toxins. Ciguatera has been one of the most difficult of the marine toxins to study. Difficulties include the unpredictable and variable nature of fish toxicity, the scarcity of toxic fish to study, the logistics problems encountered in working in some of the endemic areas, the diversity of fish species implicated, the tedious isolation procedures required due to the extremely low toxin concentration in the fishes (ppb), and by the complex nature and multiplicity of the toxin(s). Finally, research on detection methods for ciguatoxins has been impossible for many due to the absence of any toxin standards.

In the past two decades, many new ciguatoxin congeners and precursors have been described, the characteristic feature of the most potent ciguatoxin form (CTX-1B, see Fig. 23.8) is the presence of 13 contiguously transfused ether rings of five through nine members. Precursors of the more toxic forms are first produced by an epiphytic dinoflagellate *Gambierdiscus toxicus* and then transmitted to various fishes through the food chain. Difficulties in laboratory studies result from the fact that this alga does not produce CTX-1B directly. It is believed that, instead, it produces several less polar toxins and that some (for example CTX-4B, shown in Fig. 23.9) are converted to CTX-1B by partial metabolism in fish. It was originally believed that oxidized forms of the algal toxins were produced only in this way, but recently a strain of *G. toxicus* was found in Japanese waters that directly produced an oxidized ciguatoxins congener (51-hydroxy CTX3C in Fig. 23.8). *G. toxicus* also produces maitotoxin (MTX, structure not shown), a second important toxin first detected in surgeonfish. Caribbean forms of ciguatoxins have also been discovered (Fig. 23.10) but nomenclature by region may not be appropriate since these same toxins have now also been found in contaminated fish from the Canary Islands, off the west coast of Africa. A third regional category of ciguatoxins has also been discovered in the Indian Ocean.

Ciguatoxins have historically been detected by mouse bioassay. This assay is, however, time consuming and difficult to use quantitatively. It also requires too much fish tissue to be useful for analysis of often limited quantities of meal remnants important in studying outbreaks. Several alternative methods have been proposed for the detection of ciguatoxins, including HPLC-MS/MS, immunoassays, binding assays at sodium channels using radiolabeled brevetoxin, and (sodium channel targeting) cell assays such as the mouse neuroblastoma (N2A – MTT) cytotoxicity assay. For relatively rapid detection without the need for multiple toxin standards, the latter two methods are very promising and are undergoing validation studies. The N2A-MTT assay has in particular proven to be an invaluable tool, and has been modified for use in many different applications including detecting ciguatoxins in blood, the study of outbreak meal remnants, and minute fractions from LC-MS/MS runs. The latter capability confirms detection of toxins before knowing their identities. Powerful HPLC-MS/MS methods are indispensable, but the major drawback remains the need for multiple ciguatoxin standards that, for most laboratories, are hard or impossible to obtain.

In the past one of the impediments, besides the extreme paucity of ciguatoxin standards, has been the lack of targeted limits of detection implied by having no official advisory levels

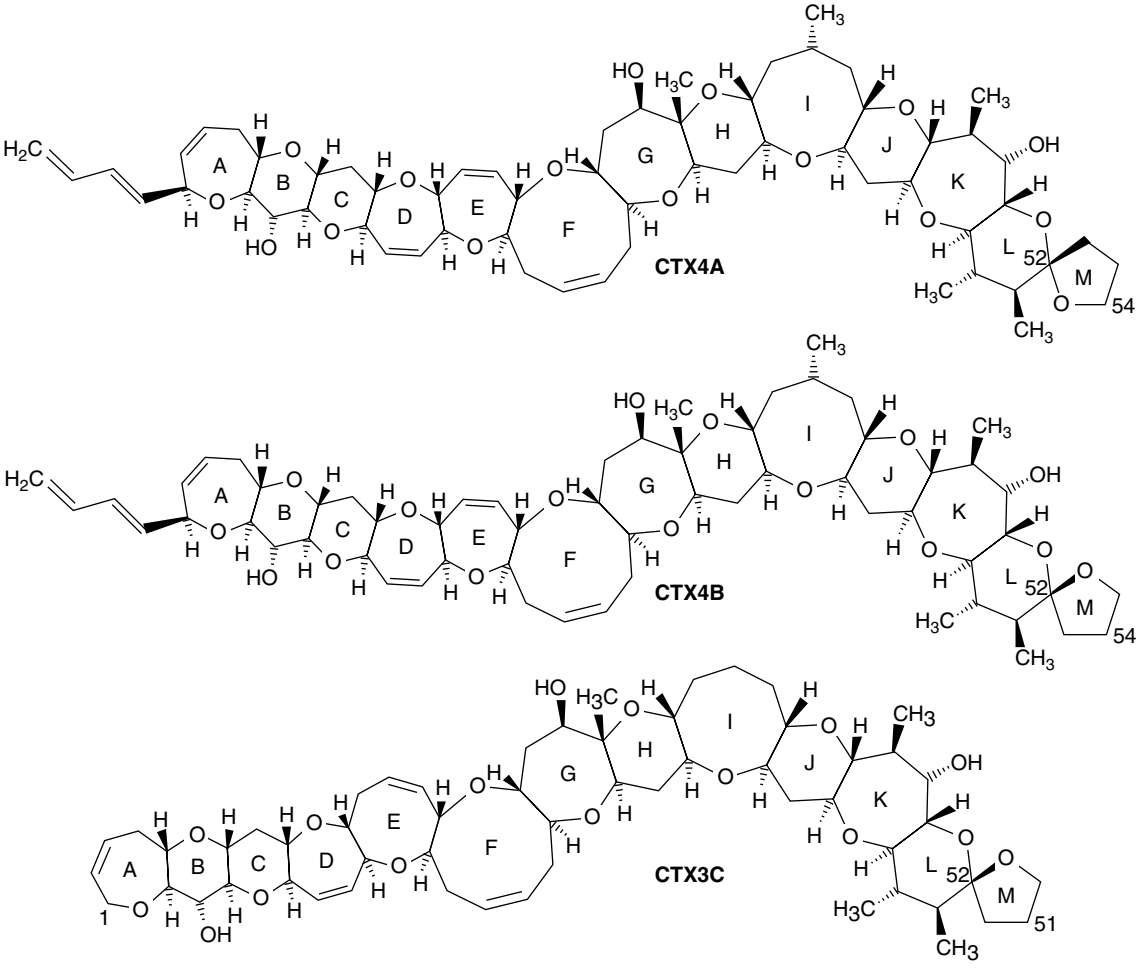


Figure 23.9 Pacific ciguatoxin precursors found in algae.

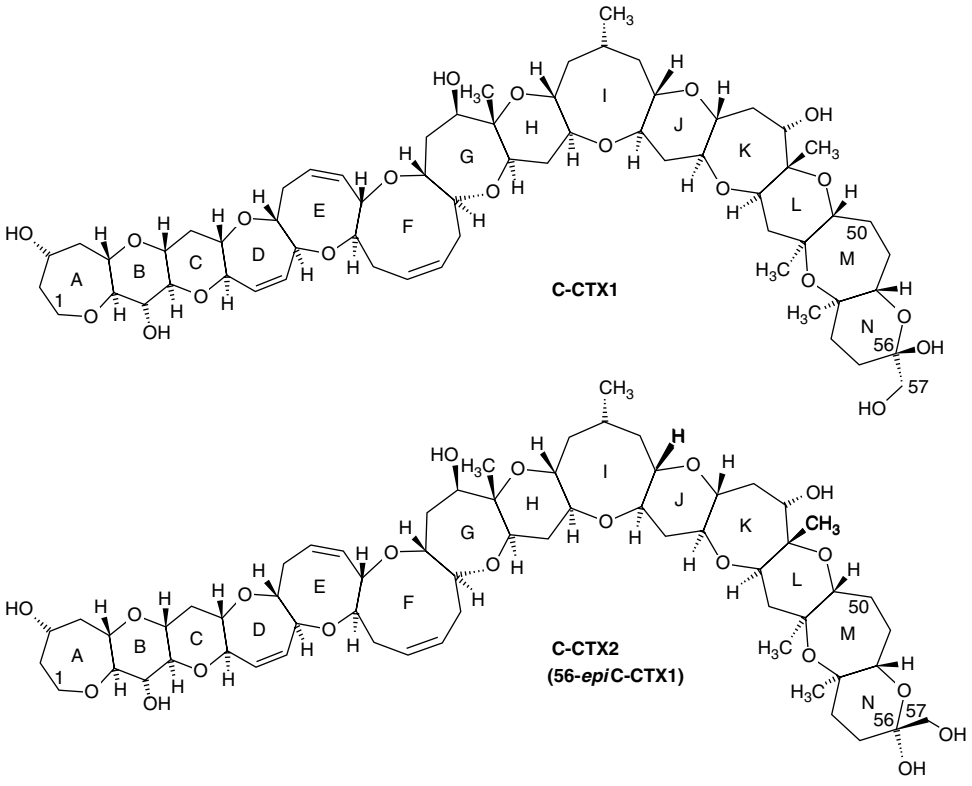


Figure 23.10 Carribean ciguatoxins implicated in illnesses.

for the ciguatoxins. The U.S. Food and Drug Administration has now addressed this need in its 4th edition of the *Seafood Hazards Guide*. The guidance levels are at 0.01 ug Pacific CTX-1/kg fish and 0.1 kg Caribbean CTX-1/kg fish. These guidance levels reflect the high potency of the ciguatoxins, and illuminate the daunting challenges of method development for the ciguatoxins. For example, many of the early attempts at ciguatoxin immunoassays including ELISAs and a commercial dipstick format test kit would have clearly been seen to have inadequate sensitivity to protect human health.

Major advances are now being made to address these new and demanding requirements. Complete synthesis of some of the ciguatoxins in Japan is now allowing the development of more effective ELISAs that use a sandwich format. The key to success of the assay is the use of synthetic fragments of the toxins in developing effective conjugates for antibody development. Refinements to the ELISA and the flow cytometry assay could bring these detection technologies and others into the ultratrace realm of the new advisory levels, and surpass the MTT cytotoxicity assay as effective tools for detecting the ciguatoxins.

23.4.2 Tetrodotoxins

Puffer fish (members of order Tetradontiformes) are popular food items in Japan, in spite of the fact that some of these fish contain potentially fatal levels of tetrodotoxin (TTX), a potent sodium-channel blocker. Tetrodotoxin is the most toxic form of a series of compounds containing the guanidinium group (Fig. 23.11). With much higher toxicity than the other forms,

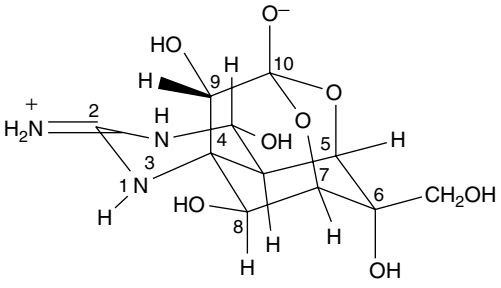


Figure 23.11 Tetrodotoxins.

TTX itself accounts for most of the total toxicity observed. Other members of the TTX family of compounds have been detected (as minor components) in species of *Fugu* puffer fish (*F. niphobles*) and *F. pardalis*. This group includes 4-epi-TTX, 6-epi-TTX, 4,9-anhydro-TTX, 11-deoxyTTX, 11-norTTX-6(R)-ol. Tetrodonic acid has also been reported. The flesh of pufferfish caught in temperate waters is believed to have low or nonexistent toxicity, but improper handling can easily result in release of the toxin from highly toxic tissues.

Symptoms of puffer fish, or tetrodotoxin, poisoning include depressed core body temperature, sweating, weak and rapid pulse, tremors, cyanosis, muscle twitching and tremors, and poor muscle coordination. At lethal doses death occurs by respiratory paralysis. In Japan alone, pufferfish poisoning is implicated in over 10 deaths per year. Certain exotic gastropods have also caused TTX poisonings and even death. TTX occurs in the ivory shell *Bablyonia japonica*, the frog shell, *Tutufa lissostoma*, the trumpet shell, *Charonia sauliae* and the lined moon shell, *Natica lineata*. The TTXs are also found in the eggs of the blue ringed octopus (*Hapalochlaena maculosa*), newts, and in certain tropical and subtropical crabs.

Many species of bacteria are capable of producing TTX and/or anhydro-TTX. It is now well established that these toxins accumulate in marine sediments. TTX-producing bacteria have also been found in deep sea sediment. It has been suggested that TTXs found in marine sediments are synthesized only by bacteria and are concentrated and deposited by benthic organisms in the food web. It appears that toxin-producing bacteria might be responsible for much of the TTX-toxicity of several marine finfish and shellfish. For example, TTX-producing bacteria have been isolated from the intestines of the trumpet shell *Charonia sauliae* which has been implicated in poisoning incidents and from the intestines of the xanthid crab *Atergatis floridus*. One study suggests that TTX found in pufferfish is a result of their exposure to TTX-producing marine bacteria. It would seem that the production of tetrodotoxins by a wide variety of bacteria, many of which are prevalent in the marine environment, could lead to TTX contamination of finfish other than pufferfish. Certainly the wide occurrence of TTX in so many different species is consistent with a bacterial origin.

23.4.2.1 *Detection methods*

The mouse bioassay for TTX is similar to that for the PSP toxins. Both toxin groups cause blockage of the sodium channel with death by respiratory paralysis. Since bioassays provide only a composite response representing the total potency of samples, initial efforts at detecting the toxins by chemical means were predicted on an assumption that only TTX was present since the other forms had not yet been discovered. As with the saxitoxins, this was soon proven incorrect as HPLC and bioassay studies soon revealed the presence of multiple toxin forms.

23.4.3 Scombroid poisoning

The symptoms of scombroid poisoning, besides including the usual gastrointestinal problems, resemble an allergic response. The main difference is that scombroid poisoning will have a virtually 100% attack rate while allergies are much lower. Another similarity is that the victims also respond to antihistamines.

Scombroid intoxication results from ingestion of certain species of fish that have not been adequately chilled. Only those species that have high levels of free (non-peptide) L-histidine are implicated. Some bacteria are known to have enzymes that can cause decarboxylation of free L-histidine to histamine. Thus, the frequent finding of elevated histamine levels in implicated fish, and finally the above response to antihistamines, has led to scombroid poisoning often being called histamine poisoning. Although histamine is somehow involved in scombroid intoxications, there may be other agents, either inhibitors which interfere with human enzymatic detoxification of histamine, or even potent histamine-like compounds that bind to the same receptors as histamine.

The commonly-used (and in the US also officially approved) method for detecting histamine in seafood is a wet chemical procedure that is performed manually. Alkaline condensation of histamine with o-phthalaldehyde (OPA) and subsequent dehydration with phosphoric acid produces a fluorescent product. Another method for detecting histamine that has 'world status' is the pre-column derivatization (dansylation) HPLC procedure officially approved in Europe. It is also a relatively laborious procedure, although it has the advantage of detecting other biogenic amines of interest in addition to histamine (to date no action level have been approved for the other amines). A major drawback of this HPLC method is that calibrations must be developed for each fish species due to matrix effects, even though the method benefits from use of an internal standard. In addition to these two reference methods, a variety of rapid test kits have been developed for histamine detection in fish including commercial ELISA methods, enzymatic microplate methods, and threshold level 'dipsticks' based on both LFIC and enzymatic principles. Flow injection analysis, using a kinetics-tuned version of the chemistry from the batch fluorescence method has also been demonstrated on a commercial instrument and remains the most rapid screening method.

23.5 Trends in seafood toxin detection

Beginning in 2005, modern detection methods are finally beginning to replace mouse bioassays in the monitoring of seafood toxins. For over 50 years, the most potent seafood toxins have been monitored by mouse bioassays. Although this approach has been successful in protecting human health, until now the lack of true reference methods has resulted in application of the mouse bioassays in spite of performance problems such as low recoveries and matrix interferences at concentrations near the quarantine level. There is also, in some countries, considerable resistance to animal use in testing. The precedent set by mouse bioassays has also placed a heavy burden on analytical method developers and on validation efforts since new chemical methods are more likely to be accepted when they can estimate the equivalent mouse bioassay response. In spite of these challenges, over the years chemical methods have been applied extensively in research. Generally, nearly all detection techniques used in routine monitoring were first used as research tools. Even research tools had only limited impact at first, partly due to initially insufficient understanding of the toxins to develop alternative methods, but primarily due to fact that the toxins were not commercially available as certified materials.

23.5.1 Toxin standards and reference materials

The lack of commercially-available toxin standards for many of the seafood toxins has delayed development of improved methods and also method validations. Even research tools had only limited impact for a long time, partly due to initially insufficient understanding of the toxins to develop alternative methods, but primarily due to the fact that the toxins were not commercially available as certified materials. This situation has improved considerably in the past decade, thanks to the development and commercial availability of certified standards and other toxin reference materials, primarily by National Research Council of Canada (CNRC). Previously, multiple toxin standards were available in sufficient quantities only for research purposes, and even then only as generous gifts to collaborating labs by researchers in Japan and the US. Particularly for the paralytic shellfish toxins, and also many of the (non-tropical) lipophilic toxins, real progress has been made and CNRC continues to expand the number of different reference materials available. Unfortunately, today some of the most lethal seafood toxins, and also the most worrisome such as the ciguatoxins and palytoxins, are still commercially unavailable. Even for research purposes in small quantities they are hard to come by.

23.5.2 Advances in HPLC, UPLC and LC-MS/MS

In the recent past, use of separation methods was with a few exceptions restricted to the less potent seafood toxins such as domoic acid. Application of separation methods like HPLC in the detection of seafood toxins, at first only in research mode, has revealed considerable detail about toxin profiles in the algae and how these are modified by their metabolism in shellfish and fish, first for the saxitoxins as described previously. Accumulation of this data, in addition to providing extensive detail in the environmental sense, has proceeded in parallel to improvements in the reliability of instrumentation like post-column reactors and other instrumentation. Even more so, detection based on mass spectrometry, particularly tandem methods, has proven to be an increasingly powerful tool in combination with HPLC (LC-MS/MS) and in the 21st century there has been a virtual explosion of rapidly evolving and competing mass detection schemes. For some of the marine toxins, LC-MS/MS procedures have also been validated and applied in routine monitoring of multiple toxin groups using a single, efficient chromatographic procedure. New Zealand and Canada were first in routinely applying LC-MS/MS in this way to the lipophilic toxins. Among the chemical methods, toxin profiles and other data provided by these HPLC fluorescence and LC-MS/MS methods have revealed the inherent challenges and performance requirements involved in developing rapid tests.

23.5.3 Rapid tests and portable instruments

In addition to LC-MS/MS and other chromatographic methods such as LC-fluorescence, efforts to develop alternative analytical methods for seafood toxins target immunochemical and receptor-based competitive binding assays, enzymatic assays, or cell assays. All of these methods offer four potential advantages. These are inherently high speed, high sensitivity, the potential for developing a field test format for screening samples, and reduced requirements for expensive or rare toxin standards. In the case of the receptor-based, enzymatic, or cell assays, in several cases there is potential for a fifth and crucial asset: the development of assays where the overall response parallels the response to that toxin group by the exist-

ing mouse bioassays. For immunochemical tests this cannot be assumed and in practice is difficult to achieve. Methods more intimately connected to toxic mechanism have an advantage here, and for these 'functional assays' relative responses of the assay to the various toxin forms at least parallel the relative mammalian toxicities so that the assay response is correlated to mouse toxicity. This very desirable property of these pharmacological assays is helpful for authorities looking to phase out their existing mouse bioassay-based monitoring programs without compromising health protection. To some extent, as with mouse bioassays, the pharmacological nature of these assays can also be said to reflect health risk and perhaps be able to detect unknown congeners, metabolites, etc. even when their chemical identities are not yet known.

23.5.3.1 *Biochemical assays*

Immunoassays and other competitive binding assays are among the most sensitive methods, and in most cases are able to detect toxins below levels of concern. Enzyme linked immunosorbent assays (ELISAs) can be developed in a variety of different formats. In a similar manner, immunoassays based on surface plasmon resonance (SPR) often use antibodies for their selective binding. These assays are now becoming available on small, portable SPR instruments. Since the sensor signal is exquisitely sensitive to mass, with surface binding essentially leading to response to a change in refractive index, for large toxins, sandwich assays using SPR can leverage nanobeads to both capture the toxin of interest and amplify the signal. Also solid-phase 'field test' immunoassays, such as those based on lateral flow immunochromatography (LFIC), have been on the market for some time for paralytic shellfish toxins and domoic acid and more test kit firms are now offering this technology including test strips for the diarrhetic shellfish toxins. Many of the seafood toxins exert neurotoxic effects by first binding to voltage-gated sodium channels (VGSC) on neurons. Receptor binding assays (RBAs) use sodium channel 'synaptosome' preparations and radiolabeled compounds in a competitive format to detect toxins binding at specific VGSC sites. In combination with the appropriate radiolabeled reagents, this methodology can be used to detect saxitoxins or tetrodotoxins (VGSC site 1) in one format, or brevetoxins and ciguatoxins (VGSC site 5) in another. The assay format used for detecting the saxitoxins in shellfish has been collaboratively studied and approved as official method 2011.27 by AOAC Int., and is now beginning to benefit developing countries in their monitoring. Although handling and disposal of radioactive materials is required, the levels of radioactivity are low enough that, in the US for example, licensing requirements for their handling have been relaxed considerably. In 2012, a new RBA format for brevetoxins was introduced that does not require radioisotopes and instead uses fluorescence as an endpoint, and in 2013 this (VGSC site 5) assay was demonstrated in preliminary studies to also respond to Pacific ciguatoxin.

23.5.3.2 *Cell assays*

In living neurons, specific binding to nerve cell receptors results in changes in ion permeability. In the presence of auxiliary toxins cytotoxicity assays, such as the N2A-MTT assay mentioned above (Section 17.3.2) for ciguatoxin detection, have been developed which exploit this binding. At the voltage gated sodium channel, binding at toxin receptor site 5 by brevetoxins or ciguatoxins leads to an increase in sodium channel permeability (these compounds are often called site 5 toxins or sodium channel enhancers). In these assays, veratridine is used as an auxiliary toxin and also increases sodium ion permeability on binding to a different (site 2)

area of the sodium channel than brevetoxin or ciguatoxin. In combination with veratridine, response to ciguatoxins and other site 5 toxins is amplified by strong synergism due to conformation changes in the ion channel. In the cytotoxicity format of these assays, ouabaine is also added to poison the sodium/potassium ATPase and thus prevent the cells from reducing the osmotic pressure due to ion flux and imbalance, and cell death occurs in a dose-dependent manner in several hours. Due to their high sensitivity and tolerance of sample matrix, cytotoxicity assays have proved invaluable in studies of the ciguatoxins in extracts from fish and also in piscine, murine, and human blood. These ultrasensitive assays have also been used to study new ciguatoxin forms in combination with LC-MS/MS, a powerful combination of structural and activity-yielding methodologies that has also supported the establishment of the first official guidance levels for the ciguatoxins by the U.S. Food and Drug Administration. Updated versions of these assays now allow observation of a response in less than an hour, using voltage-dependent fluorescent dyes in combination with flow cytometry to detect toxin-induced membrane depolarization of the cells rather than the slower process of cell death. In either the cytotoxicity or voltage-sensitive dye formats, ciguatoxins can be detected at sub-picogram levels in fish extracts. Saxitoxins and tetrodotoxins can also be detected using modified versions of the above cell assay formats. The primary difference is that these 'sodium channel blockers' bind to site 1 of the VGSC and cause a *decrease* in sodium ion permeability and so the dose-response curves are reversed versus the site 5 toxins.

Cell assays have improved precision versus mouse bioassays, but their primary advantages are vast improvements in sensitivity. For the study of ciguatoxins the sensitivity advantage is critical since parts per trillion levels of the potent Pacific forms must be detected and ciguatoxin standards are extremely scarce and expensive to produce. Finally, no purification of crude extracts is required and the assays can be completed in 1–2 work days by cytotoxicity or less than an hour using voltage-sensitive dyes.

23.5.4 Method validation

Effective health protection from seafood toxins depends on reliable and efficient analytical methodology for their detection. Approval of methods by regulatory authorities differs among the seafood-producing regions of the world, but generally all require sound statistics and rugged procedures. In the US, the Association of Analytical Communities, AOAC International, is an organization that oversees, reviews, and approves many validation studies as part of its Official Methods of Analysis (OMA) program. In Europe another group, the European Committee for Standardization (CEN), reviews interlaboratory studies as required within the European Community, while oversight of method validation studies for marine biotoxins is the responsibility of the EU Reference Laboratory for Marine Biotoxins (EU-RLMB) in Vigo, Spain. Any of the above avenues for approval could satisfy Codex Alimentarius requirements for reference methods, but traditionally AOAC official methods of analysis (OMA) have figured prominently, for example, AOAC approval of the 'Lawrence method' OMA 2005.06 for LC detection of paralytic shellfish toxins was instrumental in pushing forward the replacement of antiquated mouse bioassays with modern analytical methods, including mention of this method in EU legislation.

23.5.5 International groups and training efforts

In 2004, a large international network of regulatory and industry stakeholders and academics, called the Marine and Freshwater Toxins Task Force and Analytical Community, was established by AOAC Int. with leadership provided by the USFDA, the ISSC, and many

partners in Canada, Europe, and Asia. This group was created to accelerate and review suitable methods for validation. Beginning in 2005 there was a significant increase in the number of analytical methods attaining AOAC OMA status, including three new official (nonproprietary) methods for detecting the saxitoxins and one official method for domoic acid based on a commercial ELISA. This trend continues.

To support implementation of the new methods, there has also been an accompanying increase in laboratory methodology training courses, including courses offered by the above toxins task force in the Seattle area of Washington State, USA, on the two new official LC methods including an automated version, as well as the multitoxin (LC-MS/MS) EU method approved for lipophilic toxins including those causing DSP and AZP. The institutes and/or agencies providing training for the methodology began with Health Canada in a leading role with contributions by the USFDA and Univ Vigo, Spain, the Cawthron Institute in Nelson, New Zealand working in collaboration with the Canadian National Research Council (CNRC) the Canadian Food Inspection Agency (CFIA) and most recently, the National Oceanic and Atmospheric Administration (NOAA). Workshops on test kits, automated flow analysis and portable SPR for the seafood toxins have also been offered by the task force and include participation by an international mix of vendors, and guest instructors from academia, federal agencies, and other institutes.

23.6 Summary and conclusion

Seafood toxins are a diverse group with microbial origins, and the number of toxin congeners increasing further as they move through the food web. The most potent, and in some cases potentially fatal, seafood toxins are neurotoxins. Many of these neurotoxins exert their toxicity via the voltage-gated sodium channels of nerve cells. Fish toxins are produced by dinoflagellates and bacteria, while shellfish toxins are produced by a variety of dinoflagellates and diatoms. Shellfish toxins are managed by harvest-level monitoring. Ciguatera is one of the most challenging fishborne illnesses to manage due to the mobility of the fish, sporadic distribution of the toxin, the paucity of toxin standards, and challenges in the development of detection methodology.

For the purpose of health protection, the potency of a given toxin determines the degree of sensitivity and selectivity required to detect it. Domoic acid, for example, can be monitored adequately by HPLC with UV detection due to relatively low toxicity. In contrast, ciguatoxins and saxitoxins require much higher sensitivity. Detection of multiple lipophilic toxins by LC-MS/MS, first developed in Canada and New Zealand and long ago implemented in those countries, is now mandated by the European Union, and will completely replace mouse bioassay in the official control of these toxins in Europe by 2014. In other applications, although LC-MS/MS methods provide rich structural detail they also require costly toxin standards and thus screening tools are also useful.

For the purpose of screening large numbers of samples, several promising technologies for monitoring high-potency seafood toxins continue to be developed and refined including mechanism-related ‘functional assays’ like receptor assays and cell assays, as well as immunoassays. Increasing numbers of these promising rapid screening methods are being developed and commercialized, and as they move into validation and implementation in both the laboratory and field, will greatly improve seafood safety and allow more effective use of seafood resources.

Impressive advances in seafood toxin detection methods and major changes in toxin monitoring are occurring in the 21st century. HPLC methods are increasingly replacing mouse

bioassays, and advances in LC-MS/MS are revealing the complexities of toxin transformations in seafood as well as allowing the simultaneous monitoring of several types of toxins at once. Assays for the seafood toxins are diverse in design, most of them based on immunochemical principles, enzymes, receptor preparations, or cells. Some assays leverage the pharmacological mechanism of intoxication and so are activity-based, or 'functional' assays specific to a particular type of shellfish intoxication. In some cases, the combination of functional assays with LC-MS/MS has been very powerful in revealing new toxin forms as well as their relative potencies. Important new initiatives in analytical method validation and implementation are also in play.

Ensuring the safety of seafood is well worth the efforts involved, since it is a nutritious and healthy food source high in protein, and regular consumption of fish and shellfish is recommended by nutritionists. Consumption of certain lipids in salmon is even correlated with improvements in cardiovascular health. As with other foods such as poultry and red meat, with benefits there are always associated risks which must be recognized and managed. Thus the benefits of eating seafood outweigh the risks, with the actual risk to consumers minimized thanks to diligent monitoring. Understanding the nature of the risks involved, starting with the basic etiology and, when available, prior understanding of regional and species-specific variations in the toxins, has been the key to success.

23.6.1 Disclaimer

The views presented in this article are those of the authors and do not necessarily represent those of the U.S. Food and Drug Administration. No official support or endorsement of this article by the Food and Drug Administration is intended or should be inferred. Mention of brand or firm name does not constitute an endorsement by the U.S. FDA over others of a similar nature not mentioned.

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24 Prion diseases

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24.1 Introduction

The outbreak of the foodborne epidemic, bovine spongiform encephalopathy (BSE) in Great Britain, its spread to Europe and the link between BSE and a new human form of the disease, variant Creutzfeldt-Jakob disease (vCJD), have focussed considerable attention on prion diseases. These inevitably fatal neurological disorders, also referred to as transmissible spongiform encephalopathies (TSEs), share several hallmark characteristics, including spongiform degeneration in the central nervous system, accumulation of a structurally abnormal form of a brain protein (the prion protein, PrP) in infected animals, and lack of an immune response. Infection naturally occurs via the oral route as well as by blood transfusion and maternal routes. Prion diseases are characterized by progressive spongiform degeneration of the brain and concomitant accumulation of an abnormally structured prion protein (PrP^{Sc}). These brain diseases are always fatal; there is no specific immune response and no cure. The long asymptomatic period thwarts efforts to diagnose infected humans prior to neural damage, eliminate infected animals from the food supply or identify infected wild animals. Uncertainty over the number of humans currently infected with vCJD, extreme resistance of the infectious agent to inactivation, lack of a cure or even a preclinical diagnosis, and uncertainty over the mode of transmission of BSE to humans makes these diseases particularly vexing.

The biology of prion diseases is different from other infectious agents and includes an extended preclinical phase, resistance to traditional sterilization methods, and difficulties in the ante-mortem diagnosis of the disease. These characteristics have had tragic consequences including the exposure of the cattle population in Great Britain to contaminated feed and the transmission of the resulting bovine disease to humans. One somewhat paradoxical trait is that (with the notable exception of chronic wasting disease of deer and elk) prion diseases are not readily transmissible. Ingestion of contaminated food is the most common means of transmission, although, in the case of vCJD, the source of infection (meat, milk, processed bovine products) remains unclear.

Guide to Foodborne Pathogens, Second Edition. Edited by Ronald G. Labbé and Santos García.

Table 24.1 Animal and Human Prion Diseases

Species	Prion disease	Source of infection
Sheep	Scrapie	Infection, maternal
Cattle	Bovine spongiform encephalopathy	Contaminated feed
Mink	Transmissible mink encephalopathy	Contaminated feed
Cats	Feline spongiform encephalopathy	BSE infected tissue or meat-and-bone meal
Deer, elk, and moose	Chronic wasting disease	Infection
Human	Kuru	Ritualistic cannibalism
Human	Iatrogenic CJD	Infection
	Sporadic CJD	Unknown
	Familial CJD	PrP gene mutation
	Variant CJD	Infection – source BSE
Human	Gerstmann-Sträussler-Scheinker syndrome	PrP gene mutation
Human	Familial FFI	PrP gene mutation
	Sporadic FFI	Unknown
	Atypical prion diseases	
Sheep	Nor98	Unknown
Cattle	H-type BSE	Unknown
Cattle	L-type BSE	Unknown

Prion diseases have been identified in a number of species (Table 24.1) and include scrapie in sheep and goats, BSE, transmissible mink encephalopathy (TME), feline spongiform encephalopathy (FSE), and chronic wasting disease (CWD) in cervids. The human prion diseases include: CJD (with sporadic, acquired and familial manifestations), and two familial forms, Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI). Prion diseases have been experimentally transmitted to a number of species from nonhuman primates to rodents. Each of the prion diseases has its unique set of characteristics, including range of species that can be infected.

Scrapie is a disease of sheep and, rarely, goats that has been recognized for at least 250 years. The term 'scrapie' is derived from the pronounced rubbing and scratching of the skin, which occurs in infected sheep 3–4 years of age. Clinical manifestation of scrapie is characterized by ataxia and recumbency. Scrapie has a worldwide distribution with the notable exception of Australia and New Zealand, countries considered to be 'scrapie-free'. Although scrapie is the prototypic TSE, its etiology is unknown. Complicating both scrapie eradication and disease detection/surveillance is the recent discovery of atypical forms of sheep scrapie.

Transmissible mink encephalopathy (TME) is a rare disease of ranch-raised mink. It was first described in Wisconsin, USA in 1947 and has since been observed in Ontario (Canada), Finland, Germany, and Russia. The most recent occurrence was in Stetsonville, Wisconsin in 1985. The incubation period of natural TME is 7–12 months with clinical symptoms including hyperexcitability and, ultimately, motor incoordination. Exposure is via contaminated foodstuffs, although the source, once believed to be sheep scrapie, is not clear.

Chronic wasting disease (CWD) affects members of the family Cervidae (cervids; including deer, elk and moose) and is the only known naturally-occurring TSE in wild species. CWD was first identified in captive mule deer in 1967 (Colorado, USA) and in a cohort of captive elk in 1978 (Wyoming, USA). A contagious disease in cervids, transmission appears to occur via both direct (animal-to-animal contact) and indirect (i.e., via the environment) pathways.

Throughout the 1980s in the western United States, free-ranging herds of deer and elk became infected with CWD and the disease gradually expanded its range. Its presence in farmed cervids and subsequent vehicular transport of captive deer and elk has further contributed to the geographic spread of the disease in North America. Farmed elk in Canada (Saskatchewan) were diagnosed as CWD-positive in 1996 and, shortly thereafter, free-ranging deer and elk in the same area tested positive for the disease with continuous expansion of the disease along the river valleys. In two large geographical leaps, CWD was discovered in free-ranging deer in Wisconsin and New Mexico in 2002 (harvested in 2001) and 2003, respectively. The geographical range of CWD continues to expand; it has now been identified in captive and free-ranging cervid populations in 18 states in the United States and two provinces in Canada (www.cwd-info.org/index.php/fuseaction/about.timeline). Export of preclinical CWD-positive elk from Canada to Korea has resulted in CWD-positive cervids in the Korean deer population. With no means of controlling the disease, CWD will undoubtedly continue to spread.

There are three distinct strains of *bovine spongiform encephalopathy* (BSE), classical BSE and two 'atypical' forms (H- and L-types). First identified in 1986, classical BSE became a catastrophic epidemic in British cattle with almost 200,000 documented cases. Epidemiological studies quickly identified prion-contaminated meat and bone meal (MBM) as the source; the subsequent ban on the inclusion of bovine-derived protein supplements in ruminant feed (1986 ban) and mammalian feeds (1996 ban), led to a dramatic decline in the disease. Unfortunately, BSE-infected cattle as well as prion-contaminated MBM were exported from Great Britain prior to the identification of the disease. BSE was subsequently identified in virtually all developed countries (OIE website), albeit at much lower levels than in Great Britain. Classical BSE is declining and appears to be under control in most countries. The OIE lists 32 countries with 'controlled BSE risk' (http://www.oie.int/animal-health-in-the-world/official-disease-status/bse/list-of-bse-risk-status/). The increased surveillance of cattle for BSE resulted in the identification of atypical strains. The two forms of atypical BSE, H-type and L-type, do not appear to be linked to contaminated feed but rather occur in very old cattle (>8 years) and are, perhaps, spontaneous forms of bovine prion disease. Atypical BSE is rare and appears to have a worldwide distribution.

Human prion diseases can be inherited, sporadic or acquired. Sporadic CJD accounts for 90–95% of the reported cases of CJD, affecting one to two people per million per year. The disease generally occurs in people older than 50 years of age, although cases have been reported in individuals in their early teens to late 80s. The etiology of sporadic CJD is unknown. Familial prion diseases (GSS, familial CJD and FFI) are autosomal dominant disorders that have been linked to specific mutations in the prion protein (PRNP) gene and occur at an incidence of 1 per 10 million per year. Two prion disease characteristics have contributed to iatrogenic CJD: the presence of CJD-infectivity in preclinical patients and the resistance of these disease agents to inactivation. Iatrogenic CJD has been documented primarily by exposure to central nervous system tissue from infected individuals, specifically dura mater, corneal transplants, and cadaveric pituitary growth hormone treatment.

Kuru is a prion disease that affected the Fore cultural group of Papua New Guinea. The disease was perpetuated by ritualistic cannibalistic practices with, at its height, approximately 1% of the population affected. The incidence of Kuru declined dramatically upon the cessation of cannibalism in the late 1950s; however, due to the long incubation periods that characterize all TSEs, a few cases were still observed in the 1980s. It can now be considered a historical prion disease.

Variant CJD (*vCJD*) was first identified in 1995. As will be described below, vCJD can be distinguished from CJD based upon clinical, biochemical, and rodent transmission studies.

Whereas sporadic CJD affects individuals in their 50s or 60s, vCJD affects primarily teenagers and young adults. Biological and biochemical studies have tightly linked vCJD with BSE. It is assumed that consumption of beef products is the source of the infection. Combined with the BSE exposure risk, there is a strong genetic component to this disease. vCJD patients typically have a specific prion protein allele, met/met, at position 129. Uncertainties over the length of the incubation period, route of infection, impact of genetics and number of individuals exposed to contaminated bovine products have resulted in very disparate estimates of the future course of this prion disease.

24.2 Nature of illness caused

Prion diseases are inevitably fatal, inducing a progressive neurological disease after a long incubation period. Typical pathological features of prion diseases include spongiform vacuolation, accumulation of PrP^{Sc}, and astrocytosis, and are often accompanied by the accumulation of amyloid plaques. Prion infections have long incubation periods, taking months to develop in mink, years to develop in sheep, deer, and cattle, and years to decades in humans. For most of the infection, animals and humans appear clinically normal. This extended preclinical stage and the subsequent inability to readily identify prion infection has had severe consequences with virtually every prion disease. The consequences of the long preclinical stage include an inability to diagnose animals and humans in the early stages of the disease. Concerns of potential prion contamination of the blood supply are exacerbated by the need for (and current lack of) an ante-mortem test to identify CJD-infected humans.

The incubation period of classical BSE ranges from 2–12 years. Clinical signs of BSE initially involve a change in the animal's temperament, with the animal becoming nervous or aggressive. During the 2–6 month clinical period, the disease develops into an obvious lack of coordination, difficulty in rising, and loss of weight. Onset of the atypical BSEs occurs much later. Most of the clinically-affected atypical cases appear as downer cows.

The initial clinical signs of scrapie occur 2–5 years after infection, with changes in temperament often followed by rubbing against enclosures. As the disease progresses, the affected animals exhibit a loss of coordination, weight loss, and gait abnormalities.

Chronic wasting disease has an incubation period of at least 2 years. Initial signs are subtle and transient, difficult to ascertain even in farmed animals. As the disease progresses, deer present with ataxia, increased drooling, difficulty swallowing and muscular wasting.

Human prion diseases can be distinguished clinically. GSS is typified by chronic progressive ataxia and terminal dementia and has a clinical duration of 2–10 years. Fatal familial insomnia initially presents as insomnia followed by ataxia and dementia. Sporadic CJD affects individuals in their 50s and 60s. Death occurs within six months of the onset of the clinical stage that presents as a rapidly progressive multifocal dementia and often ataxia. vCJD clinically presents as a psychiatric disturbance with depression being a predominant feature. The clinical course that develops includes ataxia and dementia and is primarily observed in teenagers and young adults. The clinical course is more extended than classical CJD, with vCJD having a clinical course of approximately 1 year.

24.2.1 Pathogenesis

The pathogenesis of prion diseases can vary depending upon the host species and strain of the agent. All TSEs replicate in nervous tissue and exhibit the highest levels of titer and PrP^{Sc}

accumulation in the brain and spinal cord. For example, hamster-adapted strains of scrapie and TME reach levels of 10^9 LD_{50} per gram in the brains of infected animals at the terminal stage of the disease process. Infectivity accumulates throughout the course of the disease with the outcome that considerable amounts of infectious agent are present prior to onset of clinically recognizable disease.

BSE has limited infectivity outside the central nervous system. This contrasts with scrapie (when sheep are orally infected) in which infectivity is identified in the digestive tract and lymphoreticular system prior to progressing through the autonomic nervous system, to the spinal cord and brain. Studies characterizing the disease-associated PrP isoforms in various tissues from CJD and vCJD patients readily identified PrP^{Sc} in lymphoreticular tissues from vCJD patients while the lymphoreticular system is not involved in sporadic CJD. This is likely due to the oral source of the vCJD infection and suggests a greater potential of iatrogenic transmission of vCJD.

CWD is a contagious disease in cervids. Lateral transmission of CWD most likely occurs via the oral ingestion of the agent. Oral inoculation of pooled CWD-positive mule deer brains into mule deer fawns resulted in an early accumulation of CWD-associated PrP (PrP^{CWD}) in the lymph tissues that are associated with draining the oral and intestinal mucosa. Lymphoid cells associated with PrP^{CWD} in the tonsils were characterized from clinical and preclinical deer. White-tailed deer that stain positively for PrP by immunohistochemistry in the retropharyngeal lymph node were not always obex-positive. Conversely, no obex positive samples have been identified in approximately 2000 animals that were IHC-negative in the retropharyngeal lymph nodes. Detection of CWD agent in feces, urine and saliva increases the likelihood that CWD is orally transmitted.

24.3 Characteristics of the agent

The unusual biology of these diseases has been long recognized and has influenced how these disorders have been characterized. Based upon their long incubation periods and transmissibility, prion diseases were originally described as unconventional or slow viruses. The extreme resistance of these agents to ionizing and gamma irradiation combined with the inability to isolate a disease-specific microorganism prompted speculation that there existed a non-nucleic acid mode of replication. In 1968, the mathematician J. S. Griffith proposed three means by which a protein could have self-replicating properties. One of Griffith's models involved the interaction of two proteins having the same primary amino acid sequence yet differing structurally. Infectivity purification studies in the late 1970s identified a brain homogenate fraction that was enriched for infectivity. The detergent extraction and centrifugation steps resulted in the formation of fibular structures, referred to as scrapie-associated fibrils in 1981 or the similarly structured prion rods in 1982.

Biochemical characterization of the highly infectious preparations identified a protease resistant protein termed the prion protein. This glycoprotein has a molecular weight of 33–35 kDa (in the absence of protease treatment). Treatment with mild protease (50–100 ug/ml of proteinase K) reduced the protein to 27–30 kDa. Characterization of the gene encoding the prion protein quickly led to the realization that the prion protein was not unique to an undiscovered microorganism but rather was expressed in healthy, uninfected animals and encoded by a single-copy gene in the nucleus. The difference between the infection-associated (PrP^{Sc}) and uninfected isoforms (PrP^{C}) of the protein is conformational (Table 24.2). The avid aggregation and insolubility of PrP^{Sc} makes determining its secondary structure currently impossible; its

Table 24.2 Prion Protein Nomenclature

	Protease sensitivity	**Description**
PrP^{C}	Sensitive	Cellular isoform of the prion protein
PrP^{Sc}	Resistant	Disease-associated isoform of the prion protein
PrP-sen	Sensitive	Refers to protease digestion characteristics of PrP, often in the absence of transmission data
PrP-res	Resistant	Refers to protease digestion characteristics of PrP, often in the absence of transmission data
Prion rods	Resistant	Structures produced upon detergent extraction of infected tissue. Highly infectious, comprised primarily of PrP^{Sc}
Scrapie associated fibrils (SAF)	Resistant	Very similar to prion rods

structure, however, has been inferred by sequence-specific conformational preferences, antibody binding data and two-dimensional crystalline-like arrays of PrP^{Sc} or PrP^{Sc}-like molecules. The structured *C*-terminal region consists of three α–helices and two small anti-parallel β–sheets. In contrast, the PrP^{Sc} is enriched for β–sheet content in the *C*-terminal region, is prone to aggregation and is partially resistant to proteinase K (PK) digestion.

PrP^{Sc} has the ability to recruit and convert normal PrP^{C} into PrP^{Sc}. This nucleation-dependent conversion is striking because it suggests the passage of biological information from the tertiary protein structure of PrP^{Sc} onto PrP^{C}. Most interestingly, PrP^{Sc} can exist as a number of distinct conformers conferring different properties and referred to as strains. Extraordinarily, these strains can be differentiated biologically, biochemically and biophysically, apparently possessing different tertiary conformations of the prion protein despite identical amino-acid sequences.

PrP^{C} is a glycosylphosphatidylinositol (GPI) anchored protein expressed primarily on the cell surface of neurons within the central nervous system as well as on other cell types within the body. Under physiological conditions, PrP^{C} is synthesized in the endoplasmic reticulum and transported through the Golgi towards the cell surface. Like other GPI-anchored proteins, PrP^{C} is located primarily in cholesterol-rich, detergent-resistant microdomain complexes of the plasma membrane (rafts). Cell culture studies have demonstrated that, once in the membrane, some PrP molecules are released into the extracellular space, while most PrP is internalized into an endocytic compartment. The normal function of the protein is not known. There is evidence, based upon a metal-binding domain present in the N-terminal region of the polypeptide and the binding of copper to synthetic peptides, that PrP^{C} is a metalloprotein. The transition metal induces a dramatic effect on PrP biochemical properties resulting in an increased aggregation, beta-sheet content and acquisition of protease-resistance. Deletion of the prion protein gene in transgenic mice does not appear to affect the mice, with the singular exception of making them resistant to prion infection.

The human PrP is a glycoprotein of 253 amino acids. It is a cell surface glycoprotein expressed primarily in neurons but also in astrocytes and other cells. Knockout experiments in the mouse have demonstrated that it is not an essential gene and that $PrP^{-/-}$ mice, when infected with mouse-adapted scrapie, do not replicate the abnormal form of the protein, nor develop spongiform lesions characteristic of the disease.

The prion hypothesis states that the infectious agent for these diseases is a self-replicating protein. The ability to fold/aggregate synthetic prion peptides into infectious forms clearly supports this hypothesis. The synthetic prions, however, have much lower levels of infectivity

than would be predicted suggesting that only a subset of the misfolded protein has attained the infectious conformation. As in the Alzheimer's disease field, there is a controversy as to whether the aggregated PrP is the toxic form or if oligomers, in the pathway to becoming aggregated, are the more toxic moieties.

24.3.1 Genetic impact: The *Prnp* allele

The gene that encodes the prion protein (*Prnp*) is the primary and only defined genetic susceptibility factor for prion diseases. This genetic susceptibility factor was originally referred to as the scrapie incubation gene (*Sinc* allele) in mice or scrapie incubation period gene (*Sip* allele) in sheep. Both the *Sinc* and *Sip* genes were identified by classical genetic studies as having a significant effect on the incubation period of scrapie-infected animals. The advent of molecular biology clearly demonstrated that the PrP gene is the *Sinc* (*Sip*) gene. The requirement for PrP^C for successful TSE infection has been conclusively demonstrated by PrP gene ablation experiments in mice. In those experiments, removal of the PrP gene followed by experimental infection of mouse-adapted TSE agent results in no infection occurring. Replacement of a PrP gene into these null mice results in infection by the appropriate TSE agent.

In humans, polymorphisms at amino acid 129 of the prion protein impact disease onset as well as susceptibility to vCJD. Residue 129 encodes either a methionine (most common) or a valine. Approximately 38% of Europeans are homozygous for methionine, 51% heterozygous and 11% homozygous for valine. Homozygousity for either amino acid is a susceptibility factor for sporadic as well as acquired CJD. Virtually all of the vCJD cases have occurred in individuals homozygous for the methionine allele. A number of polymorphisms are linked to the familial forms of prion disease, notably fatal familial insomnia, Gerstmann-Sträussler-Scheinker syndrome and heritable CJD. These polymorphisms can either be point mutations (i.e., the P102L variant observed in GSS) or insertions (increased numbers of the octapeptide repeat region located in the N-terminal domain of the prion). Experimental studies, using transgenic mice expressing variants of the human prion protein, suggest an earlier onset of disease upon infection with the BSE agent, when the animals are homozygous for M/M. These data suggest that more vCJD cases may occur, likely in people with the M/V and V/V *Prnp* genotypes.

Of the large number of amino acid polymorphisms associated with sheep *Prnp*, three are strongly linked to the occurrence of both natural and experimental scrapie. These polymorphisms are valine or alanine at position 136, arginine or histidine at position 154 and glutamine, arginine or histidine at position 171. Valine at position 136 appears to make sheep more susceptible (and at a younger age) to natural scrapie in many breeds of sheep while an arginine at codon 171, especially those that are homozygous, have been show to be more resistant to scrapie. One method of eradicating scrapie has been based on the resistance of specific genotypes to classical scrapie. Although successful in decreasing the number of scrapie-infected flocks, it now appears that other sheep prion diseases, specifically atypical scrapie as well as sheep/goat-adapted BSE, can overcome these genetic barriers.

A number of polymorphisms in cervid *Prnp* are also linked to susceptibility/resistance to CWD. For example, we have demonstrated, via experimental infection studies in white-tailed deer, that the incubation period, following oral infection, is longer in animals with polymorphisms at amino acids 95 (Q to H change) and 96 (G to S change). Similar observations have been made with mule deer and elk, except that the polymorphisms are different with amino acid 226 being important in mule deer and amino acid 129 in elk.

There is no clear link between polymorphisms in cattle and the onset/occurrence of classical BSE. Cattle exhibit some variability in the primary sequence of the prion protein, predominantly

a difference in the number of octapeptide repeats. The majority of cattle have five copies of the octapeptide repeat, some have six copies. There is, however, no correlation between the number of octapeptide repeats and susceptibility to BSE infection. A polymorphism in the non-coding region of the bovine *Prnp* has been identified and is associated with BSE; it is thought that this variant may change the expression levels of PrP. A mutation in *Prnp* is, however, linked to an H-type BSE case in the US. In this animal, a single codon change, at amino acid 211 (E211K) was observed suggesting that the atypical BSEs may be genetic diseases.

24.3.2 Prion strains

Prion strains have been identified in virtually every species affected by this family of fatal, neurodegenerative diseases. Distinct strains of prion disease agent differ with respect to both their biological and physicochemical properties. Understanding the properties of the different strains is critical for detection/diagnosis as well as developing strategies for management approaches.

Prion strains are caused by differences in the structure of the abnormal prion protein. One of the first studies linking prion strain with protein structure identified differences in morphology of scrapie-associated fibrils, sedimentation rate, and degree of proteinase K (PK) resistance when comparing the hamster-adapted 263 K scrapie agent with two mouse-adapted scrapie agents, ME7 and 139A. A similar correlation between PrP^{Sc} structure and agent strains was observed upon adaptation of TME agent in hamsters. In the hamsters, the primary sequence of the prion protein is identical, therefore changes in the properties of the prion protein can be attributed to conformational differences in the PrP^{Sc}. DROWSY (DY)-infected hamsters become increasingly lethargic, their voluntary movements are slow and they lack coordination when aroused but do not become hyperexcitable or exhibit cerebral ataxia like HYPER (HY) hamsters. HY hamsters have an incubation period of 65 ± 1 days with brain titers of $10^{9.5}$ LD_{50}/g whereas DY-infected animals have an incubation period of 168 ± 2 days with brain titers of $10^{7.5}$ LD_{50}/g. PrP^{HY} and PrP^{DY} proteins differ with respect to sensitivity to protease digestion and proteinase K digestion sites. FTIR analysis of PrP^{HY} and PrP^{DY} suggests that different secondary or tertiary conformations exist between the two abnormal forms of the protein.

Variants in sheep scrapie have been recognized for decades, particularly upon passage of sheep scrapie into mouse models. A new sheep prion disease was identified in Norway in 1998 (Nor98). In cattle, in addition to classical BSE, two variants (H- and L-types) of atypical BSE have been identified in the past decade. Most recently, two strains of CWD (CWD1 and CWD2) were identified upon passage of different cervid isolates into cervidized transgenic mice. These two strains displayed divergent biological properties (different incubation periods and different neuropathology), yet the PrP^{Sc} produced by the two strains were indistinguishable.

In humans, different isoforms of the PrP^{Sc} are linked to different clinical manifestations of the disease. Four major isoforms of human PrP^{Sc} can be distinguished, based on Western blot analysis of the PK-resistant PrP^{Sc}. Types 1–3 are observed in classical CJD (sporadic and iatrogenic) while Type 4 is only observed in samples from vCJD patients.

24.3.3 Interspecies transmission

A characteristic of prion diseases that has had an impact both on the origin of BSE and its subsequent transmission to humans is the ‘species barrier effect’. Transmission of a prion disease to a new host species is typically an inefficient process, resulting in either no evidence

of disease transmission or an increase in the length of the incubation period in the donor host species. Subsequent passage in the new host species results in the reduction and eventual stabilization of the incubation. Some TSEs, however, are not pathogenic for some host species. For example, the TME agent does not establish an infection in several strains of mice. Experimentally, strong species barriers can be overcome through repeated passaging of the agent in the new host. For example, TME has limited pathogenicity in ferrets, initiating clinical symptoms after an extended incubation period of 24 months. Second passage (ferret to ferret) results in a reduction of incubation period to 18 months while a third passage produces a 4-month incubation that is stable upon further ferret passage. Ferret-adapted TME (4-month incubation period) has limited pathogenicity in mink, producing an almost 24-month incubation period.

It could be argued that the apparently strong sheep-to-bovine species barrier may have been overcome agriculturally in a similar manner. In this scenario, the BSE outbreak occurred via the passage and adaptation of sheep scrapie to cattle. Scrapie-infected sheep were rendered and the meat and bone meal by-products (MBM) included as a supplement to cattle rations. The physicochemical stability that characterizes these infectious agents resulted in the scrapie agent surviving the heat treatment present in the rendering process. A subset of the cattle fed scrapie-infected MBM may have had developed either subclinical or preclinical disease. These animals would have been rendered and, again, included in MBM, thus recycling infectivity in the cattle population of Great Britain. It should be stressed that there are other possible explanations for the etiology of classical BSE. The identification of a polymorphism in an atypical case of BSE (see above) suggests that classical BSE may have arisen from a genetic case of BSE.

One notable exception to the species barrier effect is the transmission of the BSE agent into other species. Experimentally, BSE has been transmitted, in addition to cattle, to a number of species including mice, mink, sheep, goats, marmosets, and macaque monkeys. It is this limited species barrier that has led to the emerging vCJD epidemic. vCJD is, in essence, human BSE. BSE is not only transmissible to a range of species; it maintains its characteristics when passaged through an intermediate species with a distinct PrP gene. In the frame of the 'prion hypothesis', BSE may represent a thermodynamically dominant (selected) PrP^{Sc} conformation that is able to productively interact with PrPs expressed in a wide range of species, thus accounting for the remarkable transmissibility of this agent into several species.

24.4 Epidemiology

The TSE landscape has shifted considerably over the past 30 years, from CJD being an extremely rare and relatively unknown disease and scrapie being an agricultural nuisance and of veterinary interest, to the outbreak of the agriculturally disastrous BSE epidemic and the realization that BSE can and has been transmitted to humans as well as the emergence of CWD. As the extent of BSE to human transmission and the zoonotic potential of CWD as well as the atypical forms of both scrapie and BSE are unknown, these diseases are of considerable concern to the public.

Determining incidence of prion diseases worldwide is complicated as there is no uniform testing. For example, in the European Union, only cattle over the age of 30 months are routinely tested for BSE. In Japan, however, all cattle slaughtered are tested. In North America, BSE surveillance emphasizes the testing of 'downer animals', i.e., cattle that are non-ambulatory. Testing for CWD is more restricted with testing generally limited to targeted areas (surrounding known CWD outbreak areas or in areas adjacent to captive farms where CWD has been identified).

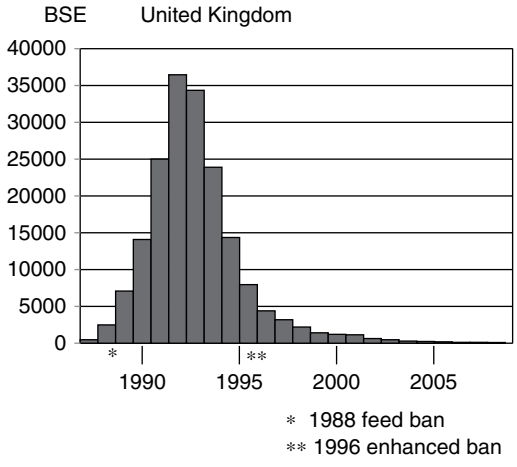

Figure 24.1 The bovine spongiform encephalopathy epidemic in the United Kingdom. The decline in cases that began in 1993 is attributed to the 1988 ban on mammalian meat and bone meal supplements being included in feed. Monthly updates of BSE in Great Britain can be obtained at http://www.maff.gov.uk/animalh/bse/. BSE has been identified (as of April 2012) in 26 countries worldwide. Notably, it has not been detected in South America, Africa or Australia. Based on data from http://www.oie.int/animal-health-in-the-world/bse-specific-data/number-of-cases-in-the-united-kingdom/

24.4.1 Bovine spongiform encephalopathy

First documented in 1986, the initial cases of BSE occurred in Great Britain in 1985, although it likely was cycling in cattle prior to that time. The disease peaked in January of 1993 with approximately 1000 new cases documented per week. There have been almost 181,000 documented cases of BSE in Great Britain (Fig. 24.1). This is certainly a considerable underestimate of the total number of infected cattle, as infected preclinical animals would be excluded. The decline in cases beginning in 1993 is attributed to the 1988 ban on the inclusion of meat-and-bone meal in cattle feed. Due to the approximate 5-year incubation period, the effects of the ban were not observed until 1993 and 1994.

The first case of BSE in North America was reported in Alberta, Canada; this animal was an import from the UK. Canada's first 'homegrown' case of BSE occurred in 2003; the US reported its first BSE case the same year (an animal imported from Canada). Canada has now had 18 cases; the US has reported three domestic cases with the most recent occurring in April 2012 in California. Two of Canada's BSE cases and one of the US's cases were atypical.

24.4.2 Scrapie

Scrapie has a worldwide distribution with the notable exceptions of Australia and New Zealand. The latter countries are considered scrapie-free. Epidemiologic studies do not provide any support of a link between scrapie in sheep and CJD in humans.

24.4.3 Chronic wasting disease

CWD is present in captive and wild populations of white-tailed deer, mule deer and elk in 18 states in the US, two provinces in Canada (Fig. 24.2) as well as in South Korea (the Korean cases were introduced by elk imported from Canada). In wild populations, CWD

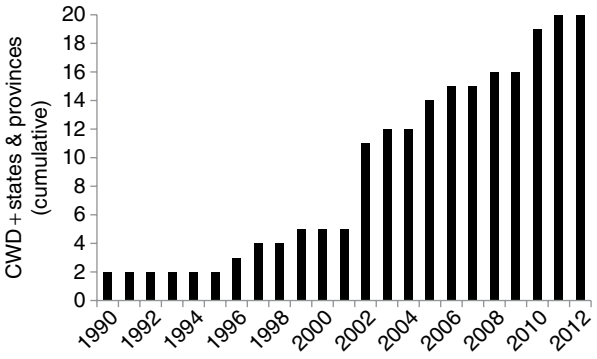

Figure 24.2 The cumulative number of US states and Canadian provinces where CWD has been identified.

abundance increases as the disease becomes more established in a region. The state of Colorado estimates CWD levels in endemic regions of the state to range from 1–25% of male mule deer and 1–17% of male elk. Not surprisingly, CWD abundance in farmed cervids can be much higher, with 79% of deer testing positive for CWD in one report.The geographic range of CWD continues to expand, due in part to the efficient animal-to-animal transmission (by as, of yet, unknown means) as well as the continued commercial movement of animals. Although there is no evidence for natural transmission of CWD to humans, there are too many unknowns for a definitive answer with respect to the zoonotic potential of CWD. The finding that CWD agent is present in muscle (venison) of infected deer suggests that humans are being exposed to it. As human prion diseases can take decades before clinical disease is observed, it is too early to rule out zoonotic transmission. As animals in endemic areas are routinely tested for CWD, it would be prudent for hunters and other consumers to avoid eating venison from animals that are test-positive for CWD.

24.4.4 Human prion diseases

The epidemiology of human prion diseases encompasses three forms: sporadic, familial, and acquired. CJD (sporadic) has an incidence of approximately one individual per million per year worldwide. It occurs in individuals in their fifth to sixth decades of life and has a worldwide distribution. The familial forms of CJD and GSS are even rarer, affecting 1 per 10 million per year.

Currently, the incidence of vCJD is waning. Not surprisingly, given the BSE link, almost every case has occurred in the UK. As of this writing (April 2012), there have been 176 cases in Great Britain (Fig. 24.3), 25 in France, five in Spain, four in Ireland, three each in the Netherlands and the US, two each in Canada, Italy and Portugal, and one each in Japan, Saudi Arabia and Taiwan. The incidence of vCJD appears to have peaked in Great Britain in 2000 with 28 documented cases. Caution should be exercised, however, when interpreting this decline. vCJD patients are of a specific age group (average age of vCJD patients is 29 years) as well a PRNP genotype (met/met at position 129). The decline in BSE as well as the removal of specified risk materials for human food should decrease, if not cease, the transmission of BSE to humans. The potential for human-to-human transmission (two cases of vCJD have been attributed to blood transfusions), however, suggests that vCJD cases may continue to occur, albeit at a much lower incidence.

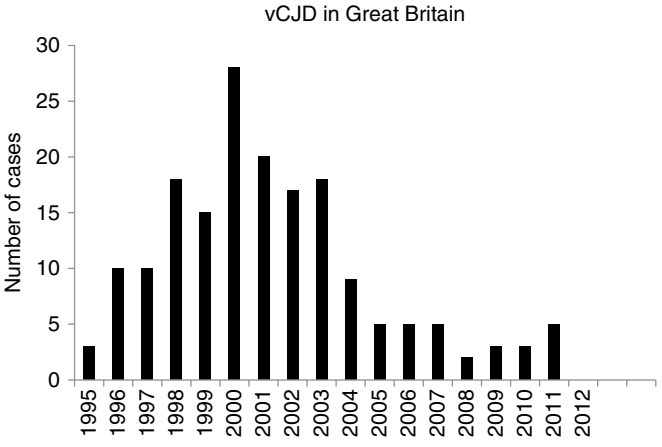

Figure 24.3 The number of vCJD cases in Great Britain. Monthly updates of vCJD in Great Britain can be obtained at http://www.doh.gov.uk/cjd/cjd_stat.htm. As of May 7, 2012, there have been no new cases of vCJD this year. Based on data from http://www.cjd.ed.ac.uk/documents/figs.pdf

24.5 Detection of the organism

One of the greatest challenges in this field is the need to develop accurate and highly sensitive methods of assaying for prion diseases. Traditional detection and/or verification of a prion infection involved the histological examination of the brain for evidence of spongiform degeneration typically combined with infectivity studies to determine transmissibility. The identification of the disease-associated form of the prion protein and the generation of PrP antibodies has facilitated Western blot and immunohistochemical approaches for the detection of PrP^{Sc}-containing tissue. It should be emphasized that the most accurate diagnosis occurs in animals and humans during the clinical stages of the disease through the examination of CNS. Brains of infected animals during the clinical phase of the disease contain the greatest accumulation of PrP^{Sc}. The earlier the stage of infection, the more difficult these diseases are to diagnose.

The Western blot analysis involves the treatment of tissue homogenates with mild levels of proteinase K (50–100 ug/ml for 1–2 hours). PrP^{C}, the only isoform in uninfected tissue, is completely degraded by proteinase K while the disease-associated form of the protein, PrP^{Sc}, exhibits resistance to the digestion (Fig. 24.4). A portion of the N-terminal region of the PrP^{Sc} isoform is cleaved during the digestion resulting in a smaller protease resistant core of approximately 27–30 kDa (Fig. 24.4). PrP^{Sc} is generally present on Western blots as three bands due to glycosylation; the bands represent unglycosylated, mono- and di-glycosylated.

Detection of prion agents in body fluids, tissues and other potentially contaminated materials has been greatly enhanced by a novel in vitro methodology, protein misfolding cyclic amplification (PMCA). In this cell-free assay, prion protein can be amplified (in a method somewhat analogous to the polymerase chain reaction) in vitro. For basic PMCA, a small seed of infectious prion agent serves as the nucleant for PrP^{C} (generally provided by normal uninfected brain homogenates). The seed and the PrP^{C} are incubated together with short bursts of sonication followed by incubation (when conversion occurs). Unlike earlier cell-free conversion procedures, the material generated in PMCA is infectious. Although the primary source of PrP^{C} is uninfected brain homogenates, researchers have successfully converted recombinant PrP^{C} to the infectious form, using PMCA, with the addition of polyanions.

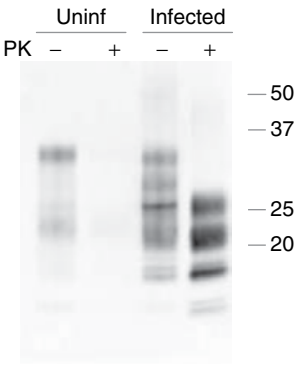

Figure 24.4 Proteinase K sensitivity of PrP obtained from brain homogenate from an infected animal (right panel) and with PrP^{C} (left panel) which is not associated with infectivity.

A method related to PMCA, quaking-induced conversion (QUIC), also provides a rapid assessment of the approximate levels of PrP^{Sc} present in a sample. This method utilizes recombinant PrP^{C} and shaking to amplify the PrP^{Sc}. The advantages of QUIC include the rapidness of the assay (approximately 2 days) and the ability to use recombinant PrP^{C} instead of brain homogenates as the source of PrP^{C}. Experimentally, QUIC has the disadvantage of not generating infectious material and it also does not appear to amplify strain-specific properties of the PrP^{Sc}.

Other advances in the detection of the prion agent include a number of ante-mortem tests developed primarily for testing for scrapie and CWD. As mentioned above, detection and diagnosis of prion diseases have generally relied on post-mortem analysis. It has recently been demonstrated that both the scrapie agents and the CWD agents can be detected using ELISA, Western blot or PMCA from the third eyelid, tonsils and rectal mucosa. In deer, CWD agent can be detected within weeks of infection suggesting utility for screening animals prior to commercial sale/movement of animals.

24.6 Physical means of destruction of the organism

Prion agents exhibit extreme biological, chemical and physical stability. Decontamination procedures that sterilize other infectious agents typically only reduce prion agent titer. For example, standard autoclaving does not eliminate prion infectivity. Dry heat sterilizes only at the extremely high temperatures (in excess of 600 °C) present during incineration. The extreme physicochemical stability of the prion disease agent is the underlying cause of the BSE epidemic as well as the iatrogenic transmission of CJD via contaminated surgical instruments. The resistance of scrapie to formalin during the preparation of a vaccine resulted in the accidental transmission of scrapie to sheep in the 1930s. Standard autoclaving (121 °C for 15 minutes) is ineffective in eliminating infectivity. Prion decontamination remains an important issue impacting biomedical applications, animal food safety and contaminated environments.

Many of the inactivation studies have been performed in the laboratory of David Taylor. Chemical methods of inactivation, including ethanol, formaldehyde, gluteraldehyde, and hydrogen peroxide, all of which exhibit efficacy in the sterilization/decontamination of microorganisms, are of little practical use with prion diseases. One hour exposure to NaOCl

solution containing 20,000 ppm of Cl_2 is suitable for inactivating TSE agents. It should be noted that there are TSE strain differences in inactivation. Richard Kimberlin and colleagues determined that autoclaving one mouse-adapted strain (strain 139A) for 2 hours at 126 °C resulted in its inactivation while a second strain (strain 22A) was not inactivated.

Chemical denaturation of infectious preparations results in a reduction of infectivity and concomitant decline in the amount of protease-resistant PrP. Our group has shown that both infectivity and the abnormal form of the protein can be regenerated upon dilution of the denaturant. It should be emphasized that the preceding experiments were performed under carefully controlled laboratory conditions. The study does emphasize the need to ensure the destruction of the protein during inactivation treatments.

24.7 Prevention/control measures

Prion diseases are always fatal. There are no drug treatments or vaccines that provide a cure. A series of compounds have been identified ('anti-scrapie drug') that extend the incubation period. However, they are not cures: they can be toxic and require continuous treatment initiated at the early stages (preclinical) of the infection. Many of these drugs are only effective if administered at the same time that infection is initiated, decreasing their utility for naturally-occurring infections or sporadic disease. As a result, control measures involve eliminating the source of infection and, in the case of humans, reducing the likelihood of iatrogenic transmission.

24.7.1 Animal diseases

With scrapie, control measures have often involved the destruction of affected animals as well as the flocks. Scrapie remains a self-sustaining disease of sheep throughout the world. Scrapie eradication programs have been apparently successful in Australia but not in North America.

CWD, being a contagious prion disease and present in both farmed and free-ranging cervids, is the most difficult of the animal prion disease to control. The presence of infectious CWD agents in body fluids such as saliva, feces, urine, as well as in infected carcasses remaining in the environment, coupled with persistence of CWD prions in the environment all suggest that eradicating CWD will not be an easy task. Furthermore, studies from our research group indicate that prions bound to soil minerals and other microparticles may be more infectious (i.e., result in onset of clinical disease more rapidly than unbound prions), potentially affecting interspecies transmission.

Classical BSE, being a foodborne disease, has declined tremendously as a result of the ban on feeding ruminant meat-and-bone meal to cattle. There is little evidence of cattle-to-cattle transmission of BSE. It is therefore argued that strict adherence to the feed ban will result in its elimination. The atypical BSEs appear not to be linked to consumption of contaminated feed and would not be impacted by feed bans.

The atypical prion diseases, both atypical BSEs and scrapie, present a new concern with respect to control and prevention. The origins of the atypical diseases are, as yet, unknown. Some researchers hypothesize that the atypical prion diseases are sporadic cases. The lack of worldwide surveillance data makes it difficult to determine the incidence of these diseases. Particularly unsettling is the demonstration that, experimentally, the species barrier for L-type BSE is lower than observed for classical BSE. Also, for Nor98, the atypical scrapie, the genetic barriers are different. Sheep bred to be resistant to classical scrapie are susceptible to Nor98.

24.7.2 vCJD

The lack of treatment for these diseases has focussed efforts to minimize further transmission of vCJD. The dramatic decline in BSE levels in Great Britain and the rest of the world as well as the general exclusion of bovine brain or nervous tissue in human food has tremendously reduced and/or eliminated further BSE to human transmission. Of concern, however, is the unknown number of humans currently incubating the disease (i.e., are preclinical). Two cases of vCJD in the UK have been attributed to blood transfusions (the blood donor was preclinical for vCJD), demonstrating the potential for iatrogenic exposure. Experimental infection of transgenic mice expressing human *Prnp* with the BSE agent suggests that infectivity is distributed at higher levels in the peripheral tissues than when the infectious agent is sporadic CJD. The combination of peripheral infectivity with the difficulties in decontaminating instruments suggests the potential for future iatrogenic transmission of vCJD.

A considerable proportion of the British population was exposed to BSE infectivity in the 1990s and estimates of the total number of potential cases of vCJD initially ranged from a few dozen to hundreds of thousands of individuals. Fortunately, there have been fewer than 200 vCJD cases in Great Britain and the disease appears to be on the decline. There are, however, still too many unknowns (route of infection, number of individuals exposed, effectiveness of CNS exclusion from meat, levels of infectivity present) to provide an accurate assessment of the future incidence of vCJD. The emergence of the atypical forms of both scrapie and BSE as well as the continuing CWD expansion suggests that prion diseases will remain a concern to public health.

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25 Forthcoming new technologies for microbial detection

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25.1 Introduction

Among the foodborne pathogens, approximately 67% of the illnesses are caused by viruses and 30% by bacterial pathogens and the remainder by parasites, molds and mycotoxins. In recent years, however, elevated public health crises are contributed to primarily by a handful of bacterial and viral pathogens including Shiga-toxin producing *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, *Campylobacter*, *Clostridium botulinum*, *Shigella, Vibrio*, and norovirus and Hepatitis A virus. In response, the US Government passed the Food Safety Modernization Act in 2010 to reduce foodborne outbreaks and to protect consumers from illnesses. This new law emphasizes increased inspection and testing. Use of advanced and reliable detection methods is essential to prevent foodborne outbreaks, illnesses, hospitalizations, fatalities, and product recalls. Detection methods are broad and, depending on the type of microorganisms or food/samples to be tested, certain methods are more effective than others. Essentially, the chosen method must be accurate and reliable with least chance for false result. Furthermore, the test result must be obtained relatively quickly so that a decision can be made whether to hold or release the product for retail distribution. This is particularly important for foods with short self-life such as high-moisture-containing soft cheeses, processed meats, salads, fruits and vegetables, and other ready-to-eat food products. Rapid results also allow processors to take corrective actions during food processing so that product losses are minimized.

25.2 Contemporary detection approaches

Depending on the length of time (sample–to–test result) required to perform a test, methods are arbitrarily grouped as culture-based (3–14 days or longer), rapid (24–48 h), ultra rapid (8 h or less), and real-time or near real-time screening (seconds to minutes). The culture-based method is lengthy and involves many steps including sample enrichment, selective

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enrichment, isolation of pure culture and identification by biochemical or genetic methods, but is considered the 'gold standard'. It is reliable, accurate and has a very low chance for false-result. The 24–48 h method has an initial sample enrichment step, similar to the culture-based method followed by detection using PCR or immunoassay omitting the culture isolation and confirmatory testing steps. The approach for ultra-rapid testing methods is similar to 24–48 h methods but it incorporates a real-time quantitative PCR method or highly sensitive biosensor method that have a detection limit in the range of 10^2–10^4 cfu so that prolonged culture enrichment is not necessary. Sometimes, these methods are prone to yield false results originating from the physiological state of the target microorganism at the time of detection. Food contains chemical preservatives, salts, spices and flavoring agents, and is stored under vacuum or oxygen-limiting or modified atmospheric environment, and at low temperature. These stress-generating conditions may affect microbial growth and the level of microbial gene or protein expression, which often is the target for antibody or PCR-based detection. The real-time methods are generally based on spectral imaging systems used on-line or in-line for monitoring products for pathogens during production and/or processing. These methods are not pathogen-specific and generally are used to monitor gross changes in physical appearance of a carcass during slaughter or fecal/soil contamination of fruits and vegetables. Such samples are removed from the line to prevent tainted products from entering into the human food supply chain.

Specificity of these methods relies on the reagents and probes used, which may include nucleic acid, aptamer, antibody or antibody-like biorecognition molecules: receptor proteins, lectins, bacteriophage proteins, selective antimicrobial agents, chromogenic differential growth media, etc. Nucleic acid-based polymerase chain reaction (PCR) assays, antibody-based dipstick (lateral flow immunoassay), and immunoassays are often used in conjunction with culture based methods to obtain results in 24 to 48 h. Assay sensitivity for these methods also vary substantially depending on the probes, number of target pathogens and sample matrices. PCR-based detection methods are now fully automated, where a thermocycler is used to amplify the target gene with intercalated fluorophor allowing rapid interrogation/detection of amplicons. This method also allows quantitative estimation of the total number of microorganisms present. Also, it can detect live cells, when the mRNA is targeted as template. PCR assay is highly sensitive, requiring a minimum of 50–10^2 cfu to give a positive result but are prone to failure due to food-associated PCR inhibitors. For dipstick assays, an aliquot of heat-treated enriched sample is applied to the sample application well on the lateral flow device, and the appearance of a band in about 10–15 min, would indicate a positive result. The dipstick method is user-friendly but requires a large number of bacteria (10^7–10^9 cells). Automated immunoassay platforms (VIDAS and miniVIDAS) marketed by bioMerieux, on the other hand, are able to detect bacterial pathogens at a lower range (10^5–10^6 cfu/ml).

25.3 New generation detection methods

Detection technology platforms are evolving at a very fast pace. In recent years, a breakthrough in detection technologies is taking place in molecular- and biosensor-based methods. In molecular methods, genome-wide microarray to detect multiple pathogens in a single run is becoming attractive. With strategic design of a universal microarray chip, one can target multiple different food pathogens that could be used to test many food samples. A complete or partial genome sequencing approach is also recognized as a powerful detection tool. Recent development in new generation high throughput sequencing instruments such as

Table 25.1 Class of Biosensors

Class	Biosensor type
Optical	Surface plasmon resonance (SPR)
	Fiber optic sensor
	Light scattering sensor
	Flow cytometry
	Fourier Transformed infrared
	Raman
Electrical or electrochemical	Impedimetric biosensor
	Amperometric
	Potentiometric
	Conductiometric
Mass-based	Piezoelectric – quartz crystal microbalance (QCM)
Miscellaneous (functional biosensor)	Mammalian cell-based biosensor
	Bacterial cell-based sensor

454 pyrosequencing, Illumina sequencing and SOLiD (Sequencing by Oligonucleaotide Ligation and Detection) techniques show potential for use in the future for foodborne pathogen detection and identification yielding results in a short time. The sequence information also could be used to track a pathogen to its source to implement proper corrective actions.

Biosensor-based assays are designed to provide results quickly with improved sensitivity. A biosensor is comprised of a biological recognition element combined with a transducer that emits a signal in the form of electrical, optical, or mass (Table 25.1). Data processing and interpretation is aided by a computer. Recognition molecules (antibody, aptamer, lectins, bioreceptors, bacteriophage) provide specificity. Even though numerous biosensor methods based on micro/nano technology platforms exist, their fundamental operating principles are very similar. The majority use similar biorecognition molecules as non-biosensor-based methods. Therefore, specificity/selectivity or performance often depends on the recognition molecules used, but biosensors may have increased sensitivity with a detection limit in the range of 100 cells or nano to pico gram quantities of toxins. Another important element for biosensor methods to be successful is a requirement for a highly sophisticated sample preparation step to separate micro-organisms/toxins from food matrices before analysis. The food matrix and its components (lipids, salts, proteins, enzymes, antimicrobial preservatives) can interfere with performance or can incapacitate an instrument. Therefore, filtration, affinity separation, immunomagentic separation, and dielectrophoresis (DEP) are commonly used for sample preparation prior to biosensor-based analyses. In this chapter, select biosensor platforms that have the greatest potential to be used as future biosensor tools for microbial detection are discussed. These include fiber optic sensor, surface plasmon resonance sensor, Fourier transform Infrared spectroscopy (FTIR) and Raman, light scattering sensor, and impedimetric sensor.

25.4 Surface Plasmon Resonance

A Surface Plasmon Resonance (SPR) sensor is one of the most matured biosensor technologies used for microbial detection. In principle, SPR monitors molecule–molecule interaction at real-time. The fundamental layout of the sensor is comprised of a thin metal film on a prism, which acts as a transducer. Gold is widely used in SPR biosensors since the surface

chemistry for immobilization of biorecognition molecules (probes) on gold is well established. When the analyte binds to the biorecognition molecule, it changes the refractive index at the metal-dielectric interface in the prism-coupling system. This generates surface plasmon resonance, resulting in the wavelength shift of the incident light, which is detected using a photodiode array or a Charge Couple Device (CCD) camera. Surface plasmons are also known as evanescent waves as they are localized at the metal dielectric interface and decay after a certain distance. The height of the evanescent wave is approximately one-fourth of the wavelength of the incoming light that strikes the prism surface. This essentially restricts the size of the analytes that could be reproducibly detected using the SPR platform; usually proteins/toxins are detected more efficiently than bacterial cells.

Although SPR is considered a label-free system, in fact, it still requires a biorecognition molecule to provide specificity, thus it is rational to designate it as semi label-free to distinguish it from spectral-based biosensors that do not require any labeling reagents such as FTIR, Raman and light scattering (see below).

SPR is widely used in life science and pharmaceutical research, and in recent years it has been used in food safety and security, diagnostic screening and monitoring of environmental pollutants. The selectivity/specificity of an SPR is primarily dependent upon the choice of recognition molecule and the immobilization chemistry employed. Continuous real-time monitoring of the sample for pathogens and regeneration of the sensor's surface is facilitated using a mild acidic solution wash.

SPR instruments are available commercially and several of them are used for foodborne pathogen detection. A list of commercial systems include Biacore (GE Healthcare), Spreeta/SensiQ (ICx Nomadics), SPR spectroscope (Multiscop™, Optrel GbR, Germany), Reichert SR7000 (Reichert Analytical Instruments, Depew, NY), resonant mirror-based IAsys (Thermo Labsystems, Cambridge, UK), and PlexArray™ (Plexera Biosciences LLC).

SPR has been used to detect cells of *L. monocytogenes*, *E. coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *E. coli* and *Brucella abortus* and microbial toxins such as enterotoxins from *Staphylococcus*. Generally, the limit of detection for bacterial cells is in the range of 10^5–10^7; however, it is highly sensitive for toxins or smaller molecules which can be detected at the pico- to nano-gram quantities.

25.5 Fiber optic biosensor

Fiber optic sensors use similar principles as SPR and utilize a laser excitation to generate an evanescent wave to quantify biomolecules immobilized on the optical waveguide instead of a prism. The fundamental layout of the assay is based on sandwich immunoassay where the biorecognition molecule is first immobilized on the waveguide to bind to microbes. A fluorescence (Cyanine 5 or Alexa-Fluor 647)-labeled second antibody or biorecognition molecule is used as a reporter that binds to the target microbe. When the laser (635 nm) is applied, light travels through the optical waveguide, undergoes total internal reflection (TIR) and the fluorophore on reporter molecule bound to analyte is excited, generating an evanescent wave. The signal propagates back to the fiber and is detected in real time by a fluorometer. A commercial fiber optic sensor was developed by Research International (Monroe, WA) and marketed as Analyte-2000. Later, a portable semi-automated system called RAPTOR™ with microfluidic set-up was built and has been used for detection of foodborne pathogens and biothreat agents including *Bacillus anthracis*, *Francisella tularensis*, *L. monocytogenes*, *E. coli* O157:H7, *Salmonella*,

Vaccinia virus, as well as staphylococcal and botulinum toxins from various food matrices. Overall reported detection limits for fiber optic sensors for most foodborne pathogens are in the range of 10^3–10^4 cfu/ml; however, employing improved biorecognition molecules, the detection of pathogens at 10–100 cells/ml is possible.

25.6 Light scattering sensor

Light scattering uses a photon source, ranges from ultraviolet (10 nm–390 nm) to infrared (750 nm–1 mm), as an interrogating agent for the specimen under test. The source of the photon to be used largely depends on the physical characteristics of the sample, since light scattering has been widely used in the semiconductor industry, atmospheric science, astrophysical science, and biology. When incident light interacts with the sample, the optical properties such as intensity, polarization, and phase are modified. There are two types of light scattering phenomena: (i) elastic or (ii) inelastic. In elastic scattering, the incident photon wavelength and secondary scattered wavelength are the same, while in inelastic scattering, a frequency shift occurs between the incident and scattered photon, yielding a net energy exchange in the scattering process. In general, elastic scattering produces stronger signals while the inelastic scattering produces weaker signal (for example, Raman scattering) but has more information on the material composition.

A novel elastic scattering system, BARDOT (BActerial Rapid Detection using Optical scattering Technology) using a diode laser (635 nm) has been used for analysis of biological samples, primarily the bacterial cells and colonies, without the need for any specific labeling reagents such as nucleic acid or antibody probes, fluorophores, etc. Hence this elastic scattering system is a label-free detection system and it does not destroy the sample integrity. This system, however, requires pure cultures or isolated colonies on solid agar surface where the integrity of the colony morphology is maintained. In this system, when a laser shines on the center of a bacterial colony growing on a petri dish, it generates a distinctive and reproducible scatter pattern that is captured by an image-capturing CCD camera. The scatter pattern is analyzed by measuring the variation in the total intensity, local intensity in a certain direction, and polarization of photons from the scattered patterns. Scatter images are stored in the image library and image analysis software based on a quantitative image processing algorithm Zernike polynomial and a support vector machine allow identification of the test organism. The scatter patterns are found to be unique for different bacteria and can be used to differentiate (identify) bacteria at the genus, species and even serovar level. Furthermore, the versatile discriminatory power of BARDOT is possible due to the availability of various selective and differential solid agar media for different microorganisms. BARDOT has been extensively evaluated for its ability to detect or identify foodborne pathogens: *Listeria monocytogenes*, *Salmonella enterica*, *Vibrio* spp., and *Escherichia coli* from food. Potential methods of applying BARDOT to identify *Campylobacter*, *Staphylococcus*, *Streptococcus*, and *Bacillus* from food are in progress.

The BARDOT-based detection approach fits well with the culture-based detection method, allowing rapid detection of most pathogens with an initial contamination level of 1 cfu/25 g under 24 h starting with the food sample. The major advantage of BARDOT over other systems is that it can screen multiple colonies in a plate in one run (scanning of a petri dish may take 1–2 min depending on the number of colonies to be scanned) and preserve the colony integrity for other use. Since BARDOT can generate phenotypic fingerprint patterns of colonies on the petri plate, it also can be used to monitor food hygiene, effectiveness of sanitizers

on food processing equipment, and to study bacterial community. Commercially-available BARDOT (Advanced Bioimaging Systems, West Lafayette, IN) is built with an incubator that can hold over 700 petri dishes, so that the system can be programmed to scan plates periodically to look for specific pathogens.

25.7 Flow cytometry

The Flow-Cytometer is another device that utilizes the basic principle of elastic and inelastic scattering to detect microorganisms in liquid suspension. However, this set-up requires labeling of cells with antibody and/or fluorophor molecules prior to detection to provide specificity. When the sample is transported through a capillary tube, incident light from a laser creates the distinctive scattering signature of the cells labeled with fluorophor. It has been used for pathogen detection from various food matrices including milk and beverages.

25.8 Fourier Transform Infrared spectroscopy and Raman

Fourier Transform Infrared spectroscopy (FTIR) measures the spectral response of the sample to incident light in the infrared (mid IR: wave numbers 4000–400 cm^{-1} and near IR: 14000–4000 cm^{-1}),while Raman scattering occurs near IR range. These are non-destructive chemical imaging systems which generate spectral responses depending on the chemical composition of the sample under investigation. For example, bacterial cell components or substructures like proteins, polysaccharides, and membrane structures are used as targets to identify and classify microorganisms. Each test sample possesses a unique molecular weight and structure and these exhibit rotational vibrations at different infrared wave numbers, thus creating unique absorption spectra for FTIR and scattering patterns for Raman.

The FTIR consists of a light source and the detector, and it enables simultaneous measurement of all spectral responses from the interference signal and the Fourier transform technique. The unique spectrum is digitized and recorded for further investigation. The spectra generated from microbial samples generally appear very similar and the variation between the peaks and valleys is somewhat smaller. Therefore, chemometrics methods such as Principal Component Analysis (PC), Linear Discriminant Analysis (LDA), Hierarchical Cluster Analysis (HCA), Artificial Neural Network (ANN), and Canonical Variate Analysis (CVA) are used to increase discrimination and to classify the test samples. FTIR has been applied to identify and classify *Bacillus* spp., *Staphylococcus aureus*, *E. coli* including *E. coli* O157:H7, *Pseudomonas*, *Salmonella* and *Listeria* spp. including *Listeria monocytogenes*. The limit of detection of the FTIR varies depending on the species and experimental conditions employed. Most of the reported results were performed with a cell concentration of 10^8–10^9 cfu/ml to ensure positive signal with relatively low background noise. However, some researchers were able to detect a minimum cell concentration of 10^3 cfu/ml.

Raman spectroscopy uses a diode laser (785 nm) which, upon interaction with microbial cells, generates Raman scatter termed as inelastic scattering that is used for detection. Typically, Raman signal is very weak, and to amplify the signal, surface-enhanced Raman spectroscopy (SERS) has been configured using biorecognition molecules conjugated to gold or silver nanoparticles to specifically bind to target microorganisms or toxin molecules. Raman has been extensively investigated for its suitability to detect biothreat agents including *Bacillus anthracis*, *Yersinia*

pestis, *Burkholderia mallei*, *Francisella tularensis*, *Brucella abortus* and foodborne pathogens: *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella*.

25.9 Impedance-based biosensor

Changes in electrical properties due to molecule–molecule interaction could be used as a sensor to detect pathogens. In impedance biosensor, electrical impedance can happen two ways: (i) binding of microorganisms to biorecognition molecule immobilized on interdigitated electrode surface, and (ii) metabolic activity of the microorganisms to convert inert substrates such as glucose into electrically conductive by-products such as ionic molecules and acids. In the impedance-based sensor, absolute changes in conductance, impedance, or capacitance are measured at regular intervals. The specificity of the sensor arises from the biorecognition molecule used. In the former configuration, if only the binding event is assessed, the system cannot differentiate live from dead cells. However, if those bound cells are allowed to grow (if viable), they can generate electrically-charged molecules to change the conductivity of the media. The ability of this sensor to detect live cells is critical since live pathogens can only cause disease.

Commercial impedance-based systems that are used for quality assurance purposes include Bactometer® (bioMérieux, Marcy l'Etoile, France); Malthus AT Analyzer (Malthus Instruments, Bury, UK), BacTrac™ and μ-Trac microorganism growth analyzer (SyLab, Purkersdorf-Vienna, Austria) which can provide results in as early as 6–24 h. These instruments are used to monitor bacterial load and contamination levels in food or food contact surfaces or food processing facilities, providing early warning about possible microbial contamination. Usually, these systems require a high level of bacterial cells to exhibit a measurable signal. Also they lack selectivity or specificity.

Employing the same principle, a miniature microfluidic chip fabricated with interdigitated electrode arrays has been developed for detection of low levels of bacterial contamination. As indicated above, biorecognition molecules such as antibody, bacteriophage, or receptor molecules are immobilized on the electrode surface to provide specificity. Often dielectrophoresis (DEP) is used to concentrate cells on a specified site and to bring them in close proximity to immobilized biorecognition molecules. A washing step removes undesirable microbes and molecules. Application of a low conductance growth medium would promote bacterial growth onchip and their resulting metabolic action is detected by monitoring conductivity, capacitance or impedance. In recent years, microfluidic biochips are also developed to perform PCR onchip to specifically amplify the target gene of interest for detection of *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella*.

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26 Stress adaptation, survival and recovery of foodborne pathogens

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26.1 Introduction

Bacteria are ubiquitous and can be found in every conceivable environment, ranging from the foods we eat and the facilities in which these foods are prepared to every corner in nature including the highest mountains and the deepest oceans. According to one estimate, our planet is home to 5×10^{30} bacteria. In terms of the food supply, microorganisms play important roles in food fermentation (e.g., cheese, sausage), food spoilage and disease in both plants and animals, with each of these environments having a profound impact on the physiological state of bacteria. Bacterial growth and survival are dictated by the intrinsic and extrinsic factors associated with the food and the organisms' surroundings. Intrinsic properties are those that are inherent to the food or other material on or in which bacteria are found (e.g., manure, a sponge, knife blade, sink) and include the pH, nutrient content and water activity. In contrast, extrinsic parameters are associated with the external environment and include, most importantly, temperature and relative humidity with the latter directly impacting water activity which can be defined as the amount of water available to a microorganism. After ingestion, bacteria are exposed to various digestive enzymes, the highly acidic conditions of the stomach and the gastrointestinal tract which is well-colonized by a highly diverse natural microflora. Hence, bacterial survival also depends on the ability of the organism to adapt to these changing environments.

When a bacterial cell is exposed to heat, cold, acid, changes in osmotic pressure, chemical sanitizers, food preservatives or irradiation, a portion of the population will become injured to varying degrees depending on the physiological state of the individual cell (Fig. 26.1). These sublethally injured cells can enter a viable-but-nonculturable state characterized by very low metabolic activity and an inability to divide, adapt to their new surroundings, recover from the sublethal stress and regain full cell function by repairing the cellular damage that has occurred, or succumb to the injury and die.

The ability of a bacterial cell to recover from a stress depends on the severity of the exposure with the level of stress progressing from mild to moderate, severe, extreme and lethal.

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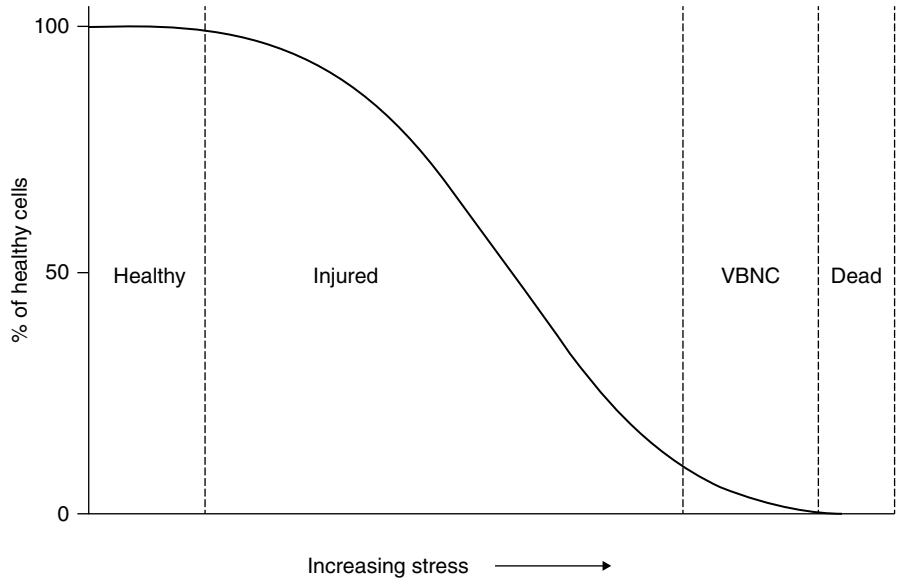

Figure 26.1 Impact of increasing stress on bacteria viability.

Following minor stress, bacterial cells will readily adapt to their new environment with the growth rate not affected. This period of adaptation is akin to the lag phase seen when a frozen bacterial stock culture is transferred to a nonselective laboratory medium and incubated under ideal conditions. Under these same conditions, a higher level of stress will result in a longer lag phase accompanied by a series of temporary physiological changes that may lead to increased stress tolerance – a condition known as transient adaptation. Exposure to moderate stress will yield a varied response that includes both healthy and dead cells along with those exhibiting various degrees of injury. Exposure to a lethal stress will typically kill the majority of the population; however, some survivors may persist due to adaptive gene mutations. Unfortunately, the relationship between the level of stress, level of injury and extent of adaptation is not yet fully understood. Hence, sublethal injury is best defined as any degree of injury short of death with such cells frequently exhibiting some degree of stress adaptation. Two types of injury are recognized – metabolic and structural. Metabolic injury is characterized by damage to proteins, enzymes and DNA responsible for normal cellular functions; whereas structural injury involves damage to the cell wall and membrane.

26.2 Types of stress and stress-induced injury

The term 'stress' refers to agents or treatments causing bacterial injury or cell death. Bacterial stress induced by adverse changes in the environment or exposure to different food processing methods can damage many structural and functional components of the cell including the cell wall, cytoplasmic membrane, ribosomes, DNA, RNA, and many enzymes including those of the tricarboxylic acid cycle (Table 26.1). Stresses are generally categorized as chemical, physical or nutritional and can occur at any stage within the farm-to-fork continuum. Although typically perceived as a rich environment for bacteria, many foods are formulated to contain one or more microbial growth inhibitors which is the basis of the

Table 26.1 Sites of Cellular Injury after Exposure to Various Forms of Sublethal Stresses

Sublethal stress	Cell wall	Cell membrane	Proteins	Ribosomes	DNA
Freezing	–	+	–	+	+
Drying	+	+	–	+	+
Freeze-drying	+	+	–	+	+
Heating	–	+	+	+	+
Gamma irradiation	+	+	+	–	+
Osmotic pressure	+	+	–	–	–
Starvation	–	–	–	+	–
High hydrostatic pressure	–	+	+	–	–
High intensity pulsed light	–	+	+	–	+

'hurdle concept' for microbial inhibition. Various extrinsic factors such as the temperature and atmosphere within the package can also be manipulated to maximize both end-product safety and shelf life.

Chemical stress can result from exposure to acid and alkaline pH as well as a wide range of preservatives and chemical sanitizers. Low pH stress is associated with DNA and RNA damage, disruption of protein synthesis, alterations in the protein profile of the cell membrane, and interference with the proton motive force which is essential for the generation of ATP. Other chemical stresses including alkalinity and chlorination can lead to changes in cell membrane permeability.

Acute acid shock and gradual acid stress are both encountered at a low pH when H^+ ions cross the bacterial cell membrane. This can also occur when organic acids diffuse across the cell membrane and lower the internal pH of the cell upon dissociation. A number of common food manufacturing/preservation methods, including the fermentations used in the production of cheese and sausage and the addition of acidifying agents and preservatives to processed foods, can also lead to acid stress. Organic acid concentrations mimicking those recommended as an intervention strategy for beef carcass decontamination have been shown to cause sublethal injury in *Salmonella* Typhimurium,

Alkaline stress can occur at pH values above neutrality. Many detergents and chemical sanitizers such as caustic soda (NaOH) and ammonium compounds are routinely used on both food- and non-food-contact surfaces in commercial food processing facilities. This is particularly true in the meat industry where highly alkaline cleaners are used to remove heavy fat and protein deposits. Floors, pipes and drains where *Listeria monocytogenes* frequently resides are also frequently exposed to alkaline-based detergents and chemical sanitizers.

Physical stresses include high and low temperatures, drying, osmotic pressure, high pressure and radiation among others. Temperature and osmotic stress are most frequently encountered during food processing and storage. Damage to and modifications of the cell membrane are associated with almost all forms of physical stress. Other types of damage include decreased control of cell membrane permeability and fluidity (low temperatures), inactivation of enzymes and disruption of the active transport of cations, sugars and amino acids (heating), ribosome and ribosomal RNA degradation (heating), and DNA damage (heating, freezing/thawing, drying).

Sensitivity of bacteria to low temperatures varies widely and is based on population density, growth temperature, cooling rate and the temperature range over which cooling occurs. Microorganisms inhabiting foods subjected to pre- and/or post-processing storage

are susceptible to cold shock. This cold shock response is divided into three stages: initial cessation of growth, resumption of growth after an adaptive period, and changes in protein synthesis. It is this cold shock and subsequent adaptation that reportedly allows *Salmonella* to survive during cold storage at 5 °C for up to 8 months.

Heat shock occurs when organisms are exposed to temperatures above their normal growth range. These temperatures may be lethal to a fraction of the bacterial population depending on their growth phase and thermal sensitivity. Conditions within both pre-processing and processing environments may induce heat shock or stimulate a heat shock response. This includes exposure to hot acid sprays used when processing animal carcasses as well as gradual heating methods that extend the come-up time such as low-temperature pasteurization of eggs, slow roasting of meat products or sous-vide processes, and the use of warming trays for meats.

Osmotic stress is intimately linked to water activity which is the amount of unbound water available for chemical activity and microbial growth. Osmotic stress can occur when shifts in external osmolarity cause water to flow either into or out of a bacterial cell. Under extreme conditions, this leads to physical damage of the cell. Changes in osmolarity can also result from the addition of various solutes including sugars, NaCl and phosphates and readily occurs during freezing, drying and rapid rehydration of foods.

Nutritional stress occurs in environments with low or no available nutrients to support biochemical metabolic activity or microbial growth. Natural environments typically have growth-limiting levels of nutrients and rapidly-changing nutrient availability. Starvation stress can occur on animal carcasses, in food, on equipment surfaces, walls, floors and in water. Microorganisms in nutritionally-deficient environments likely integrate cell density and starvation stress signals to induce cell surface modifications for the utilization of alternative energy sources. Other physiological changes include decreased membrane fluidity, loss of ribosomes and increased protein turnover. Sublethal stress can induce various morphological changes in *Salmonella*, *E. coli* O157:H7 and *Listeria* with these bacterial cells ranging in shape from round to elongated or filamentous. Such transformations, particularly those related to cellular morphology and cell surface components, also enhance bacterial adherence and may contribute to biofilm formation.

26.3 Cellular repair

The phenomenon of one type of stress response imparting protection to cells exposed to higher levels of the same stress (especially for heat) or a different stress is widely documented and referred to as ‘cross-protection’ This response also has been described as ‘stress hardening’, whereby a bacterium previously exposed to a sublethal stress is more likely to become adapted or hardened upon exposure to a subsequent stress.

Many bacterial foodborne pathogens develop some degree of thermotolerance in response to mild temperature shock. For example, heat shocking *E. coli* O157:H7 at 42 °C for 5 minutes before thermal inactivation at 55 °C was shown to increase the D-value more than two-fold. When inoculated into a non-selective laboratory medium, liquid whole egg, or 10 or 40% reconstituted dry milk, *S*. Thompson was more thermotolerant at 54 and 60 °C if held at 48 °C for 30 minutes before treatment. The impact of increased thermal tolerance on food safety becomes apparent when considering the current USDA food safety regulations pasteurization of liquid eggs at 63.3 °C for 3.5 minutes. Recent research has shown that a mixture of heat resistant strains of *S*. Enteritidis and *S*. Oranienberg will only experience

a 2.7 log reduction under these conditions and that a higher temperature of 67.4 °C for 3.5 minutes is required to achieve a 5 log reduction. These same concerns also exist for slow roasting of blade-tenderized or marinated meat and poultry products that may contain internalized pathogens.

The thermotolerance response results from the synthesis of stress proteins. Since identification of the first heat shock proteins (HSPs) in 1974, virtually every organism studied has been shown to respond to a moderate temperature shock by increased production of such proteins. Production of these stress-induced proteins increases under conditions that repress the synthesis of most other cellular proteins with these proteins playing a functional role in cellular adaptation to growth/survival limiting conditions and recovery from stress-induced damage.

Bacterial responses to stress can be general or specific. Some proteins associated with the stress response have clear functions for managing specific stresses while others play a role in general protection under multiple stress conditions. Proteins identified as HSPs can also be produced following exposure to various types of nonthermal stress including starvation, exposure to ethanol and other organic solvents, oxidative agents and high salt concentrations. Both the general stress response and several specific stress responses are adaptive responses that allow bacteria to survive, and in some cases multiply, under stressful conditions. They do so by facilitating cellular repair through specific biochemical events and metabolic processes that will differ based on the type and degree of stress. Repair of the cell membrane through lipid synthesis must occur relatively rapidly so that cells can fully repair from stress-induced lesions. Other responses to cellular damage include the synthesis of ATP, DNA and RNA, accumulation of protective intracellular compounds and re-organization of existing macromolecules (Table 26.2).

In Gram-negative bacteria, the alternative sigma subunit of RNA polymerase known as RpoS controls the expression of more than 50 genes in response to changes in temperature, pH, osmolarity and nutrient availability among others. The types of proteins produced by

Table 26.2 Bacterial Injury, Site of Injury and Repair Mechanism after Sublethal Heating, Drying, Freezing and Acidification

	Sublethal exposure			
	Heating	**Freezing**	**Drying**	**Acidification**
Evidence for sublethal injury				
Leakage of cell materials	+	+	+	–
Loss of resistance to selective agents	+	+	+	+
Selected activation of enzymes	+	+	+	+
Site of damage				
Cell wall components	+	+	+	–
Cell membrane	+	+	+	–
Ribosomes and rRNA	+	+	+	+/–
Structural DNA	+	+	+	+
Repair mechanism				
rRNA	+	+	+	+/–
DNA	–	–	–	–
Protein synthesis	–	+	+	+
ATP synthesis	+	+	+	+

bacterial foodborne pathogens, including *Salmonella*, *Listeria*, *E. coli* O157:H7 and *Vibrio* spp., in response to these various stresses have different functions. HSPs act as molecular chaperones that help cells survive by re-folding proteins, denaturing improperly assembled proteins, degrading irreparably denatured proteins, repairing DNA for cell replication, modifying cellular morphology and accumulating osmolytes to maintain or enhance protein stability. Less studied than HSPs, cold shock proteins (CSPs) are critical for maintaining membrane fluidity, sugar uptake, chemotaxis, DNA recombination, transcription and translation, and protein folding. Acid shock proteins produced by *L. monocytogenes*, *E. coli* and *Shigella* upon exposure to acidic conditions help these pathogens survive in this otherwise lethal environment with the acid tolerance response in *E. coli* O157:H7 now well documented. In response to oxidative stress, specific proteins (e.g., catalase, superoxide dismutase, exonucleases and glycosylases) are produced to prevent and repair damage to the cell and the cell's DNA. Starvation proteins produced in response to nutrient depletion enhance long-term survival by stabilizing ribosomes against degradation, transforming the shape of the cell and fostering the utilization of alternative substrates for growth.

26.4 Cross-protection

Survival under adverse environments is partially attributable to the organism's innate means of protection from destructive conditions or treatments. Bacterial cells can adapt or acquire resistance to different conditions by modifying metabolic activities, adjusting nutrient utilization patterns or by using enzymes that were previously present in a recessive role. These stress proteins, particularly those produced during heat shock and starvation, often play multiple roles in response to stress and cellular repair. Increased thermotolerance is the most characteristic physiological response of a microorganism following mild temperature shock. However, increased resistance to heat can also be induced in *E. coli* O157:H7, *Salmonella*, *L. monocytogenes* and *Enterococcus faecalis* after nutrient deprivation as well as acid and alkaline shock (Table 26.3). The phenomenon of thermotolerance is seen in both laboratory media and a wide range of food matrices. In one study, acid adaptation of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* at pH 5 led to increased thermal resistance when inoculated samples of apple, orange and white grape juice at pH 3.9 were processed at 56, 58 and 60 °C.

Table 26.3 Sublethal Exposures that Enhance the Thermotolerance of *E. coli* O157:H7, S. Typhimurium and *L. monocytogenes*

Pathogen	Sublethal exposure
E. coli O157:H7	Starvation in distilled water for 24 h at 37 °C
	Starvation in 0.85% NaCl (pH 6.6) for 48 h at 37 °C
	Acid adaptation (pH 4.8–4.9) in trypticase soy broth for 18 h at 37 °C
S. Typhimurium	Starvation in a minimal medium for 10 h at 37 °C
	Acid adaptation (pH 5.8) at 37 °C
L. monocytogenes	Adaptation in tryptose phosphate broth (pH 12.0) for 45 min at 37 °C
	Exposure to 4–8% ethanol for 1 h at 35 °C
	Starvation in 0.1M phosphate buffer (pH 7) for up to 163 h at 30 °C
	Exposure to 500 ppm H_2O_2 for 1 h at 35 °C
	Acid adaptation in trypticase soy broth (pH 4.5) for 1 h at 35 °C
	Acid shock (pH 4) in trypticase soy broth for 1 h at 23 °C

Many sublethal treatments can cross-protect against stresses other than heat. However, the ability for cross-protection following any given stress response will vary based on the specific bacterial species/strain and the magnitude and nature of the stress pretreatment. Starvation has been shown to increase freeze-thaw resistance of *Vibrio vulnificus* and *E. coli* O157:H7. Extended periods of starvation such as might be encountered on dry surfaces in food processing facilities have been shown to decrease the effectiveness of chlorine – one of the most commonly used disinfectants in the food industry against *E. coli* O157:H7. Species of *Pseudomonas* have also exhibited enhanced survival following exposure to different stresses with heat shock protecting *P. aeruginosa* against the antibiotic biapenem and starvation stress protecting *P. putida* against ethanol, heat shock and changes in osmotic pressure. Adaptation of *S.* Typhimurium to pH 5.8 reportedly increased the organism's tolerance to osmotic (salt) stress with acid, ethanol and NaCl resistance similarly increasing after adaptation of *L. monocytogenes* to 5% ethanol.

26.5 Virulence

The presence of injured microorganisms in food poses significant public health concerns. Injured cells may initially go undetected during routine quality control checks and at critical control points during manufacturing. However, repair from sublethal injury in a food may allow for growth, ensuing spoilage and the production of toxins and other virulence factors. Thus, a bacterium's pathogenicity or virulence may be considered the end result of its ability to recover from injury.

Expression of many virulence factors depends on environmental cues with many stresses intrinsic to the host's defense system being similar to those encountered in the natural environment. The induction of virulence genes is part of the adaptive response to various stresses encountered in the host. Pathogenic microorganisms, therefore, may see exposure to stress in both natural environments and food processing facilities as a signal for the expression of virulence factors. In *Salmonella*, different virulence proteins are expressed based on the type of stress exposure, including glucose starvation, low pH, elevated temperature and iron limitation. The virulence factors produced in response to stress help to facilitate rapid growth of the pathogen in host cells, systemic spread and infection of non-intestinal tissues. In one such study, *S.* Enteritidis was more virulent for mice and more invasive for chickens following sublethal exposure to acid and heat.

Environmental stresses imposed on bacterial pathogens by host defense mechanisms include changes in temperature, acidity and oxygen availability. As pathogens traverse from the natural environment through contaminated food, water or insect vectors into mammalian hosts, the sudden increase in temperature triggers a heat shock-like response that intensifies when host defense mechanisms such as fever are encountered. HSP synthesis acts to protect pathogens from various host defense mechanisms including macrophages. Development of acid tolerance from exposure to a low pH can enhance virulence by facilitating survival in the stomach, thereby decreasing the oral infectious dose. Increased acid tolerance of *E. coli* O157:H7 in acidic foods (e.g., apple cider, cheese) leading to improved survival is a major contributing factor to the low infective doses seen in foodborne outbreaks. Limited availability of oxygen in the small intestine leading to anaerobic stress can also enhance the virulence of pathogens in the gastrointestinal tract with *Salmonella* being more invasive when grown anaerobically than aerobically. Similarly, osmotic stress encountered in a host can lead to enhanced virulence of *Salmonella*, *Shigella* and *Vibrio*.

The preceding discussion indicates that alterations in cellular physiology, including the synthesis of stress proteins in response to adverse environmental stresses, strongly impact both survival and virulence. A bacterium's ability to overcome a particular environmental stress partially determines its virulence, since the response to such stress typically includes both the expression and up or down regulation of various virulence factors for survival.

26.6 Recovery and detection

Sublethal injury sustained by bacteria as a result of stress exposure increases permeability of the cytoplasmic membrane, thereby increasing susceptibility to the various selective agents used in laboratory media to suppress the growth of competing background organisms. However, when placed in a non-stressful environment, these injured cells can repair the sublethal damage which most often includes renewed synthesis ribosomal RNA, ATP and cell wall/cell membrane constituents. This ability to regain the lost cell functions resulting from injury is known as 'resuscitation' with these fully repaired cells now able to grow and divide normally.

Given the ability of sublethally injured bacterial foodborne pathogens to regain their virulence following repair, recovery of both injured and healthy cells must be considered when examining foods likely to contain stressed or injured cells (acidic, dry or processed foods) or samples collected from the food processing environment where microorganisms may be exposed to desiccation or chemical sanitizers. In the laboratory, sublethally injured cells can be resuscitated using nonselective broth and agar media. However, these media are unable to differentiate the target organism from the background microflora. After repair is complete, various selective broth- and agar-based media can be used to recover these now resuscitated healthy cells (Table 26.4). By definition, injured cells are only capable of growth and colony formation in or on nonselective media; whereas healthy cells can grow in both the presence and absence of selective agents. Hence, the percentage of injured cells in a population can be calculated as follows:

$$\%\,\text{injury} = \frac{\text{Population on selective media} - \text{Population on nonselective media}}{\text{Population on nonselective media}} \times 100$$

In microbial challenge studies, attaining a high percentage of injury (e.g., >90%) after exposing a bacterial population to a given stress in the laboratory is critical when assessing the fate of injured cells in foods and their response to subsequent stresses associated with thermal and nonthermal processing.

Table 26.4 Bacterial Growth on Different Media According to the Extent of Cell Injury

	Type of media		
Extent of injury	**Selective**	**Nonselective**	**Repair**
Healthy	+	+	+
Sublethally injured	–	+	+
Viable-but-nonculturable	–	–	–
Dead	–	–	–

The two most widely-used microbial recovery methods for foodborne pathogens – enrichment for determining the presence/absence of a particular target organism and direct plating for quantifying the numbers of the target organism present in the sample, typically employ various selective agents including antibiotics, acids, dyes, and surface-active agents that inhibit both the growth of competing background microflora and the repair and recovery of injured cells. Hence, several strategies using nonselective liquid media prior to selective enrichment and/or selective plating have been developed to facilitate the repair of injured cells which for mesophilic bacteria typically occurs within 1 to 5 h of incubation at 25 to 37 °C (Fig. 26.2). During this short incubation period, hydrogen peroxide produced by other microorganisms as a result of respiration is highly toxic to injured cells due to decreased catalase and superoxide dismutase activity. Consequently, nonselective repair media are often supplemented with catalase, pyruvate, 3,3´-thiodipropionic acid, or Oxyrase® (a commercial preparation of partially purified membrane fragments from *E. coli*) to neutralize the toxic effect of hydrogen peroxide. Including Tween 80 (a lipid and surfactant) and magnesium chloride is also beneficial for the repair of the cell membrane and ribosomes. Despite the best recovery efforts, many bacteria, including *Salmonella*, *Listeria*, *Campylobacter*, *E. coli* and *Vibrio* can enter a viable-but-nonculturable state with these difficult-to-resuscitate, morphologically smaller, less metabolically active cells able to persist in a semi-dormant state for prolonged periods of time. However, following repair, these formerly viable-but-nonculturable cells, will become fully functional and regain their pathogenicity, making their detection of major importance.

In enrichment protocols that include a repair step, the food or environmental sample is homogenized in a nonselective broth medium with this sample homogenate then incubated

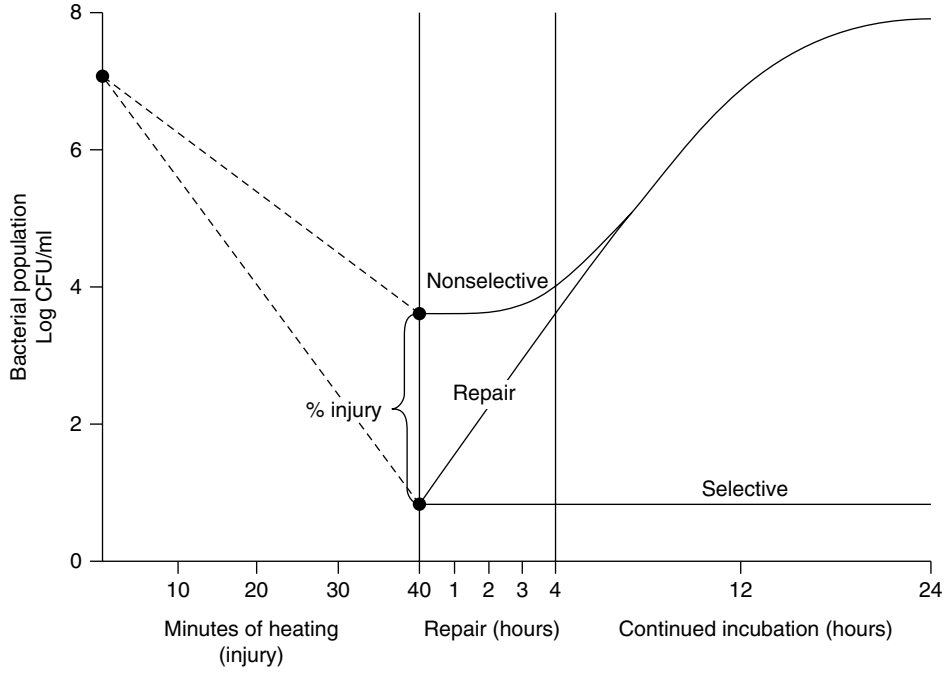

Figure 26.2 Impact of sublethal injury on resuscitation and growth of bacteria on non selective and selective media following repair.

for a period of time to allow the resuscitation of sublethally injured cells. The nonselective enrichment broth of choice is based on both the target foodborne pathogen to be detected and the type of sample being analyzed. As examples, using the standard protocol for *Salmonella* developed by the FDA, food samples are enriched for 24 h at 37 °C in trypticase soy broth, lactose broth, nutrient broth or universal enrichment broth, depending on the characteristics of the sample to be examined, prior to secondary enrichment in a selective broth or plating on one or more selective media. The FDA protocol for *Listeria* takes a slightly different approach in that food sample homogenates in Buffered Listeria Enrichment Broth containing sodium pyruvate are incubated at 30 °C for 4 h to resuscitate any sublethally injured *Listeria*, after which acriflavin, nalidixic acid and cycloheximide are added as selective agents with incubation at 30 °C then continued for an additional 44 h before plating on *Listeria* selective media. However, none of these enrichment methods can be used for enumeration since, in addition to the resuscitation injured cells, any healthy cells will also grow. Overgrowth of the target pathogen by background microorganisms can also complicate detection and recovery.

Determining the population of both healthy and sublethally injured cells in a sample is most commonly accomplished using a combination of selective and nonselective plating media in what is known as the 'agar overlay method'. When repair is to be accomplished on solid media, the homogenized sample is normally surface-plated on a nonselective media such as trypticase soy agar with the plate then incubated at 25–37 °C for 1–4 h to facilitate repair. Following this initial period of incubation, the plate is overlaid with an appropriate selective/differential agar medium specific to the target organism and re-incubated. During this second incubation period, the selective agents in the top layer will diffuse into the nonselective plating medium on the bottom, creating a selective growth environment throughout the entire plate. As a result of previous repair, these now healthy cells are no longer inhibited by the selective agents and will grow in this selective environment to form colonies characteristic of the target organism.

Several variations of this technique, termed the 'agar overlay method', have been described including the pour-overlay, surface-overlay, thin agar layer, and four compartment thin agar layer methods. In the pour-plate method, a nonselective agar medium such as trypticase soy agar or plate count agar is poured into a petri plate containing the injured target organism in the diluted sample. After solidification of the agar, the plate is incubated for 1–4 h to facilitate repair, overlaid with an appropriate selective medium and re-incubated at the desired temperature with the colonies developing beneath the agar surface. This method is most often used when low numbers of the target organism (e.g., <100 CFU/g or ml) are expected. In the surface-overlay method, the injured cells are spread-plated on a nonselective media for resuscitation during 2–4 h of incubation at 25–37 °C, after which the plate is overlaid with the selective medium of choice and re-incubated. This method, which is best suited when higher numbers of the target organism are expected (e.g., >100 CFU/g or ml) tends to yield greater recovery than the pour-plate method since sublethally injured cells are not exposed to the elevated temperature of the molten agar as in the pour-plate method.

The pour- and surface-overlay techniques just described are two-step methods that require initial plating of the sample followed by an agar overlay 1–4 h later. Using the thin agar layer method, the plating process is reduced to a single step by pouring a thin layer of the nonselective medium on top of a thicker solidified layer of the selective medium just prior to inoculation. Although more awkward, the two media can also be poured separately and later combined. After surface inoculating the nonselective agar medium for repair of injured cells during the initial hours of incubation, this layer can be removed from the petri plate and

placed on top of a selective agar to later differentiate the target organism for any competing background flora. A major advantage of these thin agar layer methods is that colonies of the target organism exhibiting typical morphologies and color reactions develop on the agar surface rather than underneath as when using the pour- and surface-overlay methods, thus greatly aiding their differentiation from the background flora. This method is preferred for the recovery of heat-, acid- and cold-injured *E. coli* O157:H7, *L. monocytogenes*, *Salmonella*, *Staphylococcus aureus*, *Campylobacter*, *Yersinia enterocolitica* and *Vibrio spp*. A modification of the thin layer technique – the four compartment thin agar layer method which uses a compartmentalized petri plate containing four different selective media overlayed with the nonselective medium, is a more efficient means for recovering different sublethally injured foodborne pathogens from the same sample.

One last alternative to the agar overlay method, namely the membrane-based support method, has also been developed for recovery of sublethally injured cells. In this method, the sample is filtered through a 0.45 μm membrane which is then placed in a petri plate containing a nonselective agar or filter pad saturated with a nonselective broth medium. After 4 h of incubation for cellular repair, this membrane is then transferred to a second petri plate containing a selective medium and re-incubated. Standard 42 mm diameter membranes and square hydrophobic grid membrane filters are both suitable with later membranes yielding 'square colonies' and a 'most probable number' count as opposed to a direct count. This membrane-based support method, which is best suited for liquids and other easily filtered samples that are suspected of containing very low numbers of the target pathogen, offers the same advantage of the thin agar layer method in that injured cells are not exposed to warm agar during overlaying.

26.7 Conclusion

Microbial contamination of foods with bacterial pathogens continues to be an ongoing concern with many thermal and nonthermal pathogen reduction strategies having been widely adopted to better ensure end product safety. When exposed to heat, acid, sanitizers, chemical food preservatives, irradiation and other forms of stress, the physiological state of the microbial population will change with different portions being killed, injured to various degrees or unaffected based on both the type of stress and length of exposure. Increased resistance of these injured cells to the same stress or other stresses – the phenomenon known as cross-protection – poses a major threat to the safety of the food supply due to their ability to undergo repair and regain their virulence. Recovery of these sublethally injured cells is based on their repair in a nonselective medium followed by growth in a selective environment with different protocols having been developed based on enrichment and direct plating. Given their increased resistance, detection of injured foodborne pathogens is an important concern when designing and conducting microbial challenge studies.

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27 Microbial biofilms and food safety

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27.1 Introduction

Biofilms are formed by almost every type of microorganism under suitable conditions. Biofilm food-associated organisms include food spoilage microorganisms, such as *Pseudomonas* sp. and thermophilic sporeformers, and pathogens, including the genera of *Bacillus*, *Cronobacter*, *Campylobacter*, *Vibrio*, *Listeria*, *Escherichia* and *Salmonella*. Simplistically, biofilms are microorganisms growing on a solid surface. However, biofilms are generally defined as matrix-enclosed bacterial populations that adhere to a surface and/or to each other, producing a dynamic environment in which the component microbial cells appear to reach homeostasis, optimally organized to make use of all available nutrients.

Throughout natural ecosystems, biofilms can be found on almost any surface with a high enough level of moisture to support growth. Interfaces where biofilms may grow in food processing environments include solid/liquid, gas/liquid or, in the case of solid foods, at the gas/solid interfaces. Over the past 15 or more years, researchers have realized that bacteria growing on surfaces, either alone or in a community containing a diversity of different organisms, have a greater resistance to a large variety of environmental stresses. Thus, the biofilm physiology and organization enable organisms to survive within the food processing environment. In order to control the threat of environmental bacterial contamination,-cleaning and sanitization of this environment is indispensable to assure safety of all commercially-produced foods.

27.2 Characteristics of biofilms

When growing in a biofilm, bacteria are known to have a different growth rate and physiology than their planktonic (free growing broth cultures) counterparts and may exhibit varied physiological responses to nutrient conditions. Although gases and liquid nutrients are transported to and from the biofilm matrix via diffusion, studies have indicated that biofilm

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bacteria receive less oxygen and fewer nutrients than cells in suspension. Surprisingly, this leads to advantages in growth, altered physiology, and increased resistance to a variety of stress compared with their planktonic forms. Through diffusional mass transport, biophysical interactions, and cell-to-cell interactions, commensal and mutual communities of organisms survive in the low nutrient and decreased temperature conditions that are often found in food processing and storage environments. The ability to resist antimicrobial agents is of particular concern to both the medical and food processing communities, since once a biofilm has been established on a surface, it becomes exceedingly difficult to clean and sanitize.

There are several steps in the formation of bacterial biofilms: (i) transport; (ii) initial adhesion; (iii) substrate attachment; and (iv) micro-colony formation (cell-cell adhesion) leading to mature biofilms consisting of cells and a surrounding exopolymer matrix with the last step being the dissemination or disruption of the biofilm (Fig. 27.1). The first step in biofilm formation consists of the transport of the organism to a solid surface. This can occur via motility of the organism, diffusion of the organism through the environment or natural or forced convection in the system. Biofilm-forming bacteria may use all of these mechanisms at one time or another. It is well documented that flagella mutants often have lower biofilm production under static conditions, indicating that under these conditions flagella are involved in active cellular transport to surfaces and this has also been observed in listerial biofilm formation. The role of flow conditions on the attachment and growth of cells has been investigated by various authors. Contrary to expectations, greater deposition of bacteria under both laminar and turbulent flow conditions has been observed when compared to static conditions. It has been speculated that turbulent flow may thrust bacterial cells onto the surface, thus enhancing probability of adhesion and biofilm formation.

Once bacteria approach a surface, physical interaction forces are thought to influence the initial adhesion of the organisms. Typical interactions that can take place include Van der Waals interactions (>50 nm from the surface), repulsive or attractive electrostatic interactions (2–10 nm from the surface) and hydrophobic interactions (0.5–2 nm from the surface). Van der Waals forces are due to dipole–dipole, induced dipole–dipole and induced dipole–induced dipole interactions and are always attractive. Electrostatic interactions arise because the cells and the surface may carry a positive or negative charge leading to the formation of a diffuse electrostatic layer. Bacteria, as well as most natural solid surfaces, generally have an overall gross negative charge but the origin of the overall charge is due to the combination of various charges from functional groups on the membrane constituent molecules, such as amino, carboxyl, phosphate and, less commonly, sulfate groups and capsular macromolecules. Ultimately, the magnitude of the electrostatic interactions is influenced by the nature of the environment, e.g., pH, ionic strength, valency of present counterions and nature of the solvent. Hydrophobic interactions in water are much stronger than Van der Waals attraction at small separation distances, and it has been suggested that hydrophobic interactions between the cell surface and the solid substrate may be responsible for overcoming the repulsive electrostatic interactions. This strict physicochemical approach, however, should not be over-interpreted. The bacterial surface is an extremely complex entity and contains a multitude of molecules that not only carry a variety of charges but are also more or less hydrophobic. In addition, the nature and composition of bacterial surfaces can vary greatly between different species. The fact that a single bacterial strain can adhere to a variety of surfaces with differing surface energies indicates that this simplified physicochemical interaction model is most likely not entirely correct. Strategies that attempted to prevent bacterial attachment by engineering the surface to be more or less

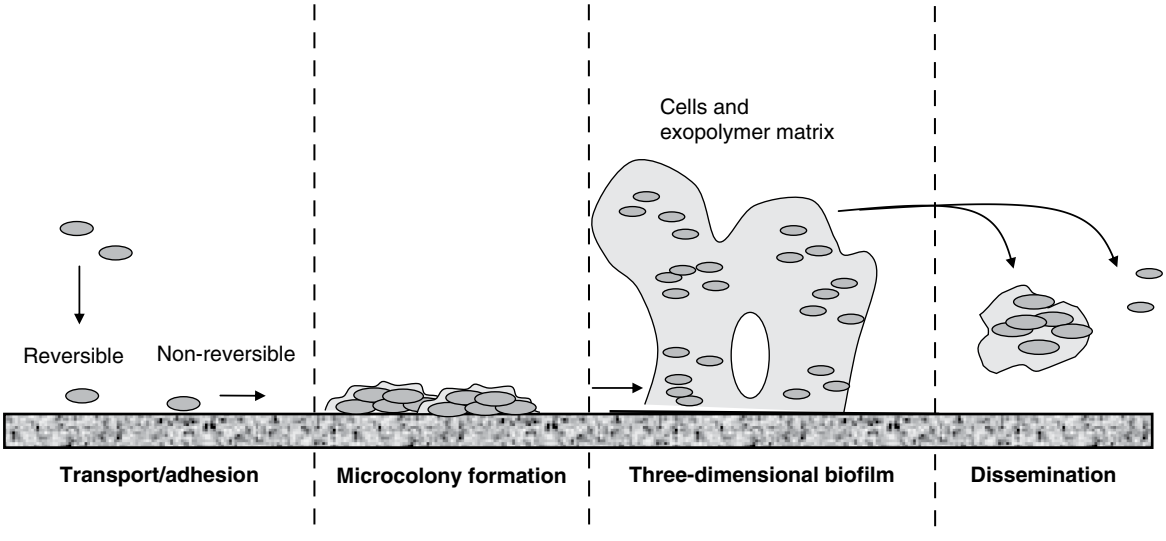

Figure 27.1 Steps in development of biofilms on solid surfaces. *From* McLandsborough et al., 2006. Biofilms; at the interface between biophysics and microbiology. Food Biophysics 1, 94–114. © Springer Science + Business Media Inc. 2006.

hydrophobic have not led to the desired results. A large variety of bacterial cells has no difficulty attaching to both hydrophobic and hydrophilic surfaces.

After the initial adhesion occurs, bacteria begin to anchor themselves to the surface by synthesizing extracellular polymeric substances (EPS) that facilitate irreversible bacterial attachment to a surface and help maintain the microcolony and biofilm structure. Interestingly, the presence of pre-adsorbed proteins on a surface prior to inoculation generally reduced the adhesion of *L. monocytogenes* regardless of the surface composition or free energy, although others have reported that the type of food soil or preconditioning film can influence the final cell density of this organism within the biofilm; therefore, there may be a difference upon initial adhesion and ultimate density of biofilm formation. EPS have been shown to enhance nutrient capture and resistance to environmental stress and antimicrobial agents. When mature, biofilms exist as a structured matrix with a network of vertical and horizontal channels to allow liquid flow to guarantee a supply of nutrients and disposal of waste products that are generated as part of the natural respiration activities of cells. The composition and structure of the extracellular polymeric matrix can vary greatly depending on the microorganism(s), their physiological status, the nutrients available, and the physical conditions present.

In the food processing environment, biofilms often are harbored in hard-to-clean areas such pitted or scratched surfaces and rough welded joints, so that initial adhesion of bacteria may actually be a function of physical entrapment of bacterial cells within rough surface topography. Once adherence occurs, if adequate nutrients and moisture are present, biofilms will eventually form. Once biofilms develop, these communities become pockets of microbial social life. There is growing scientific evidence that, within a biofilm, bacteria can communicate, interact and exchange genetic material using a variety of mechanisms which include quorum sensing (diffusible extracellular molecules which function to induce synchronized behavior of a population of bacteria), bacterial conjugation (genetic exchange), bacteriophage (viruses specific for bacteria) and outer membrane vesicles (OMV, membrane sections which can export DNA and other molecules).

27.3 Biofilm production by foodborne pathogens

27.3.1 Biofilm formation by *Listeria*

Among foodborne pathogenic bacteria, biofilm formation by *L. monocytogenes* has been studied extensively. In pure laboratory systems, *L. monocytogenes* has been observed to produce fibrils and extracellular material production when attached to a variety of surfaces. The production of extracellular polymeric substances with carbohydrate components and extracellular DNA has been observed in biofilm growth. The surface growth has been shown to have a distinct three-dimensional structure in the form a honeycomb or groups of aggregates surrounded by voids. In addition, the biofilm thickness can vary, with lower cell levels in the center of the biofilm and higher cell densities at the edges. It has been observed that the growth rate of *Listeria sp*. in biofilms was slower, required a greater levels of phosphate and produced a stringent response that was not produced by planktonicly- (or broth) grown cells. In addition, cell-to-cell communication has been shown to be involved in initial adhesion of *L. monocytogenes* to surfaces using the *agr* system (an early stationary phase quorum sensing system that was initially characterized in *Staphylococcus aureus*). This indicates that, in laboratory systems, *L. monocytogenes* has exhibited the three-benchmark biofilm

characteristics: extracellular material, three dimensional structure and altered cellular physiology, along with cell-to-cell communication which indicates multicellular coordination of a single cell organism.

27.3.2 Persistence of *L. monocytogenes* in the processing environment

With *L. monocytogenes*, almost all researchers and regulators agree that a major source of this organism is from the food processing environment. However, what is not clear is whether some strains of *L. monocytogenes* can survive better than others under the stress found in processing environments. Many studies have sampled the food processing environment and designated strains either transient (only isolated a single time) vs. persistent (isolated more than once) and tried to correlate the frequency of isolation to the genetic subtypes or lineages of the organisms. Many researchers have tried to look at the ability of the organisms to produce biofilm within the laboratory environment, along with persistence and genetic fingerprints. While there is a spectrum in the ability of pure cultures of *L. monocytogenes* to adhere and grow on surfaces under laboratory conditions, these results do not consistently correlate with genetic subtypes or persistence of isolates from the food processing environment. It has been argued that, although it was common that strains (as defined by ribotyping or PFGE typing) were isolated multiple times from a processing environment, these strains had no special qualities, such as resistance to stress, or increased adhesion, but instead were isolated repeatedly due to the organism's ability to grow within 'harborage sites' and the inability of cleaning to fully dislodge and destroy them.

However, recent studies indicate there may be a number of explanations to strain persistence, such as tolerance to disinfectants, or adaptation to the food processing environment. Recently, a strain of persistent *L. monocytogenes* was isolated, and when characterized using phenotypic arrays it was found to have higher resistance to quaternary ammonium compounds (QAC), when compared to non-persistent isolates. Sequences of transcribed RNA in the presence of quaternary ammonium compounds showed that persistent strains differentially up-regulated many genes involved in peptidoglycan biosynthesis, thus suggesting that regeneration of the peptidoglycan in the cell wall may function to prevent accumulation of QAC within the bacterial cell. In addition, there is evidence that nutrients from food conditioning films in the food processing environment, along with exposure to environmental bacteriophages, may contribute to adaptation and growth of *L. monocytogenes* within a given food processing plant. This was supported by the observation that *L. monocytogenes* isolates containing the *comK* prophage produced biofilms at greater cell densities when in the presence of meat or poultry soil or 'conditioning films' from their respective isolation environments. The junction fragment sequences (the transition regions where the chromosome and the prophage DNA sequences abut) were conserved among strains from each processing plant, however genetic heterogeneity (single nucleotide polymorphisms) was observed within the *comK* prophage between isolates from the same processing plant. This was interpreted as an indication that the processing plant environment is a factor influencing the adaptation of the strains. Whether or not these genetic polymorphisms are responsible for the greater cell densities in the presence of plant-specific conditioning films has yet to be shown. Nevertheless, these studies indicate that the food processing environment may select for bacterial strains that have a genetic advantage for survival under these less than optimal conditions.

It is important to remember that, in most foods and in food processing environments, the biofilms present will be very complex, i.e., they may be composed of multiple species that form a community of microorganisms that may or may not behave in a similar manner as pure laboratory systems. Similarly, studies of biofilms under laboratory conditions usually do not take into account that biofilms in food processing environments may contain proteins and fats derived from improperly cleaned or sanitized processing areas as an integral part of their structure. Experimental evidence suggests that the formation of a multispecies biofilm is advantageous. For example, higher numbers of *L. monocytogenes* were counted in biofilms that were co-cultured with *Pseudomonas sp*. Others have observed that when *Listeria innocua* was co-cultured with *Pseudomonas aeruginosa* in a bioreactor, the levels of *L. innocua* were two $\log_{10}$ higher at day 4 than when grown in monoculture. In other cases, the adhesion of *Listeria monocytogenes* is limited in the presence of other bacteria. Recently, other researchers have observed that a mixture of *L. monocytogenes* serotype 4b strains that produce lower cell numbers within a mono-strain biofilm could survive and be present in higher numbers when co-cultured with a 1/2a serotype strain. Understanding how *L. monocytogenes* interact and survive in mixed strain biofilms will provide important insights into the ability for these organisms to survive in the food processing plant for large periods of time.

27.4 Detection of biofilms in the food environment

For quality control purposes, there are a number of reasons to look for and monitor bacteria in the food processing environment. General culturing (such as standard plate counts) may be used to assess and monitor a processor's cleaning and sanitation scheme. Alternately, if there is a specific and recurrent problem spoilage organism in a processed food, the quality control department may sample the processing environment looking for an environmental source. Culturing may also be used as part of environmental monitoring for a HACCP plan. For example, the USDA Food Safety and Inspection Service requires under regulation 9 CFR 430.4, that ready-to-eat meat processors that do not have a post-lethality treatment and antimicrobial additive in their product, must monitor the presence of *L. monocytogenes* or an indicator organism (such as *Listeria sp*.) in the post-lethality processing environment.

When sampling bacteria from the environment, the sampling results are only as accurate as the number of samples taken and the sampling locations. In processing plants, common sites of post-processing *L. monocytogenes* contamination are filling or packaging equipment, conveyors, collators used for assembling product for packaging, racks for transporting product, hand tools, gloves, and freezers. Bacterial harborage sites are often places that are difficult to get to and clean – and by definition these sites are also locations that are very difficult to sample, especially on a regular basis. Thus, there is often a possibility of false-negative results – in that *Listeria sp*. is present in the environment, but the sampling plant did not find the harborage site. Floor drains are often a location where there is an accumulation of cleaning run-off and food waste and therefore can be a location that are common harborage sites of pathogens such as *L. monocytogenes*.

The detection of bacteria within the environment can be performed by using sterile swabs or sponges moistened in buffer and swabbing surfaces within the processing environment. With either test, it is important to collect the sample in a buffer that contains the appropriate neutralizing agent for the disinfectants used in the processing plant. Commonly-used neutralizing agents include: polysorbate 80 and lecithin for phenolics and quaternary ammonium sanitizers (QUATS); sodium thiosulfate for halogen-based sanitizers (sodium

hypochlorite and chlorine dioxide); and sodium bisulfite for glutaraldehyde-based sanitizers. Alternatively, a universal neutralizing buffer or broth can be used, such as the Dey/Engley (D/E) buffer which contains a mixture of neutralizing agents. Once bacteria have been removed from the swab, by mixing in a buffer using a test tube mixer or from sponges, and by agitation in a bag buffer within a laboratory paddle blender (such as a Stomacher®), the bacteria can then be cultured. This may be a straightforward plate count or coliform count. If you are looking for a specific organism or indicator (such as *L. monocytogenes* or *Listeria sp*., respectively) then samples must undergo selective enrichment followed by a rapid detection method (PCR or immunoassay) or selective and differential plating. If there is a recurring spoilage problem or an outbreak situation, often cultures from the processing environment are purified, identified to species, and then further characterized to the subspecies level by ribotyping or PFGE typing, so to identify definitely an environmental source of an organism. Culturing from the processing environments tends to take time (48 h–96 h) and can be expensive. In addition, most manufacturers do not want to be culturing bacteria on location, especially enrichment of pathogens, so that samples are either sent to a corporate quality laboratory away from the processing site, or to an external microbial testing lab.

27.4.1 Rapid sanitation testing

Commercially-developed rapid detection tests are used most often to confirm the effectiveness of cleaning and sanitation procedures as an indicator of the hygiene of food processing surfaces. These include the ATP bioluminescence tests and protein detection tests, both which use an integrated swab to sample processing surfaces. For the ATP luminescence tests, a surface is swabbed and then the swab is added to a solution containing the substrate of luciferin and enzyme luciferase, which produces light in the presence of ATP. The amount of light emission (above a manufacturer-set threshold) is then detected using a handheld luminometer. This enzymatic reaction occurs in seconds, allowing for a very rapid detection of ATP on food processing surfaces. There are also commercially-available protein tests that are used to test whether or not food soils have been adequately removed from surfaces. The commercially-available protein tests are usually colorimetric and consist of swabs which rapidly change color in the presence of protein. Protein tests are usually read visually – therefore do not require the purchase of a reading apparatus, so tend to be less expensive than ATP bioluminescence. It is important to note that both test formats (protein or ATP detection) are not specific to detection of bacteria or biofilms, since both detect the presence of ATP or protein from all sources (bacterial or food). The rate of rejection using either or both rapid formats generally has higher 'failure' rates than just microbial sampling alone, which is likely due to the nonspecific nature of the tests. Although these tests are not a direct replacement for microbiological testing, their rapid nature (results often in less than a minute), reduced cost, and requirement for little operator training make them ideal to use as a real-time monitoring method whether or not cleaning and sanitization was performed adequately.

27.5 Conclusions

Biofilms can protect and harbor pathogens in the food processing environment and can be a source of these organisms in post-processing contamination. Biofilms have enhanced resistance to sanitizing agents; therefore, the best control of biofilms is to prevent biofilm formation. Biofilm prevention can be done through design of processing equipment to reduce

potential harborage sites and to allow quick and easy disassembly for daily cleaning and sanitation. In addition, cleaning and sanitation should be performed to minimize cross-contamination between traditional harborage sites (such as floor drains) and processing surfaces Confirmation of cleaning effectiveness can be documented through the use of rapid methods (such as ATP luciferase testing), and culturing of environmental swabs. A combination of all these methods can be used to control and prevent biofilm formation in the processing environment

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28 Bacteriophage biocontrol

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28.1 Introduction

In 1915, when bacteriophages were discovered, A. F. Twort initially reported on a 'glassy transformation' of micrococcus germs. Today, in terms of food safety and biocontrol, sales professionals promote phages as 'natural born killers'. This is mainly because bacteriophages feature unique and efficient mechanisms to specifically target and eliminate bacteria. Accordingly, bacteriophages (literally 'bacteria-eaters') are viruses of bacteria.

The diversity and abundance of bacteriophages is striking. Phages have been isolated from many different environments and also from a diverse set of foods. Generally, a particular bacteriophage can be found in an environment as long as this is also inhabited by the corresponding host bacterium. Conspicuously, most phage isolates (96%) belong to a single taxonomic order of the tailed phages (*Caudovirales*). According to the International Committee on Taxonomy of Viruses, tailed phages are further separated into three distinct families. All members of the *Caudovirales* exhibit an icosahedral capsid (the 'head'), which contains the virus genetic material in a highly condensed form (mostly double-stranded DNA). For further classification of a phage particles, the tail structure is considered. Members of the *Podoviridae* are characterized by very short tails. In fact, many podoviral isolates appear as capsids that exhibit appendages at a particular capsid vertex only. In contrast, phages of the *Myoviridae* family have much longer straight and contractile tails. The tails of the *Siphoviridae* family members are also long, but flexible and cannot be contracted. At the distal end of the tail, a diverse set of proteins is assembled into a baseplate structure required for host cell recognition and adsorption to the target cell surface. Structurally, these appendages resemble tail fibers or are arranged in a ring-shaped structure. Other appendage proteins can hydrolyze the cell surface to enable the punctured penetration of the bacterium (capsule and/or murein hydrolyses, e.g., enzymatically active proteins). An obvious feature that separates bacteriophages from all other viruses known so far is that the phage particle itself does not enter the infected host cell but only injects its genome, leaving the empty virion outside the cell surface.

Although the morphology of newly-isolated bacteriophages easily permits their identification as Podo-, Myo-, or Siphoviruses, morphology alone is insufficient to predict whether

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or not a phage would be a good candidate for biocontrol purposes. Instead, further properties of the isolate itself, such as the host range and particle stability, should be analyzed and genome sequencing should be performed in order to better classify the phage isolate. In fact, phages which are used for biocontrol applications are well characterized and belong to different phage families. Apparently, it seems that the SPO1-like Myoviruses are especially suitable to efficiently target Gram-positive bacteria, and T4-like or the Felix O1-like Myoviruses are well suited for control of Gram-negative cells.

28.2 Bacteriophage life cycles

After adsorption and DNA injection into the host cell, phages can be separated in two different groups according to their life cycles (Fig. 28.1). Temperate phages are able to integrate their genome into the host cell chromosome, via site-specific recombination applying phage-encoded integrases. After integration, the phage becomes a prophage whose genes are mostly repressed. Hence, the silenced prophage does not multiply autonomously inside the infected cell and leaves the bacterium intact. Mediated by production of a potent transcription repressor, the bacterium becomes now resistant against another infection by the same type of phage, a phenomenon referred to as homo-immunity. Moreover, temperate phages may also transduce other genes such as pathogenicity factors into the lysogenized cell, possibly increasing the bacterium's virulence. This phenomenon is quite frequent and is referred to as lysogenic conversion. Furthermore, temperate phages often exhibit rather narrow host ranges, e.g., they are incapable of infecting all strains of any given host species. Therefore, temperate phages are not usually considered suitable for biocontrol applications. In contrast, virulent (i.e., obligately lytic) phages start to multiply immediately after infection (Fig. 28.1). When offspring particles have been assembled inside the host cell, it is quickly lysed through the action of phage-encoded pore-forming holins, and cell wall-digesting

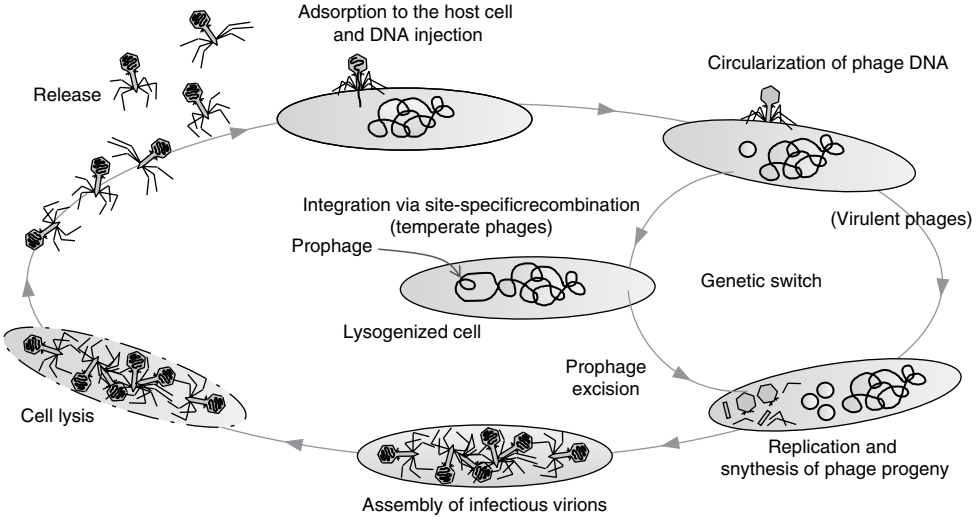

Figure 28.1 Life cycles of temperate and virulent bacteriophages. Fieseler, L., Hagens, S., and Loessner, MJ. (2011). "Bacteriophages and food safety". In "Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation" Woodhead publishing, edited by Christophe Lacroix, pp. 161–178. ISBN: 978-1-84569-669-6 (print), ISBN: 978-0-85709-052-2 (online)

peptidoglycan hydrolases (endolysins, lysins). Importantly, virulent phages frequently exhibit broad host ranges, thereby covering a larger proportion of relevant strains of a given host species. In conclusion, an ideal 'biocontrol phage' should be strictly virulent, cover a specific host range as wide as possible, does not encode any bacterial virulence factors or proteins with a potential to cause allergic reactions, and must not be able to carry out general transduction. If possible, the phage should be propagated on a nonpathogenic production strain (biosafety group 1 organism) to high yields.

28.3 Application of bacteriophages for food safety

Bacteriophages can be applied to enhance the safety of food in different ways. They can be either engineered to become reporter phages to be used for rapid detection of the host bacteria, or as a tool to specifically eliminate those target cells. Moreover, phage endolysin can also be used as a highly-specific antimicrobial agent in many ways.

The basis for the application of phages is their high specificity towards the bacterial host cell. Because phages absolutely rely on a particular host species to reproduce, mechanisms for precise host recognition have evolved. The specific recognition properties of phages are impressive and can be used to distinguish bacterial isolates. While a broad host range is considered a prerequisite for suitable 'biocontrol phages', a narrow host range enables the detection of particular bacterial strains or isolates. Hence, both features can be useful for biotechnological applications.

28.4 Reporterphages

A reporter bacteriophage is generally used to specifically detect contaminating bacteria in a given sample. The hallmark in the construction of such reporterphages is the specific introduction of a suitable reporter gene into the phage genome, either via homologous recombination or direct cloning. The gene is best placed under the control of a strong phage promoter. Upon infection of a target cell, the recombinant phage transduces the reportergene into the target cell, where it will be expressed. Reporterphages exclusively detect living cells, which is one of their main advantages. In case the reportergene is an enzyme, the catalyzed reaction can be analyzed in a bioassay, and may even serve as a quantitative measure indicating the amount of infected cells present in the sample. Reporterphages have been constructed for *E. coli*, *Salmonella*, *Yersinia pestis*, *Listeria monocytogenes*, *Bacillus anthracis*, and *Mycobacterium tuberculosis*. A brief overview is presented in Table 28.1.

28.5 Biocontrol of bacterial pathogens using bacteriophages

The potential to use bacteriophages for biocontrol purposes was recognized soon after their discovery about a century ago. Today, several bacteriophage preparations are commercially available to target bacterial pathogens such as *Listeria monocytogenes* (approved for several foods; the product Listex™ P100 features GRAS status and has been approved as a processing aid), *Escherichia coli* O157:H7 (for livestock), and *Salmonella*, *Staphylococcus aureus*, and *Xanthomonas campestris* and *Pseudomonas syringae* (approved for use as a pre-harvest application against bacterial diseases of tomato and peppers). In experimental projects,

Table 28.1 Application of Recombinant Reporterphages. Fieseler, L., Hagens, S., and Loessner, MJ. (2011). "Bacteriophages and food safety". In "Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation" Woodhead publishing, edited by Christophe Lacroix, pp. 161–178. ISBN: 978-1-84569-669-6 (print), ISBN: 978-0-85709-052-2 (online)

Phage	Target organism	Reportergene	Mode of action	Detection limit	Application
λ::*luxI*	*E. coli*	Acyl-homoserine-lactone synthase from *Vibrio* sp.	Autoinducer synthesis is detected by a reporter bacterium via a quorum sensing mechanism	In vitro 1 cfu/ml after 10 h; in situ 130 cfu/ml after 22 h	Contaminated lettuce leaf washings
λ::*luxAB*	*E. coli*	Luciferase from *Vibrio* sp.	Long chain aldehyde oxidation leads to bioluminescence (490 nm) which is detected photometrically	10^4 cfu/cm^2 after 50 min	Wipe tests in meat processing plants
PP01::*luxI*	*E. coli*	Acyl-homoserine-lactone synthase from *Vibrio* sp.	Autoinducer synthesis is detected by a reporter bacterium via a quorum sensing mechanism	In vitro 10 cfu/ml in 4 h; in situ 10^4 cfu/ml after 8 h	Contaminated apple juice
PP01::*gfp*	*E. coli*	Green fluorescent protein from *Aequorea victoria*	Gfp is a structural component of the phage particle. The adsorption of PP01::*gfp* is monitored by microscopy	n.d.	–
P22::*inaZ*	*Salmonella*	Ice nucleation protein from *Pseudomonas syringea*	InaZ mimics the lattice of ice crystals and shifts the ice nucleation temperature in a drop-freezing assay	In vitro 10 cfu/ml	–
phiA1122:: *luxAB*	*Yersinia pestis*	Luciferase from *Vibrio* sp.	Long chain aldehyde oxidation leads to bioluminescence (490 nm) which is detected photometrically	In vitro 100 cfu after 1 h	–

(*Continued*)

Table 28.1 (*Continued*)

Phage	Target organism	Reportergene	Mode of action	Detection limit	Application
A511:: *luxAB*	*Listeria monocytogenes*	Luciferase from *V. harveyi*	Long chain aldehyde oxidation leads to bioluminescence (490 nm) which is detected photometrically	In situ 1–100 cfu/ml or g after prior enrichment	Contaminated ricotta and soft cheese, pudding, cabbage, and minced meat
A511::*celB*	*Listeria monocytogenes*	Hyperthermo-stable β-glyco-sidase from *Pyrococcus furiosus*	0.3 mM 4-Methylum-belliferyl-α-D-Gluco-pyranoside (MUG) is hydrolyzed at 85 °C, pH 5.0 in a photometric CelB bioassay	In vitro 6×10^3 cfu/ml after 6 h; in situ 1–10 cfu/ml or g after prior enrichment	Contaminated smoked salmon and chocolate milk
Wbeta:: *luxAB*	*Bacillus anthracis*	Luciferase from *V. harveyi*	Long chain aldehyde oxidation leads to bioluminescence (490 nm) which is detected photometrically	In vitro 10^3 cfu/ml within 1 h	–
TM4::*luc*	*Mycobacterium tuberculosis*	Luciferase from *Photinus pyralis*	Bioluminescence of the firefly luciferase is ATP dependent and detected photometrically	In vitro 120 cfu/ml in 12 h	–

(n.d.: not determined)

Table 28.2 Bacteriophages and phage products used for the control of selected bacterial pathogens. Phages marked with an asterisk are not commercially available

Produkt or phage	Target bacterium	Treatment efficacy	Remarks
Listex™ P100	*L. monocytogenes*	3.5 log reductionup to 100%	Approved for all kinds of foods, GRAS, processing aid
ListShield™	*L. monocytogenes*	99–100%	Blend of six phages applied on foods and surfaces
EcoShield™	*E. coli* O157:H7	95–100%	Blend of three phages applied on foods, including ground beef
Salmonelex™	*Salmonella*	d.n.a.	In development
SalmShield™	*Salmonella*	d.n.a.	In development
AgriPhage™	*X. campestris* and *P. syringae*	d.n.a.	Phage blend
CP8* and CP34*	*C. jejuni*	5 log reduction in chicken cecal content after 5 days	Orally administered in chicken
ESP1-3* and ESP732-1*	*C. sakazakii*	Complete eradication at 37 °C	Applied in infant milk formula
Y2* and L1*	*E. amylovora*	In vitro 3.3 logs reduction after 24 h	Cocktail of two phages

(d.n.a: data not available)

phages were also applied to treat chicken colonized with *Campylobacter*, infant formula contaminated with *Cronobacter sakazakii*, pork adipose tissue colonized by *Brochothrix thermosphacta*, and detached apple tree flowers before infection with the plant pathogen *Erwinia amylovora*. A summary is presented in Table 28.2. Even if many promising results were obtained, the feasibility and precise conditions to treat a particular food must always be determined individually before application. Concentration of salts or other osmolytes, pH value, and temperature should be in a range which does not affect the interaction of phage and host cell. To achieve optimal coverage of the treated surface or volume the particular texture and structure of the food need to be considered. In general, 10^8 plaque forming units per ml, gram or cm^2 are sufficient to reach all possible target cells by diffusion in a reasonably short time frame and therefore reduce bacterial counts efficiently.

Even if phage application offers many advantages, bacteria may become resistant against infection by specific phages. The mechanisms of resistance can be different. Bacteria may change surface components by variation or mutation, or can acquire DNA restriction/modification systems, or clustered, regularly interspaced short palindromic repeats (CRISPR) in the bacterial genome sequences, all resulting in an abortive infection without phage multiplication. However, phage resistance does not necessarily represent an advantage or an increased fitness for the bacterium in the absence of phage. In fact, such mutations are likely to have negative or even detrimental effects. Similar phenomena were described during phage treatment of *E. coli* O157:H7 contaminated beef and during phage therapy of broiler chickens colonized with *C. jejuni*. Moreover, phages are continuously co-evolving with their hosts and adapt to bacterial defense strategies. In fact, phages can mutate at much higher frequency than bacteria and therefore maintain the efficacy of phage infection and subsequent killing. In addition, novel phages may be employed in a rotation scheme in case phage-resistant bacteria emerge. Provided that the host ranges are

non-overlapping, e.g., different phage receptors are required for phage adsorption, application of phage cocktails containing a set of different bacteriophages also reduces the probability of resistance development.

28.6 Bacteriophage protein preparations for detection and control of contaminating bacteria

Recombinant bacteriophage proteins feature a great biotechnological potential and may also be used for pathogen detection and control. The reason for this is the outstanding species and cell surface-specific binding of phage-encoded recognition proteins to the bacterial surface. Tail fiber proteins or baseplate-associated receptor binding proteins specifically bind to target bacteria during the adsorption process. Especially, tail fiber proteins can be very useful at removing bacterial surface molecules which serve as phage binding ligands (such as LPS components) from liquids.

Phage-encoded peptidoglycan hydrolases known as endolysins mediate the rapid and specific lysis of an infected host cell at the end of the infection cycle. Endolysins exhibit two distinct domains. The cell wall binding domain (CBD) mediates high affinity binding to the peptidoglycan. It is this domain which can be fused either to the green fluorescent protein or coated onto paramagnetic beads. The CBD fusion proteins can be applied to specifically label and detect bacteria, even in mixed populations or in contaminated food, because the CDB does specifically recognize and bind to its ligand from without. The enzymatically-active domain (EAD) is connected to the CBD by a flexible linker, and serves to cleave bonds in the cell wall. Accordingly, bacteriophage endolysins can be grouped into amidases, endopeptidases, glycosamidases, muramidases, and transglycosidases and, depending on the endolysin, every possible bond of the peptidoglycan can be hydrolyzed. Different endolysin domains may be shuffled and fused in a recombineering approach, yielding chimeric proteins with enhanced affinity to their cell wall ligands or altered binding specificity. For Gram-negative bacteria, the application of endolysins for detection and control is more challenging, because the bacteria are shielded by the outer membrane. Nevertheless, the approach is very promising for development into novel antibacterial treatments.

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