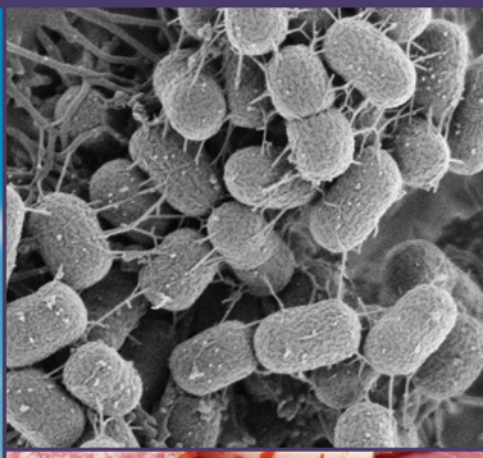
Foodborne Infections and Intoxications

Fourth Edition





Edited by J. Glenn Morris, Jr. and Morris E. Potter

Food Science and Technology, International Series

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Edited by

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Preface

Foodborne Infections and Intoxications was first published in 1969, under the editorship of Hans Riemann. In the intervening 44 years there have been two additional editions, published in 1979 (2nd edition, edited by Hans Reimann and Frank Bryan) and 2006 (3rd edition, edited by Hans Reimann and Dean Cliver). These texts have provided an outstanding scientific resource for multiple generations of students, investigators, and food safety practitioners, and have documented our changing understanding of and approach to food safety through almost half a century.

With this edition the editorship has passed on, shifting from University of California (Davis) to University of Florida (and FDA/CDC). The focus has also evolved: The current edition places a strong emphasis on estimates of disease burden, and development of risk-based approaches to food safety and food safety regulation. There remain traditional chapters on each of the major pathogens, but with an expansion to include newly recognized agents (particularly viral agents), and a focus in each "agent" chapter on understanding how the pathogen is introduced into the food supply. The microbiology is presented, but in the context of public health and disease prevention.

Development of a comprehensive text of this type is a major undertaking, and we are deeply indebted to our 72 authors, from three continents, who contributed their expertise to this task. We hope that this text will prove as useful to the current generation of food safety students and practitioners as have the prior editions, providing an ongoing, and intellectually challenging, resource for development of strategies to prevent human disease.

J. Glenn Morris, Jr.
Morris E. Potter

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Preface to the Third Edition

A quarter of a century has passed since the second edition of Foodborne Infections and Intoxications was published. Significant discoveries and developments have taken place during this time, and many journal articles and several books dealing with foodborne pathogens have been published. Some important foodborne pathogens (e.g. noroviruses, enterohemorrhagic Escherichia coli) were unknown at the time of the second edition, and organisms such as Yersinia and Campylobacter were not conclusively proven to be foodborne.

In this third edition of Foodborne Infections and Intoxications, experts present updated accounts of the known characteristics of the most important foodborne pathogens, including their host ranges and the characteristics of the diseases they cause. The present volume also has a completely revised chapter on the epidemiology of foodborne diseases, with emphasis on investigation procedures, and a new chapter on risk assessment has been added. The chapter on the effects of food-processing procedures has been expanded to include a number of newer techniques, and the chapter on food safety presents a detailed discussion of hazard analysis-critical control points (HACCP) as a tool to assure safety. Four new chapters have been added, on E. coli, Campylobacter and related organisms, Yersinia, and Listeria, in addition to a chapter on other natural toxins (not including mycotoxins).

Much new information about the detection and identification of foodborne pathogens has been presented in books and articles in recent years. Still, about half the reported foodborne disease outbreaks in countries like the US have no identified agent. Without doubt many of these outbreaks are caused by viruses, which suggests a need for virus-detection procedures that can be applied by laboratories routinely charged with testing of suspect food samples. Since sampling and testing per se do not prevent foodborne disease outbreaks, there is also a need for research to develop effective interventions against common foodborne diseases and methods to assure the implementation of such interventions; the last two chapters of the book address this need.

There is, furthermore, a need for better setting of research priorities on foodborne diseases; some diseases, like human prion diseases, are so rare that even a 90 per cent reduction in incidence would have negligible public health significance. The chapter on risk assessment describes an important tool for setting priorities.

The editors especially thank the authors for contributing their vast expertise to this book.

Hans Riemann
Dean Cliver

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Dedication

The current edition is dedicated to Hans Reimann, Frank Bryan, and Dean Cliver, who shepherded this text through three editions across almost half a century. All three were giants in the field of food safety, and their commitment and dedication to science, public health, and food safety stand as an outstanding example to those of us who follow in their footsteps.

At a personal level, we would also like to dedicate this text to our wives and families, who have provided unswerving support through our careers. They have tolerated the long hours (and the use of laptops at home during evenings and weekends to edit yet one more document), and we thank them for their valuable input, patience, and understanding.

J. Glenn Morris, Jr.
Morris E. Potter

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SECTION

1 Foodborne Disease: Epidemiology and Disease Burden

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CHAPTER

1 Estimates of Disease Burden Associated with Contaminated Food in the United States and Globally

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Introduction

Estimates of the overall burden of disease from foodborne agents are important for directing food safety policy and prioritizing interventions. However, estimating the burden of foodborne disease is challenging for several reasons. First, there are over 250 agents, including a variety of bacteria, viruses, parasites, and chemicals, that may contaminate food and cause foodborne illness. Second, transmission routes other than contaminated food may result in human infections for many of these agents. For example, *Escherichia coli* O157:H7 infections may be acquired by ingesting contaminated food or water or by direct contact with infected animals or persons. Third, a fraction of illnesses are confirmed by laboratory testing and reported to public health agencies and most surveillance systems do not attempt to determine the proportion of infections that are transmitted through food. Finally, unknown or unrecognized agents are likely to cause an important additional fraction of illnesses due to contaminated food. Indeed, many important foodborne pathogens, such as *Campylobacter* and *E. coli* O157, were only recognized in recent decades [1,2].

Surveillance for laboratory-confirmed infections provides essential information for assessing trends in diseases and detecting outbreaks. Information derived from surveillance may assist regulatory efforts to prioritize and evaluate interventions. However, because only a fraction of illnesses are diagnosed and reported, periodic assessments of the total number of illnesses, including those that are not laboratory-confirmed, are also needed to help set public health goals, allocate resources, and measure the economic impact. Several countries, including Australia, the Netherlands, the United Kingdom, and the United States, have conducted

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prospective population-based or cross-sectional studies to supplement surveillance and estimate the overall human health impact of foodborne disease [3]. In 2006, the World Health Organization (WHO) convened a meeting of foodborne disease experts that recommended the formation of the Foodborne Disease Epidemiology Reference Group (FERG) to advise WHO about how to estimate the global burden of foodborne disease [4]. The FERG began estimating the global burden of foodborne disease in 2007.

The purpose of this chapter is to describe the methods used by various countries to estimate the burden of foodborne disease. We begin by describing estimates of foodborne illness in the United States and then compare these methods and estimates with those in some other countries. We also discuss the WHO FERG initiative to estimate the global burden of foodborne disease.

Estimates of foodborne disease in the United States

In 2011, the US Centers for Disease Control and Prevention (CDC) published new estimates of the numbers of foodborne illnesses caused by contaminated foods consumed in the United States (hereafter, domestically acquired foodborne illnesses) [5,6]. Together, major known pathogens and unspecified agents transmitted by food were estimated to cause 47.8 million illnesses each year, resulting in 127,839 hospitalizations and 3037 deaths (Table 1.1).

Major known pathogens

Data from surveillance, surveys, and other sources were used to estimate the number of domestically acquired foodborne illnesses, hospitalizations, and deaths caused by 31 major known pathogens, including 21 bacterial, 5 viral, and 5 parasitic pathogens (see Table 1.1) [6]. These known pathogens were estimated to cause 9.4 million (90% credible interval [CrI]: 6.6–12.7 million) domestically acquired foodborne illnesses, 55,961 hospitalizations (90% CrI: 39,534–75,741), and 1351 deaths (90% CrI: 712–2268) each year. Norovirus was estimated to cause the most foodborne illness (58%), while nontyphoidal *Salmonella* spp. was the leading cause of hospitalization (35%) and death (28%). Seven pathogens—*Campylobacter* spp., *Clostridium perfringens*, *E. coli* O157, *Listeria monocytogenes*, nontyphoidal *Salmonella* spp., norovirus, and *Toxoplasma gondii*—were estimated to cause 90% of domestically acquired foodborne illnesses, hospitalizations, and deaths due to the major known pathogens.

Estimating illness using the "burden-of-illness pyramid"

Most known pathogens had laboratory-based surveillance data available; therefore, the total number of illnesses was estimated using the "burden-of-illness pyramid" approach (Figure 1.1). Several steps are necessary for an illness to be included in laboratory-based surveillance: the ill person must seek medical care, a specimen

Table 1.1 Estimated Annual Number of Illnesses, Hospitalizations, and Deaths Caused by Major Known Pathogens and Unspecified Agents Transmitted by Food (United States) [5,6].

	Total—All Transmission Sources[a]						Domestically Acquired—Foodborne Transmission					
	Illnesses		Hospitalizations		Deaths		Illnesses		Hospitalizations		Deaths	
Category	No.	90% CrI[b]	No.	90% CrI	No.	90% CrI	No.	90% CrI	No.	90% CrI	No.	90% CrI
Major known pathogens[c]	**37,220,098**	**28,434,745–47,630,066**	**228,744**	**188,326–275,601**	**2,612**	**1,723–3,819**	**9,388,075**	**6,641,440–12,745,709**	**55,961**	**39,534–75,741**	**1,351**	**712–2,268**
Campylobacter spp.	1,322,137	530,126–2,521,026	13,240	6,770–23,827	119	0–523	845,024	337,031–1,611,083	8,463	4,300–15,227	76	0–332
Clostridium perfringens	969,342	192,977–2,492,003	439	45–2,015	26	0–163	965,958	192,316–2,483,309	438	44–2,008	26	0–163
E. coli, Shiga toxin-producing (STEC) O157	96,534	26,982–227,891	3,268	844–7,052	31	0–173	63,153	17,587–149,631	2,138	549–4,614	20	0–113
Listeria monocytogenes	1,662	582–3,302	1,520	544–3,152	266	0–765	1,591	557–3,161	1,455	521–3,018	255	0–733
Salmonella, nontyphoidal	1,229,007	772,129–2,008,076	23,128	10,221–44,860	452	0–1,210	1,027,561	644,786–1,679,667	19,336	8,545–37,490	378	0–1,011
Toxoplasma gondii	173,995	134,593–218,866	8,889	5,383–13,203	656	409–952	86,686	64,861–111,912	4,428	2,634–6,674	327	200–482
Norovirus	20,865,958	12,842,072–30,743,963	56,013	32,197–86,569	571	331–881	5,461,731	3,227,078–8,309,480	14,663	8,097–23,323	149	84–237
Other pathogens	12,561,463	NA[d]	122,247	NA	491	NA	936,371	NA	5,040	NA	120	NA
Unspecified agents	**141,800,000**	**NA**	**258,033**	**NA**	**3,574**	**NA**	**38,392,704**	**19,829,069–61,196,274**	**71,878**	**9,924–157,340**	**1,686**	**369–3,338**
Total	**178,800,000**	**NA**	**473,832**	**NA**	**5,072**	**NA**	**47,780,779**	**28,658,973–71,133,833**	**127,839**	**62,529–215,562**	**3,037**	**1,492–4,983**

[a] *Includes all possible sources of illness, including infections acquired overseas.*
[b] *90% credible interval indicating range of uncertainty around estimate.*
[c] *Showing estimates for seven pathogens causing most foodborne illnesses, hospitalizations, or deaths.*
[d] *NA = not available.*

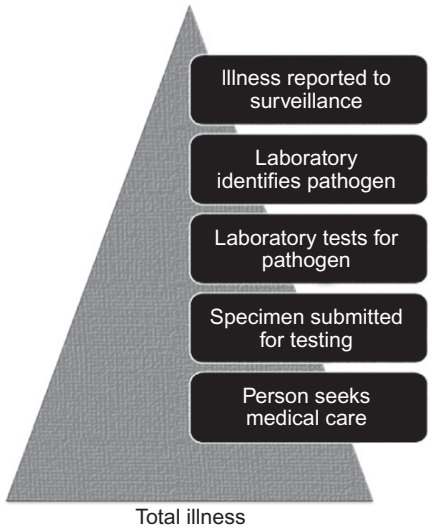

FIGURE 1.1

Surveillance steps that must occur for laboratory-confirmed cases to be reported to surveillance.

must be submitted for laboratory testing, the laboratory must test for and identify the causative agent, and the illness must be reported to the public health authorities. Estimating the frequency of cases of foodborne disease that are not reported to public health from laboratories provides insight into under-reporting in the surveillance system. Similarly, assessing differences in medical care–seeking behavior, specimen submission, laboratory testing, or laboratory test sensitivity characterizes under-diagnosis at each step of the surveillance system. Accounting for the proportion of cases missed in traditional surveillance due to under-diagnosis and under-reporting builds the "burden-of-illness pyramid". This allows for an extrapolation from laboratory-confirmed illnesses (at the top of the "burden-of-illness pyramid") to estimate the overall number of illnesses in the community (at the bottom of the "burden-of-illness pyramid"). To extrapolate, a multiplier, the inverse of a proportion, is calculated for each surveillance step. For example, if the laboratory test sensitivity of a particular pathogen was estimated to be 80%, the multiplier for this surveillance step would be 1.25 (i.e., for every case of infection diagnosed an estimated 1.25 cases would have been tested for that pathogen).

In the United States, data on laboratory-confirmed illnesses caused by 25 of the 31 known pathogens were available from one or more of five surveillance systems: the foodborne diseases active surveillance network (FoodNet), the national notifiable disease surveillance system (NNDSS), the cholera and other *vibrio* illness surveillance (COVIS) system, the national tuberculosis surveillance system (NTSS), and the foodborne disease outbreak surveillance system (FDOSS).

Similar to other countries, laboratory-based surveillance systems in the United States rely largely upon passive reports of diseases from clinical laboratories to state and local health departments, which are, in turn sent to the CDC. To assure that all laboratory-confirmed cases occurring within the FoodNet surveillance area are reported, personnel actively contact all laboratories in the catchment area. Therefore, when data were available in more than one surveillance system, active surveillance data from FoodNet were used, except for *Vibrio* spp., for which COVIS was used because of geographical clustering of *Vibrio* infections outside the FoodNet sites. Data on outbreak-associated illnesses from FDOSS were used only for pathogens with no data available from the other systems due to not being specifically reported or only manifesting as outbreaks.

Because FoodNet conducts active surveillance, the pathogens under FoodNet surveillance were assumed to have no under-reporting. Because COVIS and NNDSS are passive surveillance systems, an under-reporting multiplier (1.1 for bacterial and 1.3 for parasitic pathogens), derived by comparing the incidence of all nationally notifiable illnesses ascertained through FoodNet with that reported to NNDSS, was applied to those pathogen counts. For the five bacterial pathogens for which only outbreak data were available, an outbreak under-reporting multiplier was created by determining the proportion of illnesses in FoodNet caused by *Campylobacter, Cryptosporidium, Cyclospora*, *Listeria*, *Salmonella, Shigella*, Shiga toxin-producing *E. coli* (STEC), *Vibrio*, and *Yersinia* that were also reported as outbreaks associated to FDOSS. It was assumed that all *Mycobacterium bovis* illnesses were reported to NTSS.

To adjust for medical care seeking and specimen submission, the proportion of persons reporting an acute diarrheal illness (defined as ≥3 loose stools in a 24-hour period and lasting longer than one day or resulting in restricted daily activities) in the past month who sought medical care and submitted a stool sample for that illness were estimated using data from FoodNet surveys of the general population (FoodNet Population Surveys). Because persons with more severe illness are more likely to seek care [7], the rate of medical care seeking and stool sample submission was estimated separately for persons with bloody and non-bloody diarrhea; these proportions were used as surrogates for severe and mild presentations of most illnesses. These multipliers were derived by examining data on the proportion of patients with diarrhea seeking care and submitting specimens with different symptom profiles from population-based surveys. Multipliers for medical care seeking and stool sample submission (for those with mild and severe illness) were then applied to the pathogen-specific proportions of patients with laboratory-confirmed infections who were estimated to have severe or mild illness. Some severe foodborne diseases were assumed to have high rates of medical care seeking and specimen submission (e.g., 90% of patients with invasive *L. monocytogenes* were estimated to seek medical care and 80% were estimated to have a specimen taken for laboratory testing). Laboratory testing and test sensitivity rates were estimated using data from FoodNet and other surveys of clinical diagnostic laboratory practices.

The US estimates would not have captured mild illnesses associated with some pathogens. For example, mild cases of botulism are often recognized as part of outbreaks, but these persons seldom seek medical care and so are not captured by surveillance except during outbreaks [8,9]. Likewise, *Listeria* can cause febrile gastroenteritis but these illnesses are rarely diagnosed at least in part because *Listeria* is not detected by routine stool culture methods [10]. Cases of early spontaneous abortion or miscarriage caused by *Listeria* infection may also be under-represented.

By augmenting surveillance with information from surveys and other sources, this burden-of-illness approach has been used to estimate the overall number of illnesses caused by specific pathogens. For example, for each laboratory-confirmed case of nontyphoidal *Salmonella* infection, there were an estimated 29 cases of illness in the community that were not reported.

Alternative approaches to estimating illnesses

Infections caused by diarrheagenic *E. coli* other than STEC and Enterotoxigenic *E. coli* (ETEC), *Toxoplasma*, astrovirus, rotavirus, sapovirus, and norovirus were not routinely captured by any surveillance system; therefore, alternate approaches were used to estimate illnesses. Illness caused by diarrheagenic *E. coli* other than STEC and ETEC was assumed to be as common as illness caused by ETEC. Illnesses caused by *Toxoplasma* were estimated using nationally representative serologic data from the National Health and Nutrition Examination Survey (NHANES) from 1999–2004 [11] and an estimate that 15% of persons who seroconvert develop clinical illness [12]. It was assumed that 75% of children experience an episode of clinical illness caused by rotavirus by 5 years of age [13]; the same estimate was used for astrovirus and sapovirus. Norovirus illnesses were estimated by applying the mean proportion (11%) of all acute gastroenteritis caused by norovirus from studies in other industrialized countries [14–17] to estimates of acute gastroenteritis from FoodNet Population Surveys.

Estimating hospitalizations and deaths

Accurately estimating hospitalizations and deaths caused by foodborne pathogens is particularly challenging. National data on outpatient visits resulting in hospitalization, hospital discharges, and death certificates are likely to substantially underestimate the pathogen-specific numbers because, for pathogen-specific diagnoses to be recorded, health care providers must order the appropriate diagnostic tests, and coding must be accurate. Without detection of a pathogen, infections may be coded as non-infectious illnesses [18]. Furthermore, gastrointestinal illness may exacerbate a person's chronic illnesses through dehydration or electrolyte imbalance, resulting in hospitalization or death well after the resolution of the acute illness, so it may not be coded as a contributing factor. Therefore, for most pathogens, the numbers of hospitalizations and deaths were estimated from the proportion of laboratory-confirmed illnesses reported to surveillance where a person was hospitalized or died. Because some persons with illnesses that were not laboratory-confirmed would also have

been hospitalized and died, the number of hospitalizations and deaths was doubled to account for under-diagnosis.

Estimating the proportion of illnesses that are domestically acquired and foodborne

Data from published studies and surveillance were used to determine the proportion of illnesses acquired while traveling outside the United States for each pathogen. The remaining proportion was considered "domestically acquired." The proportion of domestically acquired illnesses that were transmitted by food for each pathogen was based on data from surveillance, risk factor studies, and a review of current literature. Assumptions about the proportion of illnesses transmitted by food have an important impact on the estimates, but data on which to base these estimates are often lacking and it is not known how representative these data are of total illnesses and if the foodborne fraction is similar across age groups. For example, the proportion of some illnesses transmitted by animals may be higher among children (e.g., *E. coli* O157) [19], and the proportion that spreads from one person to another may be higher among institutionalized elderly (e.g., norovirus) [20].

Unspecified agents

Unspecified agents causing acute gastroenteritis were estimated to cause 38.4 million domestically acquired foodborne gastroenteritis illnesses, 71,878 hospitalizations (90% CrI: 9924–157,340), and 1686 deaths (90% CrI: 369–3338) each year [5] (see Table 1.1). This unspecified agents category includes a heterogeneous group of less well-understood agents. First, there are agents, many of which cause acute gastroenteritis, that are recognized as known or possible causes of foodborne illness, but for which there were insufficient data to make reliable estimates of incidence. This category includes infectious agents such as *Aeromonas* spp., *Edwardsiella* spp., and *Plesiomonas* spp., and non-infectious agents such as mushroom and marine biotoxins, metals, and other inorganic toxins. Second, some known agents may not be routinely recognized as having a transmission route through food. For example, the detection of *Clostridium difficile* in retail meat products suggests that it may sometimes be transmitted by that route [21]. Third, there are microbes, chemicals, and other substances known to be in food that could at some time be shown to cause acute illness. Agents of foodborne illness continue to be discovered. In addition, outbreaks occur in which specimens are obtained in a timely manner yet no causative agent can be identified (e.g., Brainerd diarrhea) [22,23]. Therefore, it is likely that additional agents of foodborne illness remain undescribed and may be responsible for gastroenteritis of unknown etiology [24].

Unspecified acute gastroenteritis illnesses

To estimate the number of gastroenteritis illnesses caused by unspecified agents, the estimated number of illnesses caused by 24 major known pathogens that typically or often cause diarrhea or vomiting was subtracted from the overall number

of acute gastroenteritis illnesses estimated using data from FoodNet Population Surveys. The FoodNet Population Surveys are 12-month, random-digit-dial telephone surveys of the general FoodNet population that collect information on episodes of diarrhea and vomiting and on other gastrointestinal symptoms in the past month. The annual number of acute gastroenteritis illnesses was derived by multiplying the average monthly prevalence by 12, where an episode of acute gastroenteritis was defined as diarrhea (≥3 loose stools in 24 hours) or vomiting in the past month; both had to last >1 day or result in restricted daily activities. Persons with a chronic condition in which diarrhea or vomiting was a major symptom and persons with concurrent symptoms of cough or sore throat were excluded. The annual number of acute gastroenteritis illnesses (178.8 million episodes) was estimated by applying the average rate (0.6 episodes per person per year) from the combined surveys to the 2006 US population estimate. Subtracting 37.0 million estimated illnesses caused by the 24 known gastroenteritis pathogens left 141.8 million acute gastroenteritis illnesses caused by unspecified agents.

Unspecified acute gastroenteritis hospitalizations and deaths

The number of acute gastroenteritis hospitalizations and deaths caused by unspecified agents was estimated using a similar approach to that used for unspecified gastroenteritis illnesses. The number of acute gastroenteritis hospitalizations was estimated using data from three sources: the CDC's National Center for Health Statistics (NCHS) National Hospital Discharge System (NHDS); the Healthcare Cost and Utilization Project (HCUP) National Inpatient Sample (NIS); and combined data on hospitalizations from NCHS National Ambulatory and National Hospital Ambulatory Medical Care Surveys (NAMCS/NHAMCS). A hospitalization was considered an acute gastroenteritis episode if one of the ICD-9-CM codes listed below was listed as one of the first three diagnoses. The number of acute gastroenteritis deaths was estimated using multiple cause-of-death data from the National Vital Statistics System, where acute gastroenteritis was listed as the underlying or a contributing cause.

Acute gastroenteritis hospitalization was defined using the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) diagnostic codes 001–008 (infectious gastroenteritis of known cause); 009 (infectious gastroenteritis); 558.9 (other and unspecified non-infectious gastroenteritis and colitis); and 787.9 (other symptoms involving digestive system: diarrhea), excluding 008.45 (*Clostridium difficile* colitis) and 005.1 (botulism). Other and unspecified non-infectious gastroenteritis and colitis was included because many unknown infectious illnesses may be coded as non-infectious. Acute gastroenteritis death was defined as ICD-10 diagnostic codes A00.9–A08.5 (infectious gastroenteritis of known cause); A09 (diarrhea and gastroenteritis of presumed infectious origin); and K52.9 (non-infectious gastroenteritis and colitis, unspecified), excluding A04.7 (enterocolitis due to *Clostridium difficile*) and A05.1 (botulism).

The mean annual rate of acute gastroenteritis hospitalizations from 2000 to 2006 within each survey was 203 per 100,000 persons from NHDS, 187 per

100,000 from NIS, and 109 per 100,000 from NAMCS/NHAMCS. Using a statistical model to combine the data from the three surveys and applying the results to the United States population resulted in an estimated 473,832 acute gastroenteritis hospitalizations occurring each year. Subtracting the 215,799 estimated hospitalizations due to the 24 known gastroenteritis pathogens leaves 258,033 acute gastroenteritis hospitalizations due to unspecified agents. The mean annual death rate due to gastroenteritis was 1.5 per 100,000 persons. Therefore, 5072 acute gastroenteritis deaths were estimated to occur each year in the United States. Subtracting the 1498 deaths due to the 24 known gastroenteritis pathogens leaves 3574 acute gastroenteritis deaths due to unspecified agents.

Domestically acquired foodborne illness caused by unspecified agents

Because there were no data with which to directly estimate the proportions of unspecified agents that were both domestically acquired and foodborne, these proportions were assumed to have distributions similar to the 24 known gastroenteritis pathogens. Applying the proportion of illnesses, hospitalizations, and death from the 24 known gastroenteritis pathogens that were domestically acquired (98%, 97%, and 95%, respectively) and foodborne (25%, 23%, and 50%, respectively) yields an estimate of 38.4 million domestically acquired foodborne illnesses (90% CrI: 19.8–61.2 million), 71,878 hospitalizations (90% CrI: 9924–157,340) and 1686 deaths (90% CrI: 369–3338) caused by unspecified agents.

Estimation in other countries

Several other countries have estimated the burden of disease from contaminated food, including England and Wales, the Netherlands, New Zealand, Jordan, Greece, Australia, and France [25–30,32]. The methods vary considerably, from the assessment of incidence and health impact of a few selected diseases at a regional or subregional level to extensive consideration of all possible pathogens for the whole country. Internationally, there has been a trend towards the use of disability-adjusted life years (DALY) as a summary measure of disease in the population that combines morbidity and mortality data from acute illness and sequelae [29,31].

Australia and England and Wales are the only other countries to have conducted a comprehensive assessment of the numbers of illnesses, hospitalizations, and deaths from contaminated food, including an estimate of illness from unspecified agents. Similar to the United States, the Australian study found that norovirus was the leading cause of foodborne illness, accounting for 30% of illnesses caused by known pathogens. In the study from England and Wales, norovirus accounted for only 8% of known foodborne illnesses, primarily due to the low proportion of these viral infections that were estimated to be foodborne (10.8%) [25]. However, a reexamination of stools from the Infectious Intestinal Disease (IID) study using molecular techniques documented higher rates of infection than initially thought, which would

affect estimates of overall foodborne disease burden [14]. The incidence of gastroenteritis specifically due to norovirus infection from all sources from this re-analysis of the IID study and the recent IID2 study ranges from 45–47 cases per 1000 person years, or one episode per person every 20 years [32,33].

Nontyphoidal *Salmonella* and *Campylobacter* were estimated to be leading causes of foodborne illnesses in Australia, England and Wales, and the United States, although nontyphoidal *Salmonella* accounted for a greater proportion of illness in the United States. Recent serologic data from Europe suggests that *Salmonella* infections are far more common than estimated by "burden-of-illness pyramid" methods; however, many of these infections may be asymptomatic [34]. Another common cause of foodborne illness in all three countries was *C. perfringens.* Because clinical laboratories do not usually test stools for this pathogen or its toxin, it is typically only detected when it causes a foodborne outbreak [6,25,35]. In England and Wales, foodborne *C. perfringens* infection was estimated to be more common than foodborne norovirus infection and to cause 22% of all deaths due to foodborne disease [25,36].

The studies in Australia and England and Wales also attributed a large burden of foodborne illness to unspecified agents (73% in Australia and 48% in England and Wales versus 80% in the United States) and have estimated a similar proportion of acute gastroenteritis to be transmitted by food (32% and 26%, respectively, versus 25% in the United States). While it is difficult to direct interventions toward unspecified agents, it is important that policymakers consider the likely total burden due to contaminated food as a driver for regulatory changes [37].

While most acute gastroenteritis is mild and does not require a visit to a physician or any treatment, the annual incidence is large in most countries worldwide. In a recent systematic review, the incidence of diarrhea due to all causes in adolescents and adults in various countries ranged from 0.27–0.88 episodes per person per year [38]. Globally, the incidence of diarrheal disease is highest in children and results in significant mortality each year, particularly in developing countries [39]. While the mode of transmission is difficult to establish in developing countries, partly due to greater exposure to pathogens through unsafe water and inadequate sanitation, contaminated food plays an important role in the transmission of disease [40].

There are important benefits to assessing the national burden of foodborne illness. Knowing the burden of a specific foodborne disease helps food safety agencies prioritize intervention programs, and allows estimation of costs and benefits. In some instances, the results of an estimation process can be quite surprising. For example, the Netherlands assessed the burden of foodborne diseases using DALYs; the analysis showed that toxoplasmosis resulted in the highest burden of all foodborne diseases due to the incidence in neonates and the chronic nature of the disease [29]. This estimation of foodborne disease burden highlighted the importance of preventing *Toxoplasma* infections through food safety interventions.

Assessing and documenting the burden of foodborne disease provides weight to regulatory initiatives, which are often required to provide evidence of economic and social benefit. Estimating the burden of foodborne disease can also help national

authorities understand the performance of surveillance systems for protecting public health. Similar to the United States, Australia and England and Wales assessed the rate of under-diagnosis and under-reporting to surveillance for enteric infections. The surveillance multipliers were considerably lower in Australia, where for every case of salmonellosis reported to surveillance, 6.9 cases were estimated in the community, and in England and Wales, where for every reported case, 3.9 community cases were estimated [17,41]. Knowledge about reporting to public health surveillance has proven important in justifying regulatory measures to prevent contamination of foods, as well as in helping disease investigators understand how well the system is performing.

Global efforts

The WHO and partner organizations have made it a priority to estimate the burden of foodborne diseases at the national level and globally. The WHO training network—the Global Foodborne Infections Network (GFN; see: http://www.who.int/gfn/en/)—runs international courses for epidemiologists and microbiologists working in ministries of health to assist them with conducting surveillance and investigations of foodborne illnesses. The courses include modules on estimating the incidence of foodborne illness, which have contributed to work attributing illnesses caused by several pathogens to food and water in the Latin American region [42].

In 2007, WHO established FERG (see: http://www.who.int/foodborne_disease/burden/en/index.html) to estimate the global burden of foodborne disease [4]. As a result, WHO has initiated several complementary systematic reviews to estimate the global burden of several diseases that are commonly transmitted by food, including those caused by chemical contamination. Some of the agents included in the estimation are peanuts (allergic reactions), cyanide, *Toxoplasma*, *Salmonella*, *Shigella*, and STEC. To strengthen the evidence base for estimating the foodborne disease burden, WHO has initiated studies estimating the national burden of foodborne disease in four countries: Albania, Japan, Thailand, and Uganda. These studies will provide national estimates of the burden of foodborne disease in DALYs and will improve the evidence base for policymakers and regulatory agencies [4].

Recent efforts to estimate the global incidence of nontyphoidal strains of *Salmonella enterica* estimated that there were 93.8 million cases and 155,000 deaths annually, of which 86% might be foodborne [43]. There was a lack of data, particularly from the most populous regions of the world, which highlighted the difficulties of estimating foodborne disease at the global level. Similar to US and other efforts, this study relied on various data sources, including a novel use of rates of salmonellosis in Swedish nationals returning from holidays overseas to estimate disease incidence in destination countries, and estimated incidence for 21 different regions of the world [43]. Despite the difficulties in estimating incidence and outcome on this scale, the analysis presented convincing evidence that *Salmonella* infection results in substantial burden in both developing and developed countries.

Methodological considerations

In any assessment of the burden of foodborne illness, the first step is to identify the aim of the estimation process, as this will clarify which illnesses and agents will be included, the target population, and the intended audience. Depending on the aims, steps in assessing the burden of foodborne disease could include:

- Estimation of the incidence of illness caused by various pathogens, along with their various outcomes, such as hospitalizations, deaths, and sequelae,
- Attribution of illnesses caused by specific pathogens to foodborne transmission,
- Assessment of illnesses characterized by diarrhea or vomiting caused by unknown and unspecified agents, and
- Accounting for uncertainty by providing bounds around point estimates.

A key challenge to producing robust estimates of numbers of illnesses due to food is that it is necessary to attribute a proportion of illnesses caused by each agent to foodborne transmission. For many enteric diseases, there are multiple modes of transmission. Many efforts have relied on assessment of opinions of panels of foodborne disease experts, with modeling of uncertainty around the resulting proportions [44]. However, given the wide range of pathogens and foods, few experts have in-depth knowledge of all aspects. As a result, some expert elicitations have resulted in widely conflicting opinions [45]. In recent years, greater attention has been given to alternative means of attributing the proportion of disease due to food for various pathogens using data from outbreak surveillance, molecular subtyping, and systematic reviews of case-control studies [46]. However, many of these approaches have only been shown to work for certain pathogens, such as *Salmonella* spp., *Listeria monocytogenes*, and *Campylobacter* spp. [47–49].

For some pathogens, researchers have attempted to specifically identify the burden due to specific foods and animal reservoirs, which is very useful to agencies managing risks in the food supply [36]. A good example is the attribution of human *Listeria monocytogenes* infections in England and Wales to specific foods using a Bayesian source attribution model. This study estimated that the most important food sources of listeriosis in the whole population were refrigerated packaged foods bought in stores (23% of infections), finfish (17%), and beef (15%) [48].

Several types of outcome measures can indicate the burden of illness associated with foodborne agents, with the main ones including:

- Numbers or incidence of illnesses, hospitalizations, and deaths in a certain time period and population,
- DALYs lost—a measure of the number of years lived with a disability and the years of healthy life due to a disease in a set time period, and
- Cost—the direct and indirect costs due to a disease in a set time period.

An advantage of the DALY as a measure of burden of illness is that it collapses into a single metric, measures of disability and mortality, including those

due to sequelae. DALYs can be difficult for non-scientific audiences to understand, because they are composite metrics with no easily understood values. DALYs can be very helpful to assess the relative burden among various foodborne agents, but there are still areas requiring improvement, such as development of appropriate disability weights and incorporation of co-morbidities into disease models.

For certain outcome measures, such as cost of illness and DALYs, assumptions must be made about societal willingness to pay or about disability weights for various outcomes [50]. Comparing infectious and non-infectious disease burden using DALYs to allocate resources for public health spending may be problematic due to the short-lived nature of many infectious diseases and the pre-existing preventive programs in place focusing on food safety. Lawmakers easily understand data presented as costs, and it may be necessary to transform disease incidence and impact into costs to achieve consensus about public health interventions. However, even estimating costs for foodborne diseases associated with specific foods can be difficult due to the epidemic potential of some pathogens. For example, *Salmonella* contamination of alfalfa and other sprouted seed has resulted in many episodic outbreaks, but the direct and indirect costs are difficult to estimate on an annual basis. This has proven difficult for food safety regulators proposing food safety interventions based on cost–benefit analysis.

Conclusions

The "burden-of-illness pyramid" approach outlined in this chapter, combined with assessment of unspecified (including unknown) agents, provides a means for national governments to quantify the impact of contaminated food on the population. Conducting these assessments requires development of methods that incorporate many different types of information, such as case surveillance, population-based surveys, investigated outbreak surveillance, expert opinion, and hospitalization and mortality statistics.

Assessing the burden of disease is complex and requires consideration of the availability of data. The resulting estimates can be useful to regulatory agencies in prioritizing and explaining the impact of interventions to improve the safety of the food supply. Several countries have conducted national assessments of the burden of foodborne disease. Some have already been useful for policymakers. In the United States, estimates published in 2011 are being used to help direct policy and interventions and are contributing to other analyses, including evaluating the economic cost of these diseases, attributing illnesses to various food commodities, and estimating the burden of disease caused by sequelae. It is important to note, however, that just as these estimates addressed some of the limitations and data gaps highlighting the United States foodborne illness estimates published in 1999, there is still much future work required to address limitations and data gaps.

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CHAPTER

2

The Foods Most Often Associated with Major Foodborne Pathogens

Attributing Illnesses to Food Sources and Ranking Pathogen/Food Combinations

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Introduction

A risk-based approach to food safety, as called for by the FDA Food Safety Modernization Act and numerous national and international public health bodies, is based on the prioritization of limited resources in ways that most benefit public health [1–7]. This calls for an understanding of which pathogens in which foods are responsible for the greatest burden of foodborne disease. Identifying the most significant pathogen/food pairs involves three steps. First is the estimation of the annual incidence of disease caused by major foodborne pathogens [8,9]. Second is the estimation of integrated measures of public health—such as cost of illness or quality-adjusted life years (QALYs)—that allow for the direct comparison of diseases with very different symptoms, severities, and chronic sequelae [10]. Third is foodborne illness source attribution: estimation of the proportion of illnesses due to each pathogen that can be linked with a specific food source [11,12]. This chapter presents a systematic, national assessment of the relationship between the burden of disease from major foodborne pathogens and food consumption across the entire US food supply [13–15]. It includes a discussion of integrated measures of disease burden, an overview of methods of foodborne illness source attribution, an analysis of US outbreak data, and a ranking of pathogen/food pairs based on outbreak and expert attribution.

Integrated measures of disease burden

Estimates of the number of annual illnesses, hospitalizations, and deaths, as presented in Chapter 1, are critical but incomplete metrics of disease burden. They do not allow for the direct comparison of pathogens—such as norovirus and

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Listeria monocytogenes—with very different symptoms, severities, and outcomes. They also exclude important congenital disease and long-term health outcomes of many foodborne diseases [16]. Integrated measures of disease burden allow for comparisons between foodborne pathogens and with other public health concerns [10].

Health-adjusted life year (HALY) metrics, such as QALYs and disability-adjusted life years (DALYs), are based on the principle that health-related quality of life can be measured on a scale from 0 (death) to 1 (perfect health) [17]. HALYs are computed by multiplying the preference weight for a given health state by the duration of that health state, in years. HALY loss is measured as the difference between the HALY for baseline health (either population average or perfect health) and the HALY for an adverse health state [18]. Both QALYs and DALYs have been used for prioritization of foodborne and zoonotic pathogens (e.g., [15,19]; Kemmeren et al., 2009; [20–23]).

Economists prefer willingness-to-pay (WTP) to HALY and other monetary measures because it is consistent with welfare theory; it is based on the tradeoffs that individuals must make between health and other goods [24,25]. Cost of illness (COI), in contrast with HALYs and WTP, does not measure intangible costs (e.g., "pain and suffering"), but quantifies measurable monetary costs such as health care costs and lost productivity (e.g., sick days). COI is relatively straightforward to compute but reflects a lower bound on socioeconomic costs, whereas WTP is a more complete measure but available for few health states due to the difficulty and costs of estimating it [26,27]. COI values have been estimated for foodborne pathogens in a number of countries ([28–30]; Kemmeren et al., 2009; [21,31]).

Two recent studies that estimate disease burden in the United States for a number of pathogens based on [9,15,32] employ a modified cost of illness approach that uses traditional COI values for morbidity and WTP values for mortality in the form of a value-of-statistical life. Scharff also estimates an "enhanced cost of illness" that monetizes QALY estimates for morbidity states to approximate the "pain and suffering" captured by WTP. This approach has been heavily criticized on theoretical grounds [33–35] and based on empirical findings (Fryback and Lawrence, 1997; [36,37]). [15] also estimate QALY loss based on the EuroQoL EQ5D instrument [38].

These estimates are summarized for 14 pathogens in Table 2.1. "Basic" cost of illness estimates are roughly comparable, while Scharff's enhanced approach doubles the estimates. Table 2.1 shows that the burden of foodborne disease due to known pathogens is concentrated among a relatively small number of hazards. Roughly 90% of the loss estimated by Hoffmann, and about 85% of the loss estimated by Scharff, is concentrated in five pathogens: nontyphoidal *Salmonella enterica, Campylobacter* spp., *Listeria monocytogenes, Toxoplasma gondii*, and norovirus.

Methods of foodborne illness source attribution

There is a growing body of scientific research focused on methods for attributing foodborne illness to food sources [11,12]. The five most common approaches are

Table 2.1 Mean and Ranges of Annual Burden of Disease Caused by 14 Foodborne Pathogens*

	Hoffmann [46][a]		Scharff [80][a]	
Pathogen	**QALY Loss**	**Cost of Illness (millions of $, 2009)**	**Basic Cost of Illness (millions of $, 2010)**	**Enhanced Cost of Illness (millions of $, 2010)**
Campylobacter spp.	13,256 (7,993–26,021)	1,747 (841–4,152)	1,560 (437–4,031)	6,879 (1,134–20,129)
C. perfringens	876 (83–3,942)	309 (20–1,572)	382 (45–1,443)	466 (56–1,641)
Cryptosporidium	341 (40–1,265)	47 (3–192)	118 (21–394)	168 (21–569)
Cyclospora	10 (0–33)	2 (0–8)	11 (0–39)	17 (0–63)
E. coli O157:H7	1,660 (131–7,872)	255 (25–1,102)	607 (121–1,827)	635 (120–1,931)
STEC non-O157	149 (10–268)	24 (1–77)	101 (11–273)	154 (16–467)
Listeria monocytogenes	9,375 (1,531–23,525)	2,577 (204–6,952)	2,025 (95–6,613)	2,040 (105–6,644)
Norovirus	5,027 (2,897–7,832)	2,002 (1,140–3,147)	2,896 (1,545–4,728)	3,677 (1,424–6,912)
Salmonella, nontyphoidal	16,782 (304–44,380)	3,309 (160–8,586)	4,430 (1,479–10,881)	11,391 (2,459–29,064)
Shigella	545 (14–3,372)	121 (8–639)	257 (38–768)	1,254 (105–4,526)
Toxoplasma gondii	10,964 (6,026–16,771)	2,973 (1,763–4,437)	3,100 (1,112–5,726)	3,456 (1,019–6,606)
Vibrio, other[b]	210 (60–595)	103 (28–298)	146 (57–348)	176 (53–415)
Vibrio vulnificus	557 (294–882)	291 (154–460)	268 (54–538)	268 (54–538)
Yersinia enterocolitica	1,415 (13–8,216)	252 (3–1,412)	409 (69–1,662)	1,107 (167–3,311)
Total	**61,166 (19,397–144,974)**	**14,012 (4,352–33,034)**	**16,310 (5,084–39,271)**	**31,688 (6,733–82,816)**

[a]*Ranges for [46] reflect 90% credible intervals for incidence as reported in [78], while ranges for [80] also incorporate uncertainty in economic components.*
[b]Vibrio *other" represents non-cholera* Vibrio *species, including* V. parahaemolyticus.

analysis of outbreak data, studies of sporadic cases, comparative exposure assessment, use of microbial subtyping, and expert elicitation.

A critical concept for interpretation of attribution results is the "point of attribution," or the location in the farm to fork chain addressed by a specific analysis [11]. Microbial subtyping approaches generally focus on the point of production, such as an animal reservoir, whereas epidemiological approaches, such as outbreak analysis, are focused at the point of consumption. Because contamination can occur at many points between production and consumption (e.g., cross-contamination in the kitchen), results from these two analyses can be difficult to compare.

Another critical factor for interpretation of attribution studies is how food sources are categorized. There is no consensus scheme for attribution, and schemes depend on the attribution approach. For example, a reservoir-based approach may not distinguish between ground and whole muscle cuts of beef, though an outbreak-based method might. Likewise, a case-control study may not attribute to "chicken" but rather to "chicken prepared in a restaurant" and "chicken prepared at home." These differences in schemes make it difficult to combine findings from multiple attribution studies [12].

Epidemiologic approaches to attribution generally "work backwards" from data on human illness to estimate the role of foods. Outbreak data—compilations of foodborne outbreak reports with known pathogen and food vehicle—have been analyzed for attribution for many countries and regions [14,39–45]. For each pathogen, the number of outbreaks or outbreak cases due to a particular food category is divided by the total across all foods to obtain an attribution percentage. The key advantage of outbreak-derived attribution is that data are available for many pathogens at the national level across time, allowing for comparison of a wide swath of pathogens. The major disadvantages are misclassification bias (outbreaks are uncharacteristic of sporadic cases), investigation bias (differences in investigation due to outbreak size, duration, setting, or organism), detection bias (certain vehicles are more likely to be identified), and temporal or geographic inconsistency [46]. Because outbreaks identify foods at the point of consumption, food vehicles include those cross-contaminated by improper handling during food preparation.

Epidemiological studies of sporadic infections provide important attribution information that may better reflect risks in the general population [11]. Numerous case-control studies have been conducted in the USA for a number of major foodborne pathogens (e.g., [47–52]). [53] present a meta-analysis of 34 *Salmonella* case-control studies conducted between 1989 and 2003 in 11 countries. Similarly, [54] analyze 38 *Campylobacter* case-control studies from 1983 to 2004 in 14 countries. Case-control studies are "population-based" and therefore more reflective of sporadic infection, but food vehicles are rarely laboratory-confirmed. Case-control studies can have issues due to selection bias in questionnaires, recall bias of respondents, long exposure windows, acquired immunity, and the fact that case patients generally reflect more serious cases that have sought health care [55]. Case-control studies often leave a significant portion of illnesses unattributed, which impedes interpretation [10].

Comparative microbial risk assessments offer a contrast to epidemiologic approaches, as they "work forward" from data on the prevalence and/or level of microbial contamination in food sources to estimate human illnesses due to these routes [56]. Attributable fractions are estimated by comparing the results of separate risk assessments for all relevant food and non-food transmission routes [12]. Each risk assessment models pathogen growth, persistence, and inactivation along the transmission route, relies on food consumption data to estimate exposure, and predicts infections based on dose–response models [57]. Comparative exposure assessments do not predict illnesses, but rely instead on comparing estimates of exposures [12]. Examples of comparative risk assessments include those done for *L. monocytogenes* in ready-to-eat foods ([58,59]; FDA/FSIS, 2003), *E. coli* O157:H7 in beef, sheep, and pork [60], and *T. gondii* in beef, sheep, and pork [61]. Comparative exposure assessments have been conducted for *Campylobacter* [89]. These approaches have significant data requirements and modeling uncertainties; they are likely to perform best when there are limited and well-understood routes of exposure.

Microbial subtyping approaches to attribution essentially work both forwards and backwards, using microbial data from both human illness and contaminated foods and animals. They are based on the premise that animal species carry unique host-specific populations of microorganisms. By characterizing isolates of a specific pathogen by genotypic or phenotypic subtyping, the distribution of subtypes of isolates from food and animals can be compared with those isolated from humans [11]. Subtyping methods include serotyping, phage typing, pulsed field gel electrophoresis, sequence-based genotyping, antimicrobial resistance patterns, and biomarkers [62]. Because subtyping approaches involve direct linking of human and animal data, they are very powerful for certain zoonotic pathogens, but are also data- and resource-intensive [10]. Subtyping approaches cannot easily distinguish between routes of transmission, but instead point upstream to reservoirs, so they are of limited utility for assessing the role of produce in illness.

Subtyping methods of attribution have been conducted for *Salmonella* in Europe ([63,64]; Pires et al., 2011; [65–67]), New Zealand [68], Japan [69], and the USA [70]. *Campylobacter* subtyping attribution has been conducted in the UK [71], New Zealand [68,72], the Netherlands, and Denmark. [73] describe a novel Bayesian model based on typing and temporal patterns for *Campylobacter*. [74] applied the subtyping approach to *Listeria monocytogenes* in the UK, with some success.

Expert elicitation is a set of formal research methods used to provide alternative parameter estimates where there are gaps or disagreement in available data or to characterize uncertainty about existing scientific knowledge [75,76]. The elicitation of expert judgment using structured protocols is increasingly used in policy models, particularly as the methods for capturing uncertainty and validating expertise have matured [77,78]. Several studies have employed this approach to attribute illnesses to food sources [21,79–85]. Expert elicitation is sensitive to a number of biases—selection of experts, wording choice, systematic biases in existing data—so effort must be made in the elicitation protocol to minimize these effects and measure them when possible.

Analysis of US outbreak data for food source attribution

My colleagues and I have analyzed 10 years (1999–2008) of reported US foodborne outbreaks, obtained from the CDC's online repository [86], for proportional attribution of illnesses due to 12 pathogens to food sources [14,15]. As with all other outbreak attribution studies (e.g., [39–42,44]), we average multiple years of data to address data sparseness and year-to-year variability. There are four stages of analysis: determining food categories; cleaning data; coding outbreaks into food categories; and making modeling assumptions.

There is no consensus on food categories for outbreak-derived attribution, and many studies use their own scheme. Categorization entails judgment, and it was ours that the most practicable and applicable approach was to focus food categories at the point of consumption. As such, our categories are similar to those in other consumption-oriented schemes [39–41,45], and distinct from commodity or ingredient-driven approaches [42–44]. Our scheme is intended to be useful to food safety practitioners, intelligible to consumers, and consistent with guidance on risk ranking categorization [87]. Our 12 categories, as shown in Table 2.2, are: beef, pork, poultry, deli and other meats, game, seafood, eggs, dairy, produce, beverages, baked goods, and complex foods. This last category will be explained momentarily.

For the 14 pathogens we examined, we obtained data on 5830 outbreaks associated with 172,495 cases of illness. There were no outbreaks due to *T. gondii* or *Vibrio vulnificus*. In 3242 outbreaks (93,053 cases), the food vehicle was listed as "unknown" or listed too many foods to be interpretable. Thus, our data includes 2588 "attributable" outbreaks with 79,442 associated cases.

Available foodborne outbreak data do not use a standardized coding scheme for the field identifying responsible food vehicles, so text-based entries are often incomplete, use colloquial terms, or are otherwise vague. For example, an outbreak might identify only "ethnic food." We developed a coding scheme to aid categorization of food vehicles into our scheme. For simple foods in which a single food item or specific ingredient is identified, we code by that food. Many outbreaks identify multi-ingredient dishes (e.g., lasagna), however, which we classified in three ways: Those in which the vast majority of ingredients fall into a single category are lumped into that category (e.g., salsa as produce); those in which beef, poultry, pork, seafood, or eggs were the primary ingredient were classified by species (e.g., beef stew as beef, chicken pot pie as poultry, quiche as egg); and all remaining dishes (about a quarter of all attributable outbreaks) were classified as "complex foods."

Outbreak-derived attribution can be computed based on the distribution of outbreak events across foods [41,45], or on the distribution of outbreak cases across foods [39,40,42,44]. We determined that attribution based on outbreak events was likely to be more representative of sporadic cases due to biases in outbreak data to over-represent large events and the distortion caused by very large events on attributable proportions based on outbreak case counts [14]. For example, while the mode outbreak size is 2 cases, the median is 12, and the mean is 28, evincing major rightward skew. Outbreak size varies by food category: Produce averages 51 cases, dairy 36 cases, and complex foods 31 cases, while beef, pork, and seafood

Table 2.2 Foodborne Outbreaks due to 12 Pathogens, by Food Category, 1999–2008[a]

Food	*Campylobacter*	*C. perfringens*	*Cryptosporidium*	*Cyclospora*	*E. coli* O157:H7	*STEC* non-O157	*Listeria*	Norovirus	*Salmonella*	*Shigella*	*Vibrio* spp.	*Yersinia*	Total
Beef	7	118	0	0	79	6	0	47	43	6	0	0	**306**
Pork	3	30	0	0	0	0	1	32	41	0	0	5	**112**
Poultry	22	99	0	0	1	1	1	87	130	4	0	0	**345**
Deli/other meats	2	10	0	0	5	0	7	24	18	2	0	0	**68**
Game	2	1	0	0	0	0	0	1	0	0	0	0	**4**
Eggs	0	0	0	0	0	0	0	7	73	0	0	0	**80**
Dairy	61	2	0	0	8	3	6	24	35	1	0	0	**140**
Seafood	3	4	0	0	1	0	1	103	33	3	59	0	**266**
Produce	7	11	0	11	26	2	1	176	109	5	0	0	**348**
Beverages	0	0	2	0	3	2	0	23	7	0	0	0	**37**
Breads and bakery	0	0	0	0	1	0	0	89	16	0	0	0	**106**
Complex foods	13	87	2	3	19	1	3	512	116	20	0	0	**776**
Total attributable	**120**	**362**	**4**	**14**	**143**	**15**	**20**	**1,125**	**621**	**41**	**59**	**5**	**2,588**

[a]*Outbreaks due to "unknown" foods and "multi-source" are not included.*

average 23, 24, and 15 cases respectively [14]. Thus, attribution by cases decreases proportions from meat and animal products and increases to those vehicles that are traditional cross-contamination risks. Table 2.2 shows the number of outbreaks from 1999–2008 attributable to each pathogen/food combination.

Assessing the applicability of outbreak-derived attribution estimates

While outbreak data have been used as a primary source of attribution information in the USA, they may not provide satisfactory attribution for all relevant pathogens. We conducted a series of analyses to assess for which pathogens outbreak-derived attribution is inadequate, as described in detail in [14]. The results are shown in Table 2.3.

First, we examined the number of attributable outbreaks in the data set. For some pathogens, there are simply too few outbreaks with identified food vehicles.

Table 2.3 Assessing Applicability of Outbreak-Derived Food Source Attribution

	Total Number of Attributable Outbreaks (1999–2008)[a]	Ratio of Overall Incidence to Outbreak Cases[b]	Sum of Mean Difference Squared[c]	Mean Standard Deviation of Expert Estimates[d]
Campylobacter	120	1,712	3,307	0.91
C. perfringens	362	495	–	–
Cryptosporidium	4	1,152	3,622	1.64
Cyclospora	14	87	62	0.42
E. coli 0157	143	132	20	0.76
STEC non-O157	15	745	–	–
L. monocytogenes	20	55	827	0.96
Norovirus	1,125	555	701	1.77
Salmonella	621	296	218	1.02
Shigella	41	256	1,198	1.68
Toxoplasma	0	–	2,706	2.02
Vibrio spp.	59	780	9	0.25
Yersinia	5	11,909	562	1.38

[a]Number of foodborne outbreaks between 1999 and 2008 with identified etiology and food vehicle.
[b]Number of estimated annual cases [78] divided by the average annual number of foodborne outbreak cases between 1999 and 2008.
[c]Sum across foods of the mean difference squared between experts' food attribution percentages and outbreak attribution percentages based on data from 1993 to 2002 (to utilize data concurrent with the expert elicitation conducted in 2003).
[d]Mean across foods of the standard deviation among experts' food attribution percentages.

Second, we examined the ratio of estimated disease incidence in the US population as estimated by [9] to the average annual reported outbreak cases from 1999–2008. This represents the number of illnesses one would expect in the population for each outbreak case.

Third, we compared outbreak-derived attribution with the results of an expert elicitation. As part of our larger effort, [82–84] conducted an expert elicitation in 2003 for 12 pathogens to estimate attribution. The elicitation involved 45 leading food safety scientific experts identified through an iterative peer nomination process. For each pathogen, experts provided a best estimate and 90% confidence interval for the proportion of illnesses due to consumption of each of a dozen food categories. Food categories were nearly identical to those in the aforementioned outbreak analysis, with the exception of "complex foods." Experts had trouble interpreting this category due to overlaps with single food categories. Following [88], the percent attributable to each pathogen/food combination was estimated as the unweighted mean of all experts' best estimates.

To measure how closely experts agree with outbreak data, we computed the sum of the mean difference squared between outbreak and expert percentages. Because experts draw on a range of scientific evidence, and not just outbreaks, large differences point to pathogens for which outbreak data may not be representative.

Fourth, we evaluate the mean standard deviation across experts' individual attribution percentages as an indicator of scientific agreement and, therefore, as a signal of the quality of available scientific knowledge on attribution for that particular pathogen. Of note, while expert estimates for *Campylobacter* and *T. gondii* differ quite a bit from outbreak-derived estimates, experts are far more confident in their assessment for the former than the latter.

Based on the totality of the evidence, we determined that outbreak-derived estimates are insufficient for *Campylobacter*, *Cryptosporidium parvum*, *T. gondii*, and *Yersinia enterocolitica*. Thus, for the purposes of ranking pathogen/food combinations, we use expert-derived attribution estimates for these four pathogens and outbreak attribution for the other 10, as shown in Table 2.4.

Ranking pathogen/food combinations

By applying outbreak-derived and expert-based attribution percentages (Table 2.4) to estimates of cost of illness and QALY loss for 14 pathogens (Table 2.1), we estimate the public health impact of 168 pathogen/food pairs in both cost of illness and QALY loss. The combined rank for each pair is the average of the cost of illness rank and the QALY loss rank. The burden of disease is highly concentrated; the top 10 pathogen/food pairs account for over $8 billion and 36,000 lost QALYs, or about 60% of the total impacts across all pairs. The top 50 pairs account for over 90% of the total.

Figure 2.1 shows the top 10 pathogen/food combinations, sorted by combined rank. *Campylobacter* in poultry ranks first, in part because experts attribute over 70% of this pathogen to a single food category. *Salmonella* illnesses, on the other hand, are attributed in significant numbers to multiple foods, so *Salmonella* appears

Table 2.4 Selected Food Source Attribution for 14 Pathogens Based on Outbreak Data and Expert Elicitation

	Campylobacter	*C. perfringens*	*Cryptosporidium*	*Cyclospora*	*E. coli* O157:H7	STEC non-O157	*Listeria*	Norovirus	*Salmonella*	*Shigella* spp.	*Toxoplasma*	*Vibrio* spp.	*Yersinia*
Data Source[a]	***EXP***	***OUT***	***EXP***	***OUT***	***OUT***	***OUT***	***OUT***	***OUT***	***OUT***	***OUT***	***EXP***	***OUT***	***EXP***
Food													
Beef	4.4	32.6	7.4	0.0	55.2	40.0	0.0	4.2	6.9	14.6	23.2	0.0	2.2
Beverages	0.0	0.0	9.0	0.0	2.1	13.3	0.0	2.0	1.1	0.0	0.0	0.0	1.1
Baked goods	0.0	0.0	0.3	0.0	0.7	0.0	0.0	7.9	2.6	0.0	0.0	0.0	0.0
Dairy	7.8	0.6	5.8	0.0	5.6	20.0	30.0	2.1	5.6	2.4	2.4	0.0	12.2
Eggs	2.6	0.0	0.3	0.0	0.0	0.0	0.0	0.6	11.8	0.0	0.0	0.0	0.1
Game	2.0	0.3	5.4	0.0	0.0	0.0	0.0	0.1	0.0	0.0	20.4	0.0	2.0
Deli & other meats	0.9	2.8	1.4	0.0	3.5	0.0	35.0	2.1	2.9	4.9	1.7	0.0	1.8
Complex foods	0.0	24.0	0.0	21.4	13.3	6.7	15.0	45.5	18.7	48.8	0.0	0.0	0.0
Pork	4.4	8.3	2.0	0.0	0.0	0.0	5.0	2.8	6.6	0.0	41.0	0.0	71.6
Poultry	72.0	27.3	1.2	0.0	0.7	6.7	5.0	7.7	20.9	9.8	3.7	0.0	1.2
Produce	5.2	3.0	59.5	78.6	18.2	13.3	5.0	15.6	17.6	12.2	7.0	0.0	3.2
Seafood	0.8	1.1	7.7	0.0	0.7	0.0	5.0	9.2	5.3	7.3	0.5	100.0	4.7
Total	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

[a]Data source indicates whether attribution percentages are based on outbreak data ("OUT") or expert elicitation ("EXP"). Outbreak attribution based on data from 1999–2008 with known etiology and known food vehicle [4]. Expert attribution based on a 2003 elicitation [43,44].

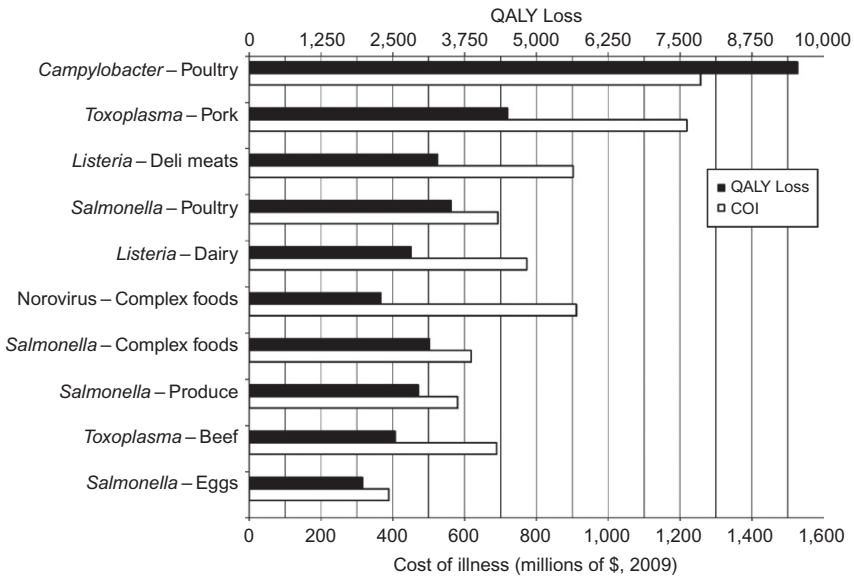

FIGURE 2.1

four times in the top 10. *Toxoplasma* in pork and beef both rank in the top 10, though it must be repeated that attribution data for this parasite is sorely lacking, and experts show significant uncertainty in their assessment. *Listeria* in deli meats ranks very highly, though outbreak data do not likely fully account for a significant decline in contamination rates of prepackaged deli meats in the 2000s.

Our efforts to rank pathogen/food pairs show that a systematic approach can be used to produce tools for food safety priority setting that are based on quantitative analysis of empirical data. The analysis serves as a starting point for understanding the relative importance of microbial hazards and their transmission routes. It suggests that although there are very many microbial hazards that can cause illness due to consumption of any number of foods, a select few pathogens and foods are responsible for an outsized portion of the burden of foodborne disease. This analysis shows the importance of including chronic sequelae and other disease outcomes not included in surveillance of acute disease. Further characterization of long-term health outcomes associated with foodborne infection is critical to measuring the full impact of these diseases. Our analysis also shows the limitations of available attribution data; outbreak-derived attribution provides useful insight into the pattern of disease, and expert elicitation can be used both to characterize uncertainty and fill data gaps when empirical data are missing, but even together they only tell part of the story. Clearly, there is much work to be done.

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CHAPTER

Microbial Food Safety Risk Assessment

3

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Introduction

Foodborne illness has major consequences worldwide, in terms of human health burden and costs to society and the food industry. Advanced laboratory detection and enumeration methods, targeted surveillance, and rapid outbreak recognition have improved our ability to identify and track foodborne disease and implement significant pathogen control measures. However, disease-causing microorganisms are capable of adapting to new niches, new vehicles of transmission, and new hosts, acquiring novel resistance and virulence mechanisms along the way. New technologies, consumer preferences, a global food trade marketplace, and shifts in population demographics add to the complexity of understanding and managing food safety risks. Microbial risk assessment (MRA) is a systematic approach to aid our understanding of complex food systems and to translate the potential presence of pathogens in the food production, processing, and preparation environments into statements of the likelihood and magnitude of a food safety risk defined in terms of adverse public health outcomes.

Since the first publication of this chapter, MRA has seen considerable advancements in analytical methods and computer simulation models, as well as the development of a variety of "risk-based" tools designed to make risk modeling and analyses user-friendly. Published risk assessments for most of the significant foodborne pathogens are found in the scientific literature or online repositories such as FoodRisk.org. Greater recognition of the value of risk assessment as a management tool and a means to predict the effectiveness of options for mitigating risk has encouraged targeted and rigorous data collection at all points of the food chain including consumer food handling practices. Through the collaborative efforts of the Food and Agricultural Organization (FAO) and the World Health Organization (WHO), considerable resources are now available to provide guidance for conducting risk assessments. The FAO/WHO Joint Experts on Microbial Risk Assessment (JEMRA) have also provided an extensive series of risk assessments on priority pathogen/food combinations that are relevant to most countries and which can serve as templates to adopt and modify using an individual country's

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industry-specific data and knowledge (www.fao.org/food/food-safety-quality/scientific-advice/jemra/en/). This chapter serves as a general introduction to MRA, and readers are encouraged to access these resources for more detailed guidance.

Background

Structured scientific risk assessment processes were introduced within US federal regulatory agencies during the late 1970s as a means of standardizing the basis for decision-making [1]. These were driven by the need for regulatory action in situations where large numbers of people were, or could be, exposed to relatively low levels of chemical substances that had been identified as hazardous to health, but only under conditions of relatively intense exposures [2]. Formally defined, risk is not only a measure of the likelihood, or probability, of something going wrong, i.e., an adverse event or situation occurring. It is also a measure of how bad it will be if something does go wrong [3]. Managing risks cannot be based on science alone, and the decision-making processes and communication of information based on risk assessment activities are described as risk management and risk communication [1,4]. These three elements are collectively referred to as "risk analysis." A recent report, *Science and Decisions: Advancing Risk Assessment*, published by the [5] provides new guidance to strengthen the scientific basis, credibility, and effectiveness of risk assessment practices and subsequent risk management decisions [6].

Although risk analysis has been applied in many fields for several decades, its adoption for microbial food safety has been relatively recent. In 1994, the US Council for Agricultural Science and Technology published a report advocating the use of risk assessment to establish priorities for managing and improving food safety [7]. The recommendations were revisited in 1998 with even stronger recommendations to base food safety policy on risk assessment and risk management practices [8]. At the same time, the World Trade Organization agreements recognized formal risk assessment as a means to evaluate the safety of commodities in international trade (i.e., plants, animals, and foods), establish consistent standards and guidelines, and resolve non-tariff trade issues [9]. The Codex Alimentarius Commission (CAC) is designated as the international body responsible for defining risk assessment principles and practices for all foodborne hazards, and for promoting overall consistency and clarity in the establishment of Codex standards through the use of risk analysis principles. Advice provided by member countries and FAO/WHO has resulted in guidance documents for food safety risk analysis for international purposes and to assist governments to establish national policies [10–15].

Many risk-based methodologies can be shared and adapted from advancements made in other fields. However, between disciplines, and even among practitioners within a field, some differences exist in terminology used to categorize activities and risk parameters. The basic framework described by NRC underlies most risk

paradigms, and the different nuances in language primarily arise from specific factors or considerations that influence the risk issue in one field versus another.

Table 3.1 lists the CAC definitions of terminology adopted for microbial food safety risk analysis [10]. *Risk assessments* are typically divided into four distinct steps: hazard identification, exposure assessment, dose–response assessment, and risk characterization [1]. For food safety hazards, the term "hazard characterization" has been introduced—a step that includes a dose–response assessment if the data are available, but allows more subjective assessments of the consequences of exposure if a dose–response assessment cannot be carried out (Figure 3.1).

Table 3.1 Definitions of Risk Analysis Terminology for Foodborne Hazards [10]

Hazard	A biological, chemical, or physical agent in, or condition of, food with the potential to cause an adverse health effect
Risk	A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food
Risk Analysis	A process consisting of three components: risk assessment, risk management, and risk communication
Risk Assessment	A scientifically based process consisting of the following steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization
Quantitative Risk Assessment	A risk assessment that provides numerical expressions of risk and indication of the attendant uncertainties
Qualitative Risk Assessment	A risk assessment based on data, which, while forming an inadequate basis for numerical risk estimations, nonetheless when conditioned by prior expert knowledge and identification of attendant uncertainties permits risk ranking or separation into descriptive categories of risk
Hazard Identification	The identification of biological, chemical, and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods
Hazard Characterization	The qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with the hazard; for the purpose of microbiological risk assessment, the concerns relate to microorganisms and/or their toxins
Dose–Response Assessment	The determination of the relationship between the magnitude of exposure (dose) to a chemical, biological, or physical agent and the severity and/or frequency of associated adverse health effects (response)
Exposure Assessment	The qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant

(*Continued*)

Table 3.1 (Continued)

Risk Characterization
The process of determining the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse effects in a given population based on hazard identification, hazard characterization, and exposure assessment

Risk Estimate
Output of risk characterization

Transparent
Characteristics of a process where the rationale, logic of development, constraints, assumptions, value judgments, decisions, limitations, and uncertainties of the expressed determination are fully and systematically stated, documented, and accessible

Uncertainty Analysis
A method to estimate the uncertainty associated with model inputs, assumptions, and structure/form

Risk Management
The process of weighing policy alternatives in the light of results of risk assessment and, if required, selecting and implementing appropriate control options, including regulatory measures

Risk Communication
The interactive exchange of information and opinions concerning risk and risk management among risk assessors, risk managers, consumers, and other interested parties

Food Safety Objective (FSO)[a]
The maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP)

Performance Objective (PO)[a]
The maximum frequency and/or concentration of a hazard in a food at a specified step in the food chain before the time of consumption that provides or contributes to an FSO or ALOP, as applicable

Performance Criterion (PC)[a]
The effect in frequency and/or concentration of a hazard in a food that must be achieved by the application of one or more control measures to provide or contribute to a PO or an FSO.

[a]*CAC, 2011.*

While risk assessment is the scientifically based platform for risk analysis, it must be highly integrated with management and communication activities to be a useful and valid methodology to inform decision-makers and others. *Risk management* is the decision-making process, in consultation with all interested parties, considering other factors in addition to the science, and selecting the appropriate action to be taken. This also encompasses preliminary risk management activities, including identifying the food safety issue and defining the problem, commissioning a risk assessment if warranted, clearly articulating the questions the risk assessment should address, and formulating policies to guide the risk assessors in their work [11,14,17].

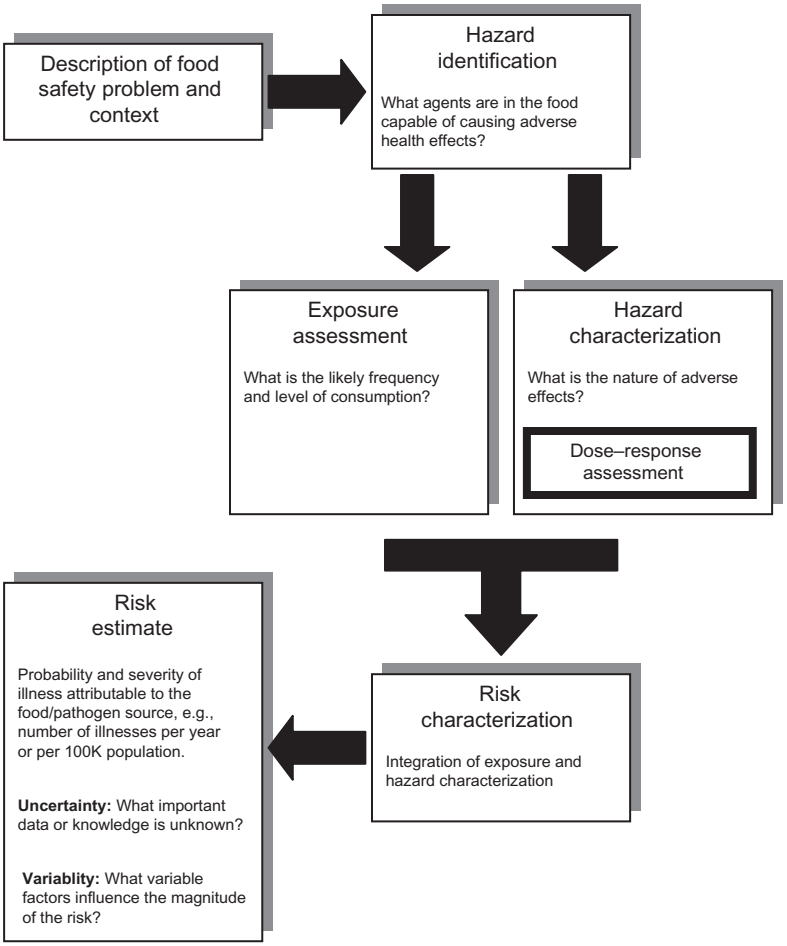

FIGURE 3.1

Steps of microbial food safety risk assessment.

Adapted from [16].

Risk communication is an interactive exchange of information and opinions throughout the risk analysis process with all stakeholders, including the explanation of risk assessment findings and the basis of risk management decisions. The scientific process of risk assessment should be objective and fact-finding, and not influenced by other management considerations. However effective communications between managers and analysts may be, it is crucial to ensure that the right questions are being addressed, that time limitations and resource constraints are acknowledged, that the approach taken to assess the risk is appropriate to the magnitude and urgency of the risk issue, and that the limitations of the assessment are understood by managers and stakeholders [15,17,18].

Managing microbial food safety risks

Evaluations of risks associated with foodborne hazards in the past have most often been general considerations of the hazard, routes of exposure, handling practices, and/or consequences of exposure. Many policies and regulations concerning microbial food safety hazards have been largely based on qualitative information and observations, and often focus on only one segment of the food chain to control and/or eliminate food hazards. Increasingly, it has been acknowledged that effective food safety risk management strategies must consider all steps in the food chain in order to optimize control options, and, equally important, that there is no such thing as "zero risk" [19]. Management decisions must be made about the acceptability, or tolerability, of a risk, which is both a societal and scientific issue, in order to most appropriately allocate limited resources to minimize unacceptable risks to society. In order to do this, some measure of the likelihood and magnitude of the public health burden is needed in order to balance against the costs associated with invoking new regulatory or other action.

The hazard analysis critical control point (HACCP) approach is a risk management strategy that has been applied successfully for decades by the food industry for processes that are designed to reduce or destroy microbes, and to prevent the introduction and/or recontamination of product. Key steps in the processing, distribution, marketing, and commercial preparation of foods, which are critical to the safety of the product (i.e., critical control points or "CCPs"), are monitored and controlled. In general, the HACCP approach has been and continues to be effective and efficient. However, the efficacy of HACCP is limited by its inability to quantify the potential combined result of multiple control-point deviations, and to relate the operation of an HACCP system to a measurable public health outcome [20]. Very low levels of pathogens that may be present in prepared foods, such as *Listeria monocytogenes* in ready-to-eat meats, are largely undetectable by sampling and testing [21]. Such low numbers may present a very small risk, however, under conditions of temperature abuse, pathogen numbers, and consequently risk increase. Pathogens are often present on raw foods of animal origin, such as *Salmonella* in raw poultry, but such products are intended to be cooked (and thus ideally eliminated) by the consumer. These types of situations pose challenges in determining how "risky" a food may be at the point of consumption, and whether that risk is acceptable or not. Simply identifying and describing the potential threat is insufficient information. The value added through the use of risk assessment is in the inference of the likelihood of adverse outcomes by combining a representation of the risk-generating system (e.g., one or all stages of the food chain) with formal rules of inferring probability to arrive at logical conclusions about the likelihood and magnitude of the risk [18].

International trade agreements also underline the need to quantify the level of safety ultimately achieved by a food safety system. The same degree of safety can be achieved using alternative processes and different national and industry control systems, but this must be demonstrated by quantifying the end product risk to consumers. Hence, use of risk assessment offers flexibility to food industries in designing their processes to achieve the specified food safety goal, and regulatory systems established that are appropriate for a nation's food supply and population rather

than attempting to create identical food control programs among trading partners and within individual industries.

An important international advancement has been the introduction of new "metrics" to help industry and the governments that oversee them formulate public health-based targets for control measures in food production and processing. These are referred to as "food safety objectives" (FSO), "performance objectives" (PO), and "performance criteria" (PC) (Table 3.1, [11,21–23]). Together with traditional controls and measures, such as process and microbiological criteria, these new metrics are linked to a measure of public health (e.g., numbers of illnesses in a population or risk per meal) defined as the appropriate level of protection (ALOP) through the risk assessment process.

Beyond determining the current status of food safety and consumer protection resulting from a food production system, a valuable attribute of the risk assessment process is that it can be designed to quantitatively evaluate the effectiveness of potential interventions and risk mitigation actions, at any point from farm to consumer, before actually implementing any changes to the system. Similarly, risk models can be created to investigate alternative "future scenarios," for example, how potential changes in climate conditions may produce potential new threats in the food supply. Such applications help to identify those points where risk management efforts should focus, where to allocate resources to maximize the benefits of risk reduction actions, and where more research or epidemiological investigation may be needed to fully understand a current or future problem [24].

And finally, an underlying premise of risk assessment is transparency, in that the rationale, logic of development, constraints, assumptions, value judgments, decisions, limitations, and uncertainties of the determination are fully and systematically stated, documented, and accessible for review [10]. This aids in discussions about risk and risk management among stakeholders, particularly when a risk issue is contentious, and supports communication with all interested parties, including consumers.

The risk assessment framework

Hazard identification

The first step in risk assessment is to describe the association between the microbial pathogen(s) in a food and human illness [10]. A hazard identification step is primarily a brief qualitative description of the risk issue, providing the association between the pathogen(s) in a food and human illness, and the background, evidence and rationale for the assessment [16]. It is here that the scope of the assessment is defined: what parameters are included or excluded, the population of concern, and the nature of the public health impact that is being considered for the risk estimation measure.

Exposure assessment

The output of exposure assessment is an estimate of the likelihood of a consumer being exposed to the pathogen in the food and to the numbers of the organism

(dose) in a single serving at the time of consumption [10]. The first step is to produce a description of the food pathway, which may include all or only some of the stages of production, processing, distribution, handling, and consumption [16]. Contamination sources, the likely numbers that may be introduced and at what frequency, and the influence of factors that affect the distribution, survival, growth, inhibition, or inactivation of the organism are considered. Exposure estimates typically must incorporate predictive microbiology models to characterize the behavior of pathogens under various extrinsic (e.g., temperature) and intrinsic (e.g., pH) conditions presented by the food and by normal food handling practices [25,26]. Amounts of food consumed and frequency of consumption are typically considered in the assessment. Guidelines provided by [27] provide a practical framework and approach for undertaking exposure assessment of microbiological hazards in foods in the context of an MRA or as a stand-alone process.

Hazard characterization

Hazard characterization describes the nature and probability of adverse human effects as a function of viable pathogen numbers ingested [10,28]. Health consequences are greatly affected by the physiological and immunological status of the host as well as the nature of the pathogen. The population of concern may be all consumers or may specifically consider only those at increased risk such as young children and/or immunocompromised subpopulations [29,30].

The dose–response assessment specifically refers to a mathematical relationship that translates the number of organisms ingested into a probability of an adverse outcome. Traditionally, it has been assumed that there is a threshold number of organisms, or a minimum infectious dose (MID), that must be ingested before infection or disease occurs. This implies there is no effect below some exposure level, but above that level the effect is certain to occur. However, improved surveillance and detection methodologies have shown that adverse outcomes can happen in even healthy adults at very low exposures. Thus, the biological feasibility of a non-threshold infection mechanism has become widely accepted. This states that ingestion of even a single infectious organism, capable of surviving and multiplying within the host, has the potential to cause infection and, subsequently, illness and other sequelae. This is known as the single-hit concept and implies that, as long as even one cell is ingested, there is some probability, though possibly very small, of infection.

Once ingested, a number of outcomes are possible: the pathogen is simply excreted or does not survive in the host; it may infect (colonize) but not produce any overt symptoms of illness, resulting in a carrier state; once infected, there is a probability of acute illness. Once ill, there is a probability of more severe outcomes, such as septicemia, chronic syndromes, and death. Factors that influence these processes include the pathogen's resistance to the host's defense mechanisms, its ability to colonize, and its virulence mechanisms; the host's immunological and physiological status; and the effects of the medium in which the pathogen was ingested, e.g., protection against gastric acidity afforded by high-fat food.

FAO/WHO [31] discusses these factors in detail and provides guidance for the format and information to be included in a hazard characterization. Inclusion of a fitted dose–response curve and mathematical form of the relationship depends on the quality of data available and the goodness of fit to the data.

Risk characterization

The final step in risk assessment combines the information generated in hazard identification, exposure assessment, and hazard characterization to produce a complete picture of the assessed risk ([10,28]). Risk managers and risk assessors at the beginning of the work should have defined the form of the risk assessment outcome, and the types of questions to be answered in a risk characterization [32]. The risk estimate should reflect the range of contamination of a food product, factors that might affect growth or inactivation of the pathogen, and the variability within the food chain and of the human response to the microbial pathogen. Risk characterization should also provide insights about the nature of the risk which are not captured by a simple qualitative or quantitative statement of risk. Such insights include, for example, a description of the most important factors contributing to the average risk, and a discussion of gaps in data and knowledge. Sensitivity analysis is a valuable technique to identify those factors in the MRA that have the greatest influence on the risk estimate.

The risk assessor may also include a comparison of the effectiveness of alternative methods of risk reduction for consideration by the risk manager. Sources of uncertainty in the data or understanding of the system, the rationale for any assumptions made, and data limitations must be clearly presented and should be understood by the risk manager. Every effort should be made to compare the results produced against independent observed data if such data are available. At the very least, the assessment should undergo rigorous peer review to ensure that the results are reasonable and plausible, and that the data, models, and assumptions were appropriate. The conclusions from a risk assessment should be both defensible and reproducible.

Risk assessment approaches

Formal assessments require time, expertise, and data. Not all food safety issues or all management decisions require risk assessments, and in some cases not all elements of an MRA are needed. For example, there may be circumstances where only an exposure assessment is appropriate. The MRA may focus on only one part of the food chain or may describe the entire farm-to-fork continuum [27]. The food safety concern may be about a specific food produced by a specific process or an entire commodity group—such as the risk of salmonellosis from all eggs produced in a country or region. The assessment may focus on a specific pathogen or consider all microbial hazards associated with a foodstuff. Detailed, comprehensive assessments are generally recommended when significant decisions must be made about implementation of new control measures and regulations, or to resolve major safety

or trade disputes. In industry, risk assessment is useful to help make decisions about new products and/or processes when significant uncertainty or variability is present.

An important first step is the process initiation. Stating the reason for doing the assessment at the beginning of the work is necessary to define the context, scope, and parameters of the assessment and should reflect the importance of the activity [6,11,17]. The output form and possible output alternatives of the risk assessment should also be defined at the onset. Finally, choosing the most suitable approach to a specific risk assessment problem will depend on the problem being investigated, the time needed for the work, and the availability of data.

Risk profile, defined under the risk analysis as part of the "preliminary risk management activities," is a description of the food safety problem and its context [11]. The information assembled presents in a concise form the current state of knowledge relevant to a food safety issue and the food safety policy context that will influence future possible actions. In this perspective, a risk profile may be considered a narrative type of assessment, or a "preliminary" MRA, but it may also include, in addition to scientific information, other considerations such as public perceptions and trade impacts associated with the issue. Such a document may be sufficient for decision-making in itself, or it will help to identify what is needed for further detailed analysis and in what form.

Using the risk assessment framework and the steps of exposure assessment, hazard characterization, and risk characterization, different strategies can be taken to assemble and analyze relevant information [32], In very broad terms, an MRA may be described as qualitative or quantitative depending on the representations of data and assumptions. Qualitative MRA relies on descriptions or categorizations, such as high, medium, or low, for exposure measures and hazard effects to estimate the likelihood and magnitude of risk and the importance of factors affecting the risk. Quantitative assessments rely on numerical data and mathematical analyses. Risk models include both the schematic, or conceptual, representation of the real-life events under investigation, as well as a description of the relationship between two or more elements in the conceptual model. In quantitative analyses, the relationships are defined by mathematical equations, and mathematical tools are used to estimate risk as a function of one or more inputs. Semi-quantitative risk assessment provides an intermediary level between the descriptive evaluations of qualitative risk assessment and the numerical analyses of quantitative risk assessment by evaluating risks with a score. This approach is most useful in providing a structured way to rank risks according to their probability, impact, or both (severity) to help managers set priorities must be set among competing risks.

Quantitative risk assessments can further be characterized by how data are analyzed, either by using point estimations or probabilistic (stochastic) distributions to represent the inputs [33,34]. Briefly, point estimates are single values such as the mean of a data set, and such analyses generate a single value for the risk estimate (Figure 3.2). A simplified example would be to consider the mean concentration of *Salmonella* in a contaminated product, the mean prevalence of such product, and an average amount of the food that a consumer would eat to calculate a single value for

likely exposure. By considering this information in conjunction with a single value for the dose that would likely result in illness, the assessor arrives at the risk estimate. In some situations, point-estimate determinations can be appropriate, for example, if the purpose is only to determine how large or how small the potential risk may be, or if there are sufficient data and little controversy on the issue. However, by choosing single numbers for inputs, the assessor unavoidably ignores uncertainty and variability in the risk estimate. Point estimations provide little insight into the influence of individual risk factors and how likely or unlikely it is that a worst-case high-risk situation will occur, and they convey an unrealistic sense of certainty in the risk estimate.

However, foodborne illness is a complex, multi-factorial phenomenon, and most risk assessors have preferred probabilistic, or stochastic, methodologies. These incorporate the variability present in the food chain pathogen and host populations and allow representation of the uncertainty in our knowledge. The independent variables that influence risk in any system do not have discrete, fixed values but rather are characterized by a range of values. Alternatively, the values may not be known with certainty and may have to be estimated. The range of possible values can be characterized by a minimum, maximum, and some central most-likely value, such as a mean or median, but in reality the possible values form a continuous spectrum of values, some of which are more likely to occur than others, i.e., they form a distribution (Figure 3.2). Many distributions can be described by a unique mathematical equation, and those equations are used instead of the fixed variable values in the mathematical conceptual model. The calculated solution, or outcome, of the risk model is also a distribution based on all the possible combinations of circumstances and thus shows the range of values that may be possible and the likelihood of their occurrence.

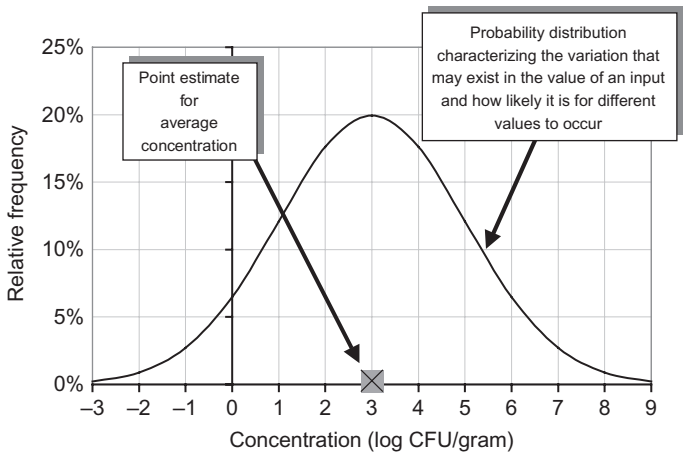

FIGURE 3.2

Comparison of a point estimate and the probability distribution for a data set.

For calculating the outcome of many variables in a system, the entire field of risk assessment has been greatly advanced through the introduction of computer modeling software that simplifies probabilistic calculations. Monte Carlo simulation is a mathematical technique often used for probabilistic analyses, using software such as @Risk™ (Palisade Corp., Newfield, NY) and Analytica™ (Lumina Decision Systems, Inc., Los Gatos, CA). Monte Carlo analysis literally simulates, or reproduces, events or sequences of events that could occur in the real physical system. The analysis involves the construction of the model of a process, defined by the input parameters, the mathematical relationships between parameters, and the assigned distributions (Figure 3.3).

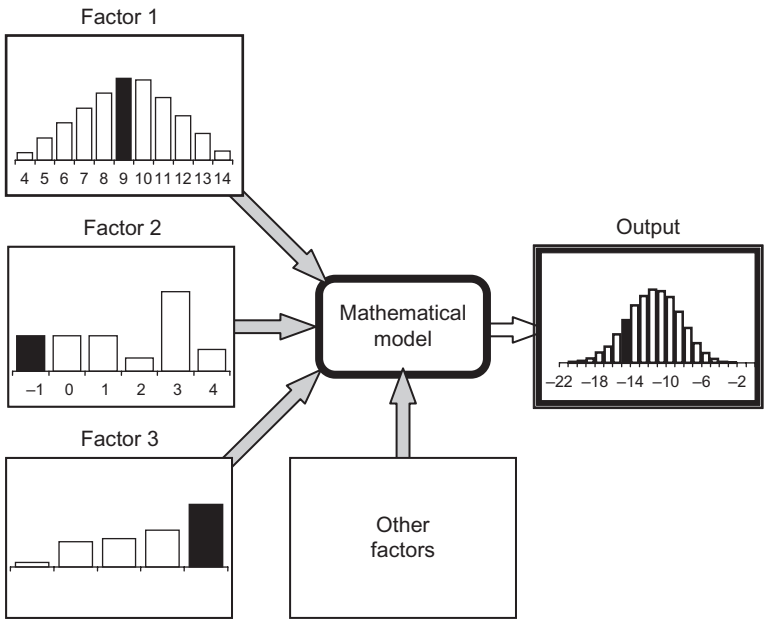

FIGURE 3.3

Illustration of a Monte Carlo simulation model for probabilistic quantitative risk assessment. In each iteration of the model, a single point value is randomly selected from the distributions defined for each input factor (bold columns in the graphs for each factor). For the exposure assessment, factors include prevalence and concentration of the pathogen in the food at different stages in the production, and consumption data such as frequency and amount eaten. The dose–response assessment would also be a major factor in a full assessment. These values are used in the calculation of the single output value in, say, the probability of a contaminated serving or the number of people ill. The next iteration selects a new set of single values from each distribution, calculating a new output value. Repeated thousands of times, the distribution of the output is constructed from the individual input distributions and the mathematical relationship between the parameters.

MRA is typically a modular process, describing whatever segments of the food chain are of interest. Within each stage, several submodules may exist to describe specific elements. Ideally, common elements can be adapted or transposed between risk assessments created for different purposes. For example, a farm-to-fork risk assessment for a raw end product might use the same on-farm production and primary processing modules as an assessment for processed product using the same raw material. Production and slaughter modules may be similar for beef in different countries; however, time and temperature characteristics of the product distribution chain may differ and would be modified by using national data to account for the differences. The dose–response model for any one pathogen and a specific health outcome may be adopted for characterizing the risk from the same pathogen but in a different commodity, with consideration for individual food factors that might alter the survival of an ingested pathogen.

Summary

Risk assessment is a systematic process for the collection, organization, and analysis of data that can be brought to bear to assist in rational, objective decision-making processes. As a structured inquiry into the hazard, exposure, and dose–response parameters, the risk assessment document itself serves as a database of relevant information and a record of assumptions and decisions. The field of MRA is still evolving, with novel approaches and improved data, to provide better tools and information in support of risk management and risk communication activities to ensure and enhance the safety of the food supply.

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CHAPTER

4 Development of Risk-based Food Safety Systems for Foodborne Infections and Intoxications

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Introduction

There is widespread support for the idea that food safety systems should be science- and risk-based (see, e.g., [1–7]). A science base is intended to assure that the causes, sources, and pathways for foodborne illness are well understood. A risk base uses the science base but goes beyond it. It is intended to develop frameworks that allow decision-makers to think systematically about sets of risks and to select actions to reduce risk overall and improve public health.

At their heart, risk-based systems are a means of developing best practices. Their development allows participants, including international agencies, governments, industry, and non-governmental organizations, to propose and vet approaches to food safety assurance including system goals, risk ranking and prioritization, choice, design, and implementation of interventions to improve food safety, and evaluation of system effectiveness. These best practices allow benchmarking as decision-makers compare their own work to the quality of the science and decision tools being used by their peers. They can also facilitate challenges to decisions that are not based on best practices. Overall, the effect of risk-based systems should be the improved effectiveness of food safety assurance activities.

The development of risk-based systems is a work in progress across many public and private parties around the world. This chapter uses one of these efforts, the description of a risk-based system published in *Enhancing Food Safety: The Role of the Food and Drug Administration* [8], as a basis for discussing the elements of such systems. This report was written by a Study Committee established by the National Academies to conduct a review of the Food and Drug Administration's role in ensuring safe food. The Study Committee found that a comprehensive framework showing the steps in a risk-based food safety system did not exist, so it created one. This chapter particularly focuses on how risk-based systems can be

Foodborne Infections and Intoxications.

used for government regulatory choices, and discusses the need for the development of data and analytical capabilities to support such systems.

Building blocks for a risk-based food safety system

Current efforts to develop risk-based systems rest on several building blocks that have been developed over time. The risk analysis framework has been the main vehicle for the development of thinking about and best practices on how to make regulatory decisions for assuring food safety [9–11]. This framework is based on the triad of risk assessment, risk management, and risk communication. It has been used extensively, with risk assessment becoming particularly well established (see, e.g., [12–14]). The risk analysis framework is spread over several areas of the risk-based systems discussed below but has largely been applied to study a specific risk or small set of risks on a case-by-case basis.

The Agreement on the Application of Sanitary and Phytosanitary Standards (the SPS Agreement) of the World Trade Organization, which entered into force in 1995, has been a spur over time for governments to base food safety regulation on science and to think about risk-based food safety systems. The Agreement has allowed countries to formally challenge each other's regulations but probably more importantly, in terms of overall impact, has led to increased transparency through the requirement to notify changes in SPS policy and self-discipline in regulatory decision-making (see, e.g., [15]).

Another building block for risk-based systems is work on ranking food safety risks based on public health impacts (see, e.g., [16–20]) and also on additional factors such as consumer perceptions, societal sensitivity, and market impacts [21,22]. In the United States, the FDA Food Safety Modernization Act of 2011 calls for several steps to strengthen the risk basis of regulatory practices. These steps are broadly consistent with risk-based systems but the Act does not require the FDA to establish such a system.

A risk-based system uses all of these building blocks and adds others. The *Enhancing Food Safety* report uses a working definition for a risk-based approach as a "systematic means by which to facilitate decision-making to reduce public health risk in light of limited resources and additional factors that may be considered" ([8], p. 79). Risk-based systems should support an on-going cycle of activities that inform each other. The entire system should be guided by strategic planning (e.g., for food safety regulation in a particular country). The system will need to identify which food safety risks are most important to address. This risk prioritization can be accomplished through a two-step process (as envisioned in *Enhancing Food Safety*), with risk ranking based on public health being followed by risk prioritization based on the public health ranking plus additional information and factors. Alternatively, risk prioritization can be done in a single step using multi-criteria decision analysis to consider public health and other factors at the same time, using weights provided by decision-makers. Risk-based systems require more broadly based analysis

of proposed interventions to address priority risks than is commonly undertaken by government agencies. This needs to be followed by design and implementation of the selected interventions. For specific risks, the decision could be to do nothing based on available interventions. A risk-based system ends one cycle and begins a new cycle with monitoring and review of the outcomes of the system.

Risk-based food safety systems are broad and ambitious. *Enhancing Food Safety* identifies a sound list of attributes that a risk-based system should have ([8], pp. 79–81):

- Proactive based on a strategic management plan.
- Data driven.
- Grounded in principles of risk analysis.
- Employs analytical methods to rank risks based on public health impact.
- Incorporates deliberation with key food safety stakeholders.
- Considers factors such as consumer perception, public acceptance, market impacts, and environmental impacts in decision-making when appropriate.
- Employs analytical methods to prioritize the allocation of limited resources to manage risk most effectively.
- Employs measures to evaluate the efficacy of the risk management program on a continuous basis.
- Performs all these functions in a systematic and transparent manner with involvement of stakeholders.

This list sets out important best practice goals to be aimed for in the development of risk-based systems.

Elements of a risk-based food safety system

The elements of risk-based food safety systems are not standardized at this point in time. They will be elaborated on as well as tailored by different users, much as the risk analysis framework and specific parts of it, such as risk assessment, were developed. This section uses the risk-based system presented in *Enhancing Food Safety* ([8], Chapter 3) as scaffolding for exploring the major elements of such systems. As shown in Figures 4.1 and 4.2, the report identified and described six major steps in a risk-based food safety system. In general, the steps identified for a risk-based system are iterative and may be undertaken in a different order in specific situations. These systems also must have extra capacity built in to deal with emergencies and crises; if not the management system will frequently be diverted from its main job of managing the overall profile of food safety risks and mitigation activities.

Step 1: Strategic planning

The first requirement of a risk-based food safety system is an overall strategic plan that defines the scope of activities (e.g., the set of food safety risks) that will be

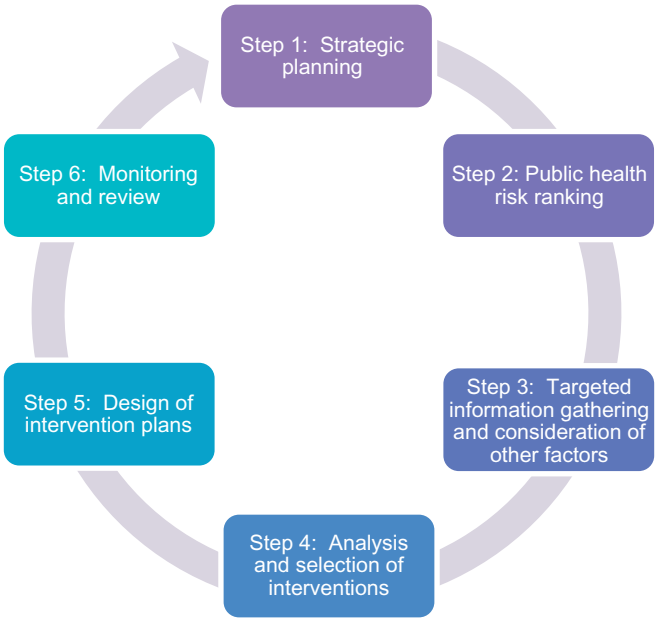

FIGURE 4.1

Major steps of a risk-based food safety system.

Adapted by Barbara Kowalcyk from [8].

managed by the system. The broader the scope of risks that will be incorporated, the more likely it will be that the system can make sound decisions on risk prioritization and allocation of resources to risk mitigation across risk-food combinations. Of course, the tradeoff is that the broader the scope, the more complex the management problem.

The strategic plan should start with the identification of goals for public health protection (public health objectives) because they are the central focus of the system. These objectives should be developed in consultation with stakeholders. Public health objectives may be stated as direct public health outcomes (e.g., reduction in foodborne illness rates associated with a particular pathogen). Frequently such objectives may be accompanied by goals for intermediate outcomes (e.g., reduction in pathogen contamination rates in the food supply) that are thought to be reliably associated with public health outcomes. Agencies may also set operational goals, for example improved inspection efficiency, which are intended to facilitate the achievement of public health protection.

Other objectives beyond public health may be included in the strategic plan depending on the public or private party developing the plan. For example, a food safety agency in a strongly export-oriented country may include an objective focused on assuring continuous access to export markets around the world. Another

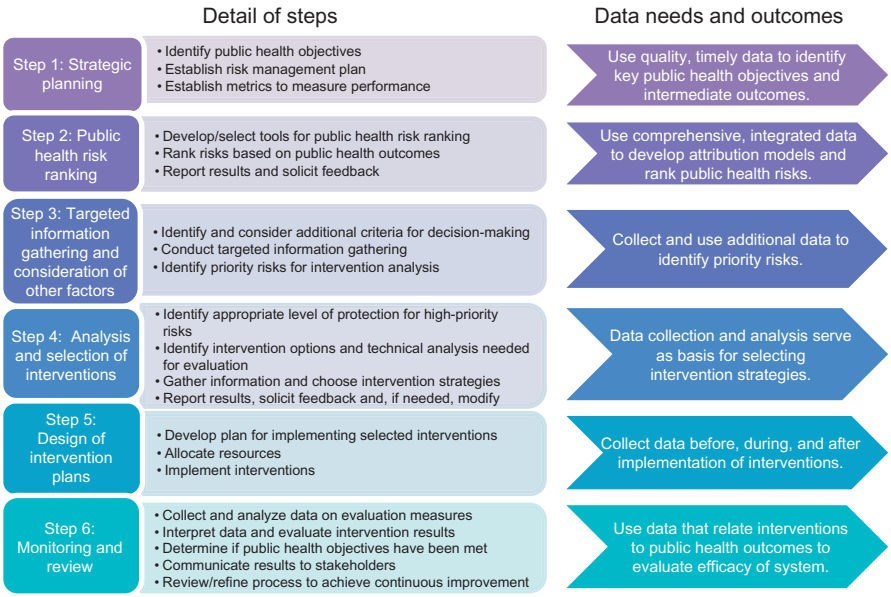

FIGURE 4.2

Details of a risk-based food safety system.

Adapted by Barbara Kowalcyk from [8].

food safety agency could define an objective around avoiding disruptions in domestic food markets, including loss of consumer confidence and sales, due to foodborne illness outbreaks. The strategic plan's objectives should explicitly include all the outcomes that decision-makers will seek to influence through the management system.

Strategic planning should include the development of a risk management plan that encompasses the general strategy as well as specific strategic plans, for example, for data, policy development, research, and inspection or other interventions. The strategic plan should also articulate a regulatory philosophy and general principles for regulatory activity ([8,23], Chapter 4). The regulatory philosophy and general principles are needed to address the reality that food safety is jointly produced and "is a responsibility shared between suppliers, farmers, food handlers, processors, wholesalers and retailers, food service companies, consumers, third-party organizations, and government (federal and state) agencies" ([8], p. 121), both domestically and internationally. The strategic plan cannot delineate the responsibilities of all parties in all situations because the assignment of responsibility will differ depending on, for example, the source of the risk, how it changes along the food chain, and how controllable it is and at what cost at different intervention points. What the strategic plan must do, however, is set out an overall philosophy about preferred approaches

(e.g., command and control regulations, industry responsibility for preventative measures, consumer education) and a road map of the factors that will have an influence and how they will influence the choice of interventions, including the option to do nothing.

Finally, the strategic plan must establish metrics to measure the performance of the system on all of its objectives, again in consultation with stakeholders. The establishment of metrics at the start of the process is important because they set the context for all subsequent steps in the risk-based system. They serve as a touchstone at all stages to assure that the system is well targeted to assuring food safety and attaining system objectives.

Step 2: Public health risk ranking

As discussed above, there are two major approaches to identifying the most important risks to be addressed by a food safety system. One is to sort risks based first on public health risk and then to bring in additional information and additional factors, if desired, for a final sorting. The risk-based system presented in *Enhancing Food Safety* [8] takes this approach, calling the first stage public health risk ranking and the second stage risk prioritization. This breakdown is followed in the discussion here. Alternatively, risk prioritization can be done in one stage where multiple factors including public health are weighted and ranked using multi-criteria decision analysis [22].

Public health risk ranking is a process for measuring and comparing the public health outcomes of different food safety risks. A comprehensive system facilitates this measurement and comparison across different types of risk including microbiological, chemical, pesticide, allergen, and other risks. *Enhancing Food Safety* defines public health risk ranking as "a formalized process that involves comparing the relative risk of multiple hazards, including foods" ([8], p. 87).

Public health risk ranking requires the development and selection of tools for measurement and analysis in consultation with stakeholders. Several tools have been developed for ranking in recent years. They generally fall into two groups in terms of the type of data they use [8]. Top-down approaches use surveillance- or outbreak-based epidemiological data to assess the level of risk associated with hazards, foods, or hazard-food pairs. Top-down approaches have used direct measures of burden of illness (e.g., quality-adjusted life years [QALYs] and disability-adjusted life years [DALYs]) and expert elicitation, as well as providing monetary measures of impact based on cost of illness associated with disease burdens and willingness to pay for reduced illness. The main advantage of top-down approaches is that they directly measure public health outcomes and impacts of interest. The main disadvantages are that surveillance data remain incomplete and attribution of disease burdens to particular hazards and foods is frequently difficult.

Bottom-up approaches develop data through quantitative risk assessment techniques that follow hazards from their sources through the supply chain to predict

public health outcomes. The main advantage of bottom-up approaches is the quality and detail of information they generate about risks. In addition, the detailed approach often yields insights into intervention points that may be targeted to reduce the risk. The main disadvantage of the bottom-up approach is that the analysis is resource-intensive, meaning that only a limited number of risks are fully studied, making overall risk ranking infeasible. A promising approach for public health risk ranking is to use top-down approaches for overall ranking, informed by bottom-up analysis that goes into more depth for specific risks. This more in-depth analysis can also be part of a third step in the risk-based system as discussed below.

The alternative methodologies available for public health risk ranking have different levels of data intensity and analytical requirements. They may result in somewhat different rankings. These differences in rankings can be useful in providing insights into which factors most drive the rankings, allowing users to form judgments of the relative reliability of the rankings. In the risk-based system developed in *Enhancing Food Safety* [8], the result of public health risk ranking is a first step in a two-part process of identifying risks that are of high priority to be addressed by the food safety system. The methodology for and results of public health risk rankings should be reported and feedback should be gathered.

Step 3: Targeted information gathering and consideration of other factors

Ranking across food safety risks based on public health outcomes provides inadequate guidance to set priorities to be addressed in risk-based systems. Targeted information gathering is needed to fill in knowledge gaps in regard to public health outcomes and also in regard to a range of information included in risk assessment. Targeted gathering of additional information may involve the assembly and organization of existing data or additional research and activities to collect base-line, surveillance, or survey data. Targeted information gathering may also be done through qualitative, quantitative, or semi-quantitative risk assessments.

In addition, while public health is the central objective of risk-based food safety systems, governments in consultation with stakeholders may identify and use additional criteria in risk prioritization. Data will need to be gathered and analyzed for these additional criteria as well. Examples of additional criteria that could be considered for risk prioritization include how consumers perceive the risk as it relates to them personally, how sensitive society is to the risk (e.g., society may be more sensitive to risks that affect small children and the elderly), the market impacts of the risk (e.g., effects on international trade), or environmental impacts (see, e.g., [22]). The explicit consideration of factors or criteria beyond public health in risk-based systems may be controversial for government agencies, particularly if their mandates are primarily (or even solely) to protect public health. However, these other factors should be considered explicitly, because if they are not, they will enter the risk prioritization process in an ad hoc manner, resulting in inconsistent decisions.

Risk prioritization is the outcome of Step 3 as described here or, alternatively, of a combination of Steps 2 and 3. In either case, multi-criteria decision analysis tools should be used to weight factors to be considered in order to identify priority risks for intervention analysis. This process may also identify risks that cannot be evaluated without improved data and analysis. Note that consideration of the feasibility of interventions is separated from risk prioritization and occurs at a subsequent step. This separation is important in order to distinctly look at priority risks, without muddling feasibility considerations into the prioritization.

Step 4: Analysis and selection of interventions

Analysis and selection of interventions is surprisingly a relatively neglected area in the development of risk-based systems. In many cases, a single intervention or a small number of interventions are identified at an earlier stage, for example in the process of risk assessment. These are then focused on without a full scan and evaluation of other possible types of interventions.

Interventions are policy instruments that can be taken to address food safety risks. They range from no intervention through self-regulation, the use of information and education, various forms of co-regulation, incentive-based structures, and direct regulation ([8,23], Chapter 4). The regulatory philosophy and road map for selecting interventions developed in the strategic plan should be used in the intervention and analysis stage to guide the mix of private responsibility and government intervention that is judged appropriate for addressing high-priority food safety risks.

Analysis and selection of interventions should be done in consultation with stakeholders and begins with identifying appropriate levels of protection for high-priority risks. These levels set the goals for interventions to achieve. The interventions considered may have been suggested during targeted information gathering and risk assessment carried out at Step 3; other candidate interventions should be identified at this stage as well.

Different technical evaluations will be required based on the type of intervention being considered. For example, a labeling intervention with warnings and a processing intervention require different information for evaluation. The technical analysis of interventions may employ risk assessment and should identify performance measures and the initial design of databases needed for evaluation. An intervention strategy or set of strategies should be chosen once information is gathered on candidate interventions. This information on interventions includes the value of likely public health outcomes, other benefits, feasibility, and costs.

Choices of interventions should be made using multi-criteria decision analysis in recognition of the different factors that interventions are intended to impact. Here, again, a process of reporting results, soliciting feedback, and making modifications is needed. Intervention analysis and selection is actually a set of parallel but related efforts as alternative interventions must be analyzed and selected for each of the high-priority risks identified in the risk-based system.

Step 5: Design of intervention plans

Selecting an intervention strategy or strategies should be followed by developing a plan for implementing selected interventions, again in consultation with stakeholders. For regulatory interventions this may involve detailed rule making. With other interventions, design may focus on educational programs or overseeing self-regulation by industry. The intervention plan should include interim measures that can be used to monitor progress in addition to the primary evaluation based on achievement of public health and other objectives.

At any point in time, a government agency charged with assuring food safety will have several vintages of interventions in its portfolio, including interventions under design, ongoing programs that are operating satisfactorily, and existing programs that are slated for overhaul. The design and implementation of intervention plans involves the allocation of resources across this portfolio of interventions, with a goal of achieving the maximum results from the expenditure of the available resources.

Step 6: Monitoring and review

The final step of a risk-based system collects and analyzes data to evaluate the effectiveness of the adopted interventions and of the system as a whole. This step should assess whether the evaluation measures, including the public health objectives, defined in the strategic plan have been achieved and communicate the results of this analysis to stakeholders. It may also focus on interpreting data and evaluating whether the interventions chosen have resulted in desired intermediate outcomes. This step provides feedback to the strategic planning process as well as the risk ranking/prioritization and intervention analysis/implementation steps in order to achieve continuous improvement in the system.

The overall risk-based decision process

Risk-based food safety systems are intended to guide, organize, and lend discipline to the food safety assurance activities of governments and private parties. The steps in this process may be laid out somewhat differently by different users but should include strategic planning, risk ranking/prioritization, invention planning and implementation, and evaluation. The development of risk-based systems is a very substantial undertaking that demands better data and analytical capabilities at all steps in its development.

Challenges in implementing risk-based food safety systems

The adoption of risk-based food safety systems faces several challenges going forward. The foremost overall challenge is whether regulatory agencies can achieve the new system through comprehensive overhaul of their systems or whether they pursue incremental change. If through comprehensive overhaul, the most promising

approach is to build a parallel, new management system that would be switched to when it is in place. If an incremental approach of adopting parts of the risk-based system over time is used, the ultimate goal of a risk-based system may not be reached. The pull of current crises frequently derails overall progress toward a risk-based system. A second overall challenge to risk-based systems is that they result in a significantly more transparent regulatory system that rests on the sharing of data and analyses with multiple stakeholders at several points in the system. Transparency can be uncomfortable but is likely to improve decision-making.

Risk-based systems are intensive in their requirements for the planning and implementation of data collection, for the integration of information, and for analytical capability. As an example of these requirements, the right-hand column of Figure 4.2 shows the data needs and outcomes associated with each of the six steps of the risk-based system as developed in *Enhancing Food Safety* (see [8] and Chapter 5 for extensive discussion of data and analytical needs associated with risk-based systems). The identification of key public health objectives and intermediate outcomes in Step 1 requires the use of high-quality and timely data that set baselines. Public health risk ranking in Step 2 requires data that can reliably attribute public health burden and related costs to specific risk and food sources, while Step 3 requires further, targeted data for consideration of other factors and for risk prioritization. Steps 4 and 5 require specific data on interventions for analysis, design, and planning of evaluation systems. Monitoring and review at Step 6 take data generated to evaluate progress toward public health and other objectives before strategic planning begins again.

Overall, risk-based systems may require more data collection, although this is not clear. Risk-based systems certainly do require more targeted and better integrated data collection. This is particularly challenging because many government agencies have ongoing data collection programs that are not well connected to an overall risk-based strategic plan. There is a temptation to repurpose these legacy data systems to serve system purposes for which they were not designed and on which they cannot deliver.

Figure 4.3 shows the scope of the data needs for risk-based food safety systems as identified in *Enhancing Food Safety*. The report finds that an integrated information structure to support such systems should be made up of surveillance, food, behavioral, and economic data. In moving toward adopting risk-based systems, most if not all regulatory agencies will find they currently lack sufficient analytical capacity to operate these systems. There will be a very significant need for building analytical capabilities in strategic planning and evaluation, risk ranking, risk prioritization, intervention analysis, and decision-making tools including multi-criteria decision analysis ([8], Chapter 11).

Summary

Risk-based food safety systems take the next step beyond risk analysis in the development of approaches to food safety assurance by providing an overall approach that integrates strategic planning, public health risk ranking, broader risk

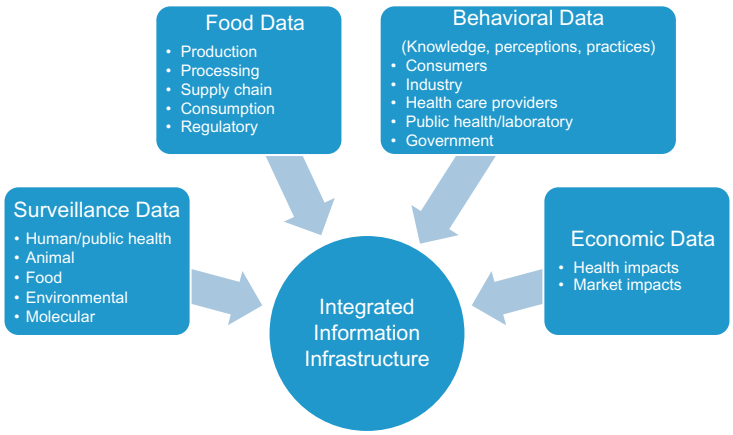

FIGURE 4.3

Data needs for an integrated information infrastructure to support a risk-based system.

Source: Barbara Kowalcyk based on [8].

prioritization, intervention analysis and design, and monitoring and evaluation. The development of risk-based systems is challenging but promises more effective use of resources to attain the public health and other objectives of food safety assurance systems.

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SECTION

Foodborne Infections: Bacterial

2

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CHAPTER

Pathogen Updates: *Salmonella*

5

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Introduction

Salmonella is an important cause of foodborne disease in humans throughout the world and is a significant cause of morbidity, mortality, and economic loss [1–6].

The majority of infections are transmitted from healthy carrier animals to humans via contaminated food. The main reservoir of zoonotic *Salmonella* is the gastrointestinal tract of warm-blooded animals including food-producing animals. Although other sources are recognized, its transmission to humans in most parts of the world occurs mainly through the ingestion of contaminated food [7,8]. Implicated foods are normally beef, pork, poultry, dairy products, eggs, and fresh produce, and reports confirm the transmission of strains from the animal reservoir through the food chain to the human population [9].

Since the early 1990s, surveillance programs and control methods for *Salmonella* in the farm-to-fork continuum have been implemented in an increasing number of countries worldwide [10,11]. The growing awareness of a global food market has led to international initiatives toward a global control of *Salmonella*. Still, the incidence of human salmonellosis in many industrialized countries has remained high [12]. The effort to reduce human salmonellosis is challenged by a widespread occurrence of *Salmonella* in a variety of food-producing animals (and non-food sources) and by the ability of *Salmonella* to adapt to and survive changing environmental conditions.

The disease in man

Symptoms and sequelae

Non-typhoid *Salmonella enterica* is considered one of the leading causes of gastroenteritis and bacteremia in the world [6,13]. Typically, onset of symptoms is 12 to 72 hours after exposure, and the duration of illness is 4–5 days, often followed by a period of fatigue. Most patients develop a gastrointestinal illness with acute diarrhea as the main symptom. Other common symptoms include abdominal pain or cramps, fever, chills, nausea, vomiting, pain in the joints, headache, myalgia, and

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general malaise. Illness can range from a mild to severe gastroenteritis and in some people, invasive disease that can be fatal. The infection is usually self-limiting and clinically indistinguishable from other common bacterial gastrointestinal infections. The mortality rate is usually low (<1%) for most serovars [6,94], although infections with the host-adapted serovars (e.g., *S. dublin* and *S. choleraesuis*) often lead to septicemia, requiring antibiotic treatment and with case fatality rates of 20–30% [14].

Immunosuppression and other chronic underlying illnesses, including inflammatory bowel disease, organ transplantation [98], malignancy [96,106], and malnutrition, are risk factors for severe gastroenteritis as well as bloodstream infection. Patients with HIV/AIDS are at a particularly high risk for invasive *Salmonella* infections [97,101]. Children and elderly people are also considered to be more at risk of an infection with *Salmonella* than the average adult. People receiving antacids have also been reported as having an increased risk of infection due to the increased gastric pH [15]. Since the population of both the elderly and the chronically diseased is growing, this may also contribute to the continuing high level of human salmonellosis.

Complications in the acute stage of the disease are often related to bloodstream infection, and include endocarditis or arterial infections. Localized infections include abdominal, soft-tissue, and urogenital infections [16].

Long-term sequelae such as reactive arthritis (ReA) and irritable bowel syndrome (IBS) are identified as outcomes of salmonellosis [17,18]. Persistence of bowel symptoms commonly occurs after bacterial gastroenteritis and is responsible for considerable morbidity and health care costs [99]. Both inflammatory bowel disease and IBS have been associated with salmonellosis [19,103], where IBS has been estimated to occur in around 9% of patients with a previous bacterial gastroenteritis including salmonellosis [18].

ReA is the most significant long-term sequelae following salmonellosis. When reviewing published studies [20] estimated that 8% (2.3–15%) of acute foodborne illness cases would develop ReA. Multiple joints are usually involved, most commonly the knee, ankle, and wrist. Most cases are self-limiting, but severe arthropathy may be incapacitating. Reiter's syndrome is a triad of arthritis, conjunctivitis, and urethritis. A subset of patients with reactive symptoms will develop the full triad, whereas others may experience conjunctivitis, iritis, or bursitis without arthritis. Erythema nodosum is another well-known reactive consequence. As for some other bacterial gastrointestinal infections, reactive symptoms are correlated with the HLA-B27 antigen and it is reported that up to 20% of patients who are HLA-B27 positive develop reactive arthritis after salmonellosis outbreaks [107].

Incidence and burden of human salmonellosis

Human salmonellosis is the second ranking foodborne disease in most European countries, only exceeded by campylobacteriosis. The EU notification rate in 2010 was 21.5 cases per 100,000 population, ranging from 1.9 in Portugal to 91.1

confirmed cases per 100,000 population in Slovakia. The case fatality was 0.13% among the confirmed cases for which this information was reported [10]. As in previous years, *S.* Enteritidis and *S.* Typhimurium were the most frequently reported serovars, constituting 67.4% of all reported cases where the information on serovars was provided. During the past 5 years, a decreasing trend of human salmonellosis has been observed (Figure 5.1), which is explained by a decrease in the number of *S.* Enteritidis infections [10].

Non-typhoidial *Salmonella* infections have a marked seasonality. In the northern hemisphere most infections are reported around August with a rapid decline in winter months—a pattern that is prominent for all age groups (Figure 5.2). In New Zealand, human salmonellosis is reported to peak in [21], whereas in Australia, the peak usually occurs a bit later, in March [95].

Statistics for the incidence of human salmonellosis (and other foodborne infections) are notoriously difficult to compare between countries and sometimes even within a country, as they depend on the definition of a case, the diagnostic method used, and how the information is collected and analyzed. In addition, the subjective reactions of the patients and general practitioners will influence whether a case will be diagnosed and reported. There have been attempts to calibrate *Salmonella* surveillance data at national surveillance institutes, but some research groups have attempted to equate disease in the population to what appears in official statistics [2,4,6,22–26]. The studies suggest that for every reported case of salmonellosis, between 3.8 and 38 persons in the population fell ill [14].

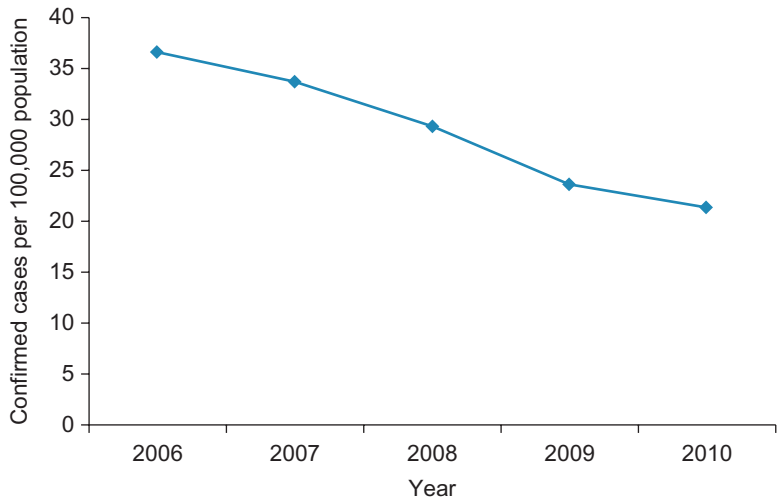
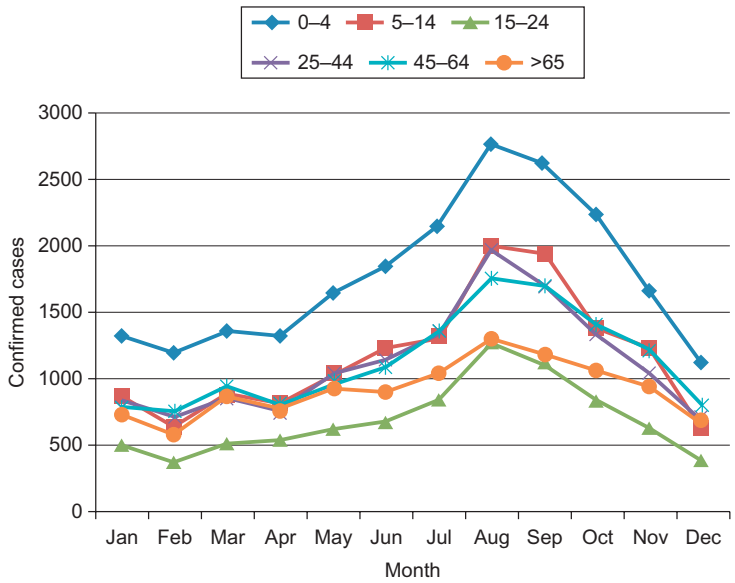


FIGURE 5.1

Trend in reported confirmed cases per 100,000 population of human salmonellosis in the EU, 2006–10 [10].



Source: TESSy data for 24 MSs: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Portugal, Slovakia, Slovenia, Spain, Sweden, and United Kingdom (N=82,642).

FIGURE 5.2

Number of confirmed salmonellosis cases in humans by month and age group in 2010 [10].

Under-reporting factors for human salmonellosis in the EU member states (MSs) have recently been estimated employing information on the risk from Swedish travelers in the EU in 2009 [27,89]. The risk of salmonellosis in returning Swedish travelers in the EU was 8.44 per 100,000 trips (95% CI: 8.18–8.71), ranging between 0.13 for travel to Finland to 94.3 for travel to Bulgaria. Based on these risk estimates, the true incidence of salmonellosis in 2009 was estimated at 6.2 (95% CI: 1.0–19) million cases in the EU and the under-reporting factor at the EU level was 57.5 (95% CI: 9.0–172), ranging from 0.4 in Finland to more than 2000 in Portugal. Based on these estimates, the European Food Safety Authority (EFSA) estimated the disease burden of salmonellosis and its sequelae to be 0.23 (95% CI: 0.05–0.6) million disability-adjusted life years (DALYs) per year and total annual costs were estimated at 2 (95% CI: 0.3–4) billion [28].

A study by Majowicz et al. [29] had the ambitious aim of estimating the global burden of human salmonellosis. By synthesizing existing data from multiple studies and surveillance reports (including prospective population-based studies, "multiplier studies," disease notifications, and returning traveler data), incidence estimates were calculated for each of the 21 Global Burden of Disease (GBD) regions, which were then summed to provide a global number of cases. It was estimated that 61.8–131.6 million cases of gastroenteritis due to *Salmonella* (ranging from

40/100,000 cases in Pacific and Central Asia to 3980/100,000 in East and Southeast Asia) occur globally each year, with 39,000–303,000 deaths. The study obviously involves a great deal of uncertainty. For instance, for 11 of the 21 GBD regions the risk estimates applied were based on returning Swedish traveler data [30] in a comparable manner with the study at the EU level described above. Both studies, therefore, assume that the incidence rate in the local population is comparable with that of Swedish travelers acquiring their infection in that region. This assumption is debatable, as travelers (mostly tourists) may consume and behave very differently than the local population and may also be more susceptible to infection due to a general low exposure level in their home country. Still, the resulting estimates at least for the EU MSs are believed to provide a better reflection of the true human salmonellosis incidence than the actual reported number of cases [28].

Epidemiology and disease transmission in humans

Salmonella can affect mammals, birds, reptiles, and insects [14]. There are numerous transmission pathways through which humans can be exposed to *Salmonella*, including a wide range of domestic and wild animals and a variety of foodstuffs including food of both animal and plant origin. Infected animals carry *Salmonella* in their feces and the usual route of infection is through fecal-oral transmission. The epidemiology of *Salmonella* is, therefore, primarily due to direct or indirect fecal contamination of live animals, food, or humans.

Despite the many efforts to prevent and control foodborne salmonellosis during the last 20 years, this pathogen continues to be one of the leading causes of human gastroenteritis. There exist many factors that contribute to this development. Among these are the adaptive ability of the pathogen itself, the changing characteristics of the population, the increasing globalization of the food trade, and changes in industrial structure and in consumer behaviors.

The occurrence of antimicrobial resistance among zoonotic *Salmonella* is an increasing problem. Antimicrobial-resistant *Salmonella* involved in human disease are mostly spread through foods, predominantly poultry meat, eggs, pork, and beef [31]. Meat is recognized as a source of human exposure to fluoroquinolone-resistant *Salmonella* spp., and high levels of extended-spectrum beta-lactamase (ESBL)-producing *Salmonella* have also been reported, particularly in poultry meat in some [32]. Such resistant strains may be associated with a significant level of human infection, depending on the pathogenicity of the strains involved and the opportunity for them to contaminate the food chain [33–36]. The control of antimicrobial-resistant bacteria in food is complicated by the fact that resistance mechanisms can be located on mobile genetic elements, such as plasmids, and thereby be transferred between different bacterial species, for instance between generally apathogenic *E. coli* and pathogenic *Salmonella* spp.

The use of antimicrobials for food animals is a major contributing factor for the selection and dissemination of resistant *Salmonella* [37,61] but also the increasing use of antimicrobials, particularly fluoroquinolones, in humans has recently

been associated with an increased incidence of infections caused by drug-resistant *Salmonella* [38]. Compared with patients infected with susceptible *Salmonella* strains, patients with multi-drug-resistant infections may be more likely to have a protracted course of disease that in addition is more severe, often requires hospitalization, and may lead to excess mortality [39,40]. This observed increase in severity can be a consequence of incorrect choice of antibiotic treatment in patients with extraintestinal infection resulting in reduced efficacy of early empirical treatment [14].

A rapidly growing international trade in live animals (including breeding animals), animal feedstuffs, raw materials, and processed foods has facilitated the introduction of new *Salmonella* types in importing countries and resulted in an increasing length and complexity of the food chain [41,42]. Concurrently, there has been an increase in the consolidation of food industries, including primary production, and mass distribution. This trend toward greater geographic distribution of products from large centralized food processors carries a risk for more widespread outbreaks affecting more people [43]. The dissemination of *S.* Enteritidis in the table-egg industry [12,44] and the occurrence of multistate and international foodborne outbreaks [45–47] are examples of this.

Traditionally, foods implicated in foodborne outbreaks have been poultry products (including eggs), red meats, and unpasteurized milk. In recent years, new types of food previously thought to be safe have emerged as sources of outbreaks. In particular these include fresh produce, which may be contaminated with animal feces during growth, harvest, and distribution. In particular, alfalfa sprouts have been implicated in large multistate or international outbreaks [48–51], and sprouts are recognized as a special problem because of the potential for pathogen growth during the sprouting process [52].

International travel has grown rapidly during the last decades. In countries with a low prevalence of *Salmonella* in their domestic livestock and food, this fact influences the national human statistics markedly. In Sweden and Norway, for instance, it is estimated that approximately 70–80% of all human *Salmonella* infections are acquired abroad [12,30,53]. Overall, around 63% of human *Salmonella* cases in the EU were reported to be acquired domestically and 11% abroad in 2010. The proportion of cases with an unknown location of origin represented around 26% of confirmed cases, but in some MSs this proportion is 100%. Data on domestic versus travel-related cases are, therefore, often incomplete, but should, to the extent possible, be accounted for, as a high proportion of travel-associated cases are likely to reduce the expected effect of national intervention strategies.

Microbiology

Classification

The genus *Salmonella* belongs to the Enterobacteriaceae family. *Salmonella* is a gram negative, facultative anaerobic motile rod; it is catalase positive and cytochrome oxidase negative, produces gas from glucose, and is able to reduce nitrate.

Salmonella is closely related to *Escherichia coli* and is believed to share a common ancestor. During evolution *E. coli*, being more closely associated with the mammals, has become lactose positive, while *Salmonella*, being more closely associated to reptiles and birds, is lactose negative. The acquisition of pathogenicity islands, with SPI-1 and SP-2 as the most prominent, conferred virulence to the bacteria.

Currently, the genus *Salmonella* is divided into two species: *S. enterica* and *S. bongori*. The species *S. enterica* consist of six subspecies: *S. enterica*, *S. salamae*, *S. arizonae*, *S. diarizonae*, *S. houtenae*, and *S. indica*, whereas no subspecies have been assigned to *S. bongori* [54].

Characterization and virulence

Serotyping is a commonly applied and important tool for classification of *Salmonella*. Seroagglutination of outer membrane O-antigens (LPS) and flagellar H-antigens defines the serovar according to the Kaufmann-White scheme. For example, the antigenic structure O:1,4,5,12 and H1/H2 b:1,2 defines *Salmonella enterica* subspecies *enterica* serovar Typhimurium (in short, *Salmonella* Typhimurium). Within the species and subspecies, more than 2500 different serovars have been identified [55].

A few serovars are termed "host-specific." *S*. Typhi, *S*. Paratyphi A and B, and *S*. Sendai are specific to humans [14], where they cause severe systemic illness characterized by fever and abdominal symptoms (enteric/(para)typhoid fever) [16]. These serovars are usually not pathogenic to animals and are not considered to have a zoonotic potential (Table 5.1). In a similar manner, there exist a few serovars that are specific to an animal host such as *S.* Gallinarum and *S.* Pullorum in poultry. The reasons behind host specificity have not been clarified.

Another group of serovars are highly adapted to an animal host, e.g., *S. cholerasuis* in pigs and *S. dublin* in cattle. These serovars only occasionally infect humans, where they may produce no, mild, or serious disease [14,56]. Non-typhoidal, ubiquitous serovars, such as *S.* Typhimurium and *S.* Infantis, affect a wide range of animals and humans. Although these serovars in principle are non-host-adapted, strong associations between certain serovars, or subtypes within a serovar, and a given animal reservoir may occur, e.g., *S.* Enteridis in laying hens. The non-host-adapted serovars are those with principal zoonotic significance and there are epidemiological studies that suggest that the ability of these serovars to infect animals and eventually infect humans via food varies [57,58]. The vast majority of zoonotic serovars associated with human illness belong to *S. enterica* ssp. *enterica*.

Typing methods for tracing the sources of human infections

Typing methods are probably applied more extensively to *Salmonella* than to any other zoonotic pathogen, and the majority of the molecular methods developed for characterization of bacteria have been applied to *Salmonella* both for epidemiological investigation and for research. The choice of method depends on the serotype and specific

circumstances, and often more than one method is used to improve the quality of the typing. The high level of differentiation of strains obtained by genotyping is particularly useful in the investigation of outbreaks, as it helps to define groups of cases that have been infected with the same strain from the same source [14,59]. Subtyping is also increasingly being applied for tracing the sources of sporadic *Salmonella* cases, i.e., for source attribution purposes [60] as discussed in a later section.

The subtyping systems based on lysis of *Salmonella* from a panel of *Salmonella* bacteriophages (phage typing) as described by Anderson et al. [92] and Ward et al. [105] have become internationally accepted. Phage typing is in some countries routinely applied to the more common serovars, *S.* Enteritidis and *S.* Typhimurium [10]. Phage typing subdivides serovars into phage types (PT) in *S.* Enteritidis or definitive types (DT) in *S.* Typhimurium. Examples are *S.* Enteritidis PT4 and *S.* Typhimurium DT104. Antimicrobial susceptibility testing may also be used to characterize *Salmonella* isolates and studies have indicated that there seems to be a strong association between some phage types and antimicrobial resistance patterns in particular *S.* Typhimurium strains [61]. The tracking of the global spread of *S*. Typhimurium DT104 documents the continued value of phage typing and antimicrobial resistance testing as a tool to investigate trends and patterns of *Salmonella* epidemiology [102,104].

Molecular methods based on characterization of the bacterial DNA have a considerably higher discriminatory power than the above-mentioned phenotypic methods. Pulsed-field gel electrophoresis (PFGE) is widely used for characterizing *Salmonella*, particularly in outbreak investigations. This method cuts the bacterial chromosome into large fragments by restriction enzymes and the fragments are separated by electrophoresis in a pulsing electric field. Bacteria showing the same bands on the gel are considered epidemiologically related.

More recently developed methods typically target specific areas or genes of the genome and include multilocus sequence typing (MLST) and multiple-locus variable number tandem repeat analysis (MLVA typing) [62,63]. MLST is based on sequencing of internal fragments of seven housekeeping genes. For each of the seven genes, different sequences (alleles) occur. Sequences for all seven genes are stored in an international database. To obtain an MLST type, the seven gene sequences are forwarded to the database and a sequence type is returned. If sequences for two isolates are identical they will be assigned the same MLST type. MLVA typing is based on the occurrence of repetitive small sequences in the chromosome, where the numbers of repeat elements in specific loci are measured. Strains with an identical number of sequences are considered identical. Particularly for the sequence-based methods, a whole new research area has become available as the latest technology makes it possible to perform large-scale sequencing at an affordable price. Thus far, however, advances in molecular techniques have not yet replaced the traditional phenotypic methods for epidemiological characterization of *Salmonella*, and the combined use of phenotypic and genotypic typing methods allowing for a detailed comparison of strains of

Salmonella is expected to be used in surveillance and control of this pathogen for some years to come.

Source attribution: approaches and discussion of studies

Source attribution is defined as the partitioning of the human disease burden of one or more foodborne infections to specific sources, where the term "source" includes animal reservoirs and vehicles, e.g., foods. Source attribution methods attempt to attribute the burden of disease at the population level, and do not describe causation of disease at the individual level. Methods for source attribution of foodborne diseases include the use of microbial subtyping, epidemiological approaches, e.g., the analysis of data from foodborne outbreak investigations and from case-control studies of sporadic disease, intervention studies, and expert elicitations. For a thorough review of source attribution methods see [60]. In the following sections, recent source attribution studies for human salmonellosis are presented and discussed.

Source attribution using microbial subtyping

The microbial subtyping approach involves characterization of pathogen isolates by phenotypic and/or genotypic subtyping methods as described in the Microbiology section above. The principle is to compare the distribution of subtypes in potential sources (e.g., animals and food) with the subtype distribution in humans; this is enabled by the identification of strong associations between some of the dominant subtypes and a specific reservoir or source, providing a heterogeneous distribution of subtypes among the sources. Subtypes exclusively, or almost exclusively, isolated from one source are regarded as indicators of the human health impact of that particular source, assuming that all human infections with these subtypes originate only from that source. Human infections caused by subtypes found in several reservoirs are then distributed relative to the prevalence of the indicator types. This approach requires a collection of temporally and spatially related isolates from various sources and humans, and is consequently facilitated by an integrated foodborne disease surveillance program focused on the collection of isolates from the major food-animal reservoirs of foodborne pathogens and from humans [60].

The principle of comparing the distribution of *Salmonella* subtypes found in animal and food sources with those found in humans to make inferences about the most important sources of human disease has been applied by several research groups [64,65]. A Bayesian model developed to attribute human salmonellosis in Denmark [66] has regularly been improved to include data on antimicrobial susceptibility [31] as well as data from multiple time periods [58]. The model attributes domestically acquired laboratory-confirmed human *Salmonella* infections caused by different *Salmonella* subtypes (e.g., serotypes, phage types, antimicrobial-resistant profiles) as a function of the prevalence of these subtypes in animal and food sources and the amount of each food source consumed. This approach has proved to be a

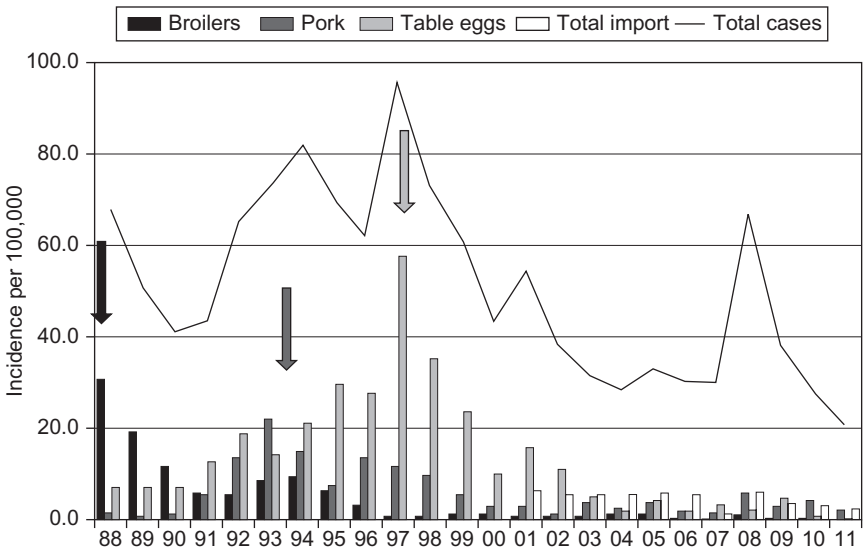


FIGURE 5.3

Effects of *Salmonella* control programs in Denmark as estimated by application of the microbiological subtyping approach on an annual basis. The arrows indicate the initiation of a new control program in broiler chickens, in pigs and pork, and in laying hens, respectively. Remaining cases were attributable to beef, imported food products, infections acquired while traveling abroad, and unknown sources.

valuable tool in focusing food safety interventions to the appropriate animal reservoir in Denmark (Figure 5.3) [67,68], and the model has recently been adapted to attribute human salmonellosis in other EU countries [69,70,91], as well as in the United States [11], New Zealand [71], and Japan [72].

The microbial subtyping approach has also been adapted to accommodate data from EU countries in a model that utilized data from the European Center for Disease Control (ECDC) and the European Food Safety Authority (EFSA) [73]. The model was applied to data from 24 MSs and attributed human sporadic salmonellosis to four animal reservoirs: pigs, broilers, layers, and turkeys. Overall estimates for the EU showed that the laying hen reservoir (eggs) was estimated to be the most important source of salmonellosis (43.8% of cases), followed by pigs (26.9%). Turkeys (4.0%) and broilers (3.4%) were estimated to be less important sources of *Salmonella*. Around 9.2% of all salmonellosis cases were reported as being travel-related, 3.6% were reported as being part of outbreaks with unknown source, and 9% of the cases could not be attributed to any source included in the model (Table 5.2).

Results varied substantially according to EU region, revealing differences in the epidemiology of *Salmonella* among regions, in the relative contribution of sources

Table 5.1 Host Ranges of Some *Salmonella* Serovars

	Serovar	Principal Host(s)	Zoonotic Potential
Host-specific	Typhi	Humans	None
	Paratyphi	Humans	None
	Gallinarum	Poultry	None
	Abortus-ovis	Small ruminants	None
	Typhisuis	Swine	None
Host-adapted	*dublin*	Cattle	Yes
	cholerasuis	Swine	Yes
Non host-adapted	Typhimurium	Many	Yes
	Enteritidis	Many (poultry)	Yes
	Infantis	Many	Yes

Adapted from [14].

Table 5.2 Proportion (%) of *Salmonella* Cases Attributed to Food Sources in the EU Regions,[a] 2007–9, Median and 95% Credibility Interval (%)

	EU	Eastern Europe	Northern Europe	Western Europe	Southern Europe
Broilers	3.4 (3.1–3.7)	7.0 (6.4–7.6)	1.2 (1.0–1.4)	2.1 (1.8–2.5)	3.1 (2.6–3.6)
Pigs	26.9 (26.3–27.6)	22.7 (21.5–23.9)	10.6 (10.0–11.1)	34.1 (33.5–34.7)	43.6 (42.5–44.8)
Turkey	4.0 (3.8–4.3)	2.2 (2.0–2.5)	7.4 (6.9–8.0)	4.1 (3.8–4.3)	7.6 (6.8–8.4)
Layers	43.8 (43.2–44.4)	59.4 (58.1–60.6)	30.0 (29.4–30.6)	41.8 (41.3–42.3)	28.4 (27.5–29.3)
Outbreak[b]	3.6 (n.a)[c]	5.4 (n.a.)	4.0 (n.a.)	2.2 (n.a.)	4.2 (n.a.)
Travel	9.2 (n.a.)	0.8 (n.a.)	34.5 (n.a.)	4.8 (n.a.)	0.7 (n.a.)
Unknown	9.0 (8.7–9.3)	2.5 (1.9–3.1)	12.4 (11.8–13.0)	10.9 (10.5–11.4)	12.5 (11.4–13.5)

Adapted from [73].
[a]EU regions as defined by the United Nations (Pires et al., 2010b). Eastern Europe: Czech Republic, Hungary, Poland, and Slovakia. Northern Europe: Denmark, Estonia, Finland, Ireland, Latvia, Lithuania, Sweden, and the United Kingdom. Southern Europe: Cyprus, Greece, Italy, Portugal, Slovenia, and Spain. Western Europe: Austria, Belgium, France, Germany, Luxembourg, and the Netherlands.
[b]Includes outbreaks with unknown source. Outbreak cases for which the source was identified were assigned to the correspondent animal sources.
[c]n.a. = not applicable: The proportions of outbreak- and travel-related cases were derived directly from the reported data (i.e., they were not estimated and consequently no Credibility Intervals were calculated).

for disease, and potentially in the efficiency of surveillance systems and data availability and representativeness. Layers were the most important source in Eastern, Northern, and Southern Europe, contributing between 30% and 59.4% of human salmonellosis, whereas pigs were the major source of salmonellosis in Western Europe (43.6%). Turkeys and broilers contributed with varying but lower proportions of reported cases. In Northern Europe, a large proportion of the reported *Salmonella* infections were reported as acquired abroad.

Adapting the Danish approach [11,66] estimated the relative proportions of domestically acquired sporadic *Salmonella* infections resulting from contamination of six food sources processed in the United States from 1998 through 2003. Results suggested that broiler chickens were the most important food source of domestic sporadic cases of salmonellosis (48%) for all study years. Additional estimated sources of foodborne illness in the USA were ground beef (28%), turkey (17%), egg products (6%), intact beef (1%), and pork (<1%). Both the EU and US analyses utilized food source data collected from points of food processing (farm and slaughter), but the US model assumed that all estimated sporadic illnesses were associated with the modeled food sources and did not attribute illnesses to travel or unknown sources.

The Danish approach was also adapted to national surveillance data from Japan collected between 1998 and 2007 to estimate the number of human *Salmonella* illnesses attributable to each of the major animal-food sources [72]. In this analysis, eggs were estimated to be the most important source of disease, being responsible for over 50% of the cases in most years. Broilers and swine were the second most important sources, depending on the year, while cattle/beef was seldom associated with human salmonellosis. The New Zealand adaptation of the Hald model included some modifications to the original approach, allowing for instance the model to be more adaptable to countries with less intensive surveillance systems [71]. The model attributed the majority of the *Salmonella* illnesses to pork (60%), followed by poultry (21%) and beef and veal (12%). Eggs (3%) and lamb (1%) were estimated as minor sources of infection.

The phenotypic typing methods currently applied to *Salmonella* isolates included in source attribution studies have limitations in their power to identify the origin of a given isolate, particularly for commonly occurring subtypes. Molecular methods based on characterization of the bacterial DNA (e.g., MLVA and MLST) have a considerably higher discriminatory power than the phenotypic methods and are increasingly being applied in outbreak investigation for pinpointing a particular source [59]. Still, the value of the DNA-based methods for source attribution of human salmonellosis needs to be assessed, and will undoubtedly challenge the optimal strategy: "one typing method that fits all needs." Very discriminatory methods are not necessarily the best solution for source attribution, where we are not looking for a single source for a particular outbreak, but rather want to relate groups of *Salmonella* strains with particular reservoirs/sources and then attribute human sporadic cases to these sources. Such a process must allow for some genetic diversity between strains from human and food sources that are epidemiologically related. It

is, therefore, expected that serotyping, phage typing, and susceptibility testing will remain useful tools for source attribution for some time and for strengthening the global *Salmonella* surveillance in general.

Source attribution using outbreak data

Another way of trying to assess the proportion of human infections that is likely to be foodborne, and the foods implicated in causing human disease, is to use data from outbreak investigations. One advantage of this method is that these data are observed at the public health endpoint and are often available in countries with less or no surveillance of sporadic cases [60]. A simple descriptive analysis or summary of outbreak data is useful for attributing illnesses to foods, but often the implicated food is a "complex" food containing several food items, where any of the items could be the actual source of the infection.

An alternative method for conducting an analysis of data from outbreak investigations was developed in the United States. In this method, food items are categorized into a hierarchical scheme according to their ingredients (Figure 5.4) [100]. Foods that contain ingredients included in only a single food category are considered "simple foods," while foods that contain ingredients that are members of multiple food categories are considered "complex foods." As an example, steak is a simple food, whereas meat loaf is a complex food. Each implicated food is assigned to one or more mutually exclusive food category, according to its ingredients. For outbreaks that have implicated a simple food item, all illnesses are attributed to that single category. For outbreaks that have implicated a complex food item, illnesses are partitioned to each category in the complex food according to the proportion of illnesses attributed to each of those categories in outbreaks caused by simple foods. As a result, illnesses in an outbreak due to a complex food item are attributed to a category in the implicated complex food only if that category has been implicated in at least one outbreak due to a simple food. The number of illnesses attributed

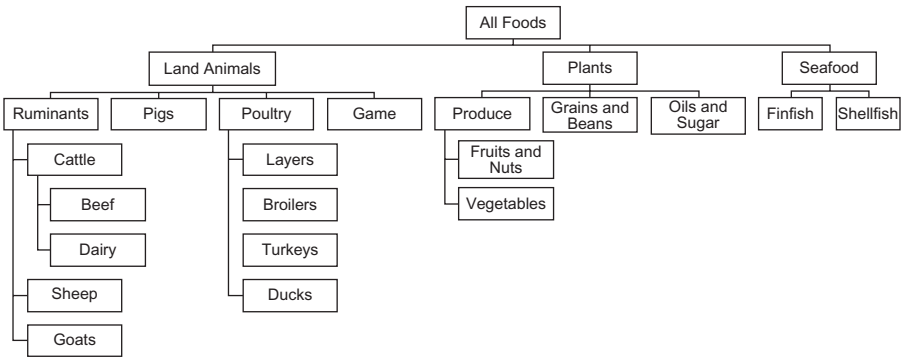


FIGURE 5.4

Hierarchical scheme for categorizing food items into commodities [73].

to each category is then summed and used to determine the percentage of disease attributed to each category [74].

This method has been adapted to attribute human salmonellosis in Europe [75]. Based on foodborne outbreak data reported by the EFSA for the years 2005 and 2006, the authors estimated that the most important food sources were eggs (32%) and meat and poultry meat (15%), but also that a large proportion of cases could not be linked to any source. The EU model was recently updated with foodborne outbreak data reported in the period between 2007 and 2009 [73]. In this study, eggs were still the most important source of disease followed by pork, chicken, the general category "meat and poultry," and dairy products (Table 5.3). In both studies, the proportion of *Salmonella* outbreaks attributed to an unknown source varied substantially between regions. When comparing source attribution results between EU regions excluding outbreaks with an unknown source (i.e., only accounting for outbreaks with known sources), the proportion of disease attributed to food sources varied. Source attribution estimates for eggs were higher in Eastern Europe (84.3%) and Southern Europe (73.8%). Pork followed in importance in Western Europe (16.6%), whereas vegetables were estimated to be a major contributor for salmonellosis in Northern Europe (18.5%). Chicken and dairy products were estimated to be of less, but more equal, importance in the regions [73].

Table 5.3 Proportion of *Salmonella* Outbreaks Attributed to Food Sources in the EU, 2007–9, by Year, Median and 95% Credibility Interval (%)

	2007		2008		2009	
Eggs	56.1	[56.0,56.3]	61.6	[61.4,61.7]	34.5	[34.3,34.6]
Dairy	2.2	[2.1,2.3]	2.9	[2.7,3.0]	1.2	[1.1,1.3]
Goat Milk	0	[0,0]	0	[0,0]	0	[0,0]
Meat	1.1	[1.1,1.12]	3.3	[3.3,3.4]	1.3	[1.3,1.4]
Poultry	0	[0,0]	0.2	[0.2,0.2]	10.9	[10.9,10.9]
Chicken	3.5	[3.5,3.5]	2.3	[2.2,2.3]	2.6	[2.5,2.6]
Ducks	0	[0,0]	0	[0,0]	0	[0,0]
Turkey	0.5	[0.5,0.5]	0.04	[0.03,0.06]	0.3	[0.3,0.3]
Beef	0.5	[0.5,0.6]	0.6	[0.5,0.7]	0.6	[0.6,0.6]
Pork	5.4	[5.4,5.4]	6.1	[6.1,6.1]	1.8	[1.8,1.8]
Lamb	0.2	[0.2,0.2]	0		0	
Mutton	0		0		0	
Game	0		0		0	
Fruits/Nuts	0.2	[0.2,0.2]	0.2	[0.2,0.2]	0.01	[0.005,0.03]
Vegetables	2.4	[2.3,2.4]	1.0	[0.8,1.1]	1.5	[1.4,1.6]
Grains/Beans	0.8	[0.7,0.9]	0.7	[0.5,0.8]	0.4	[0.3,0.4]
Oils/Sugar	0.9	[0.8,1.0]	0.2	[0.1,0.3]	0.8	[0.7,0.8]
Seafood	0.8	[0.8,0.8]	2.4	[2.4,2.5]	0.9	[0.8,0.9]
Water	0.5	[0.5,0.5]	0		0	
Unknown	25.0		18.5		43.5	

Adapted from [75].

A similar model based on outbreak data and which is able to consider complex foods was also applied to attribute human foodborne illnesses to specific sources in Latin America and the Caribbean [73]. Data from 20 countries and covering the period from 1993 to 2010 were collected. In general, eggs, meat products, vegetables, chicken, grains and beans, and pork were the most important sources of salmonellosis in the whole period. When excluding outbreaks with an unknown source, results showed a substantial increase from the 1990s to the 2000s in the proportion of disease attributed to the sources eggs (from 16.8% to 43.3%) and pork (3.7% to 9.1%), and minor increases in the relative contribution of vegetables (10.2% to 11.6%) in the same period. In contrast, the proportion of disease attributed to meat products (29.2% to 8.9%) and chicken (12% to 5.6%) decreased in the same period.

The same method for analysis of outbreak data was applied to achieve source attribution estimates for Japan [73]. Data covered the period from 2000 to 2009 and attributed disease to food sources and water. *Salmonella* source attribution estimates suggested that eggs were the most important food source during the whole study period, and that the proportion of disease attributed to this source increased in the second half of the decade. Among outbreaks with a known source, vegetables followed eggs in importance, accounting for 13.2% of disease from 2000 to 2004 and for 16.8% from 2005 to 2009. Grains and beans accounted for 11.8% and 12.4% of disease respectively in the same time periods. All remaining food sources were shown to be of minor importance for salmonellosis in Japan; however, over 80% of reported outbreaks could not be attributed to any source.

A statistical analysis of data collected from 1996 to 2005 through the Canadian foodborne outbreak surveillance system has also been performed [76]. Only data from outbreak investigations identifying both the agent and the food vehicle were used. The results indicated that produce was the most frequent cause of *Salmonella* outbreaks (28.9%), followed by poultry (14.5%), other meats (14.5%), dairy products 9.2%, and seafood (6.6%). Eggs caused 5.3% of all *Salmonella* outbreaks, and in 13.2% the implicated food was multi-ingredient, where the responsible ingredient was not identified or estimated through modeling.

Among other limitations, using outbreak data for attribution means that the quality of evidence varies between data sources, and classification schemes for the data are not consistently used. Also, large outbreaks, outbreaks associated with point sources, outbreaks that have short incubation periods, and outbreaks that cause serious illness are more likely to be investigated. Likewise, certain food vehicles are more likely to be associated with reported outbreaks than others, which can lead to an overestimation of the proportion of human illnesses attributed to a specific food.

An important factor to consider is that illnesses included in data from outbreak investigations may not be representative of all foodborne illnesses. The fraction of the burden of foodborne disease that is associated with outbreaks varies between pathogens but is typically smaller than the correspondent to sporadic disease. Consequently, the extrapolation of source attribution estimates obtained through an analysis of data from outbreaks to the overall burden of disease should be made

with care. Pires et al. [75], however, concluded that the approach seemed useful for attributing human salmonellosis, but not campylobacteriosis [75]. The latter because there are relatively few reported outbreaks of campylobacteriosis and the relative importance of the implicated sources seems to differ between outbreaks (e.g., water) and sporadic cases (e.g., poultry meat).

Source attribution using systematic review of case-control studies

Case-control studies of sporadic infections are the most commonly applied approach for identifying possible exposures for sporadic foodborne disease including salmonellosis. Culture-confirmed case patients and a representative group of asymptomatic individuals (controls) are interviewed, and the frequency of exposures among cases and controls is compared and the association between disease and a certain exposure is quantified by calculating the odds ratio (OR). The proportion of cases attributed to the exposure can then be calculated and is defined as the population attributable fraction (PAF) [77]. The population attributable fractions can be used to attribute the human disease burden to specific sources.

Numerous case-control studies of sporadic infections of diseases commonly transmitted through food have been published, as reviewed by [78,79], and case-control studies are considered a valuable tool to identify potential risk factors for human infections, including sources and predisposing, behavioral, or seasonal factors [80]. Limitations of case-control studies include misclassification due to immunity, which may reduce attributable risk or even suggest protection. Likewise, misclassification of exposures due to lack of accuracy of recall may lead to an underestimation of the burden of illness attributed to specific exposures. Most studies only explain a small fraction of all cases, and cases may reflect a mixture of possible sources of exposure, which can make it difficult to distinguish between these exposures. Lastly, statistical power to determine the importance of common exposures often requires enrollment of many participants.

A systematic review (SR) of published case-control studies of sporadic infections can, in addition, provide an overview of the relevant exposures and risk factors and may identify geographical, temporal, or age-related differences regarding the most important exposures [60]. An SR follows a rigorous search strategy to identify all relevant peer-review case-control studies for a hazard, studies being conducted in a variety of countries and time periods, designed with different settings, and potentially focused on specific age groups within the population. Data from the different studies are then combined in a meta-analysis, where risk factors can be stratified according to predefined source-categorization schemes, location of exposures, and, if appropriate, frequency of exposure. The weighted summary statistics of several case-control studies may then be combined with estimates of the burden of disease caused by that hazard to estimate the burden of disease attributed to each exposure.

Recently conducted an SR of case-control studies and a meta-analysis to identify risk factors for salmonellosis [78]. Data from 35 case-control studies from

11 countries were included. Investigated risk factors represented consumption of foods, direct contact with live animals, environment transmission, predisposition, and behavioral factors. Results showed that international travel (OR 6.5), intake of antacids (OR 2.9), pre-existent medical condition (OR 2.8), previous intake of antimicrobials (OR 2.23), eating raw eggs (OR 2.78), and eating in a restaurant (OR 2.74) were the most important risk factors for human salmonellosis in the overall study population. Consumption of undercooked or raw eggs and chicken in a restaurant were the only food items identified as relevant for human disease in the analysis, and environmental routes (both drinking and recreational waters), direct contact with pets and farm animals, and various predisposition factors proved to play a role in human salmonellosis (Figure 5.5). The results of the analyses focusing on serotypes suggested that traveling abroad and consumption of eggs are particularly important risk factors for *S*. Enteritidis infection, while previous intake of antimicrobials was the only risk factor identified for *S*. Typhimurium. Available studies did not allow for an analysis by region or age group.

An SR of epidemiological studies can only be undertaken if a sufficient number of studies focusing on the same hazard have been conducted and made publically available. Also, once a SR based on all relevant studies has been conducted, a new review or update of a previous review will only add to the existent knowledge if results from a substantial number of new case-control studies are available for inclusion.

Other approaches for source attribution

Comparative exposure assessment, intervention studies, and expert elicitations are other approaches that have been identified as useful for source attribution [60]. However, due to the different limitations of these approaches, they are generally only applied when data from outbreak investigations or subtyping data are unavailable or inappropriate to use. These approaches will therefore only be described in brief.

The comparative exposure assessment approach for source attribution makes use of stochastic modeling techniques similar to those used in quantitative microbial risk assessments. Nevertheless, the two methods differ in objectives and level of detail. A risk assessment typically aims to describe the complex dynamics of a pathogen in a single food commodity in the farm-to-consumption continuum, and then predict the public health impact of intervention strategies. In contrast, a comparative exposure assessment attributes illness to the point of exposure, taking into account the different transmission routes from the same reservoir. This allows for the investigation of different pathways of exposure from the main reservoirs, as well as the estimation of the relative importance of each. However, the method requires many parallel exposure assessments to be made and is therefore data- and labor-intensive, even though the modeling is relatively simplistic.

Modeling of the animal contact and environmental transmission routes requires information on contact frequencies and the probability of ingestion of the pathogen

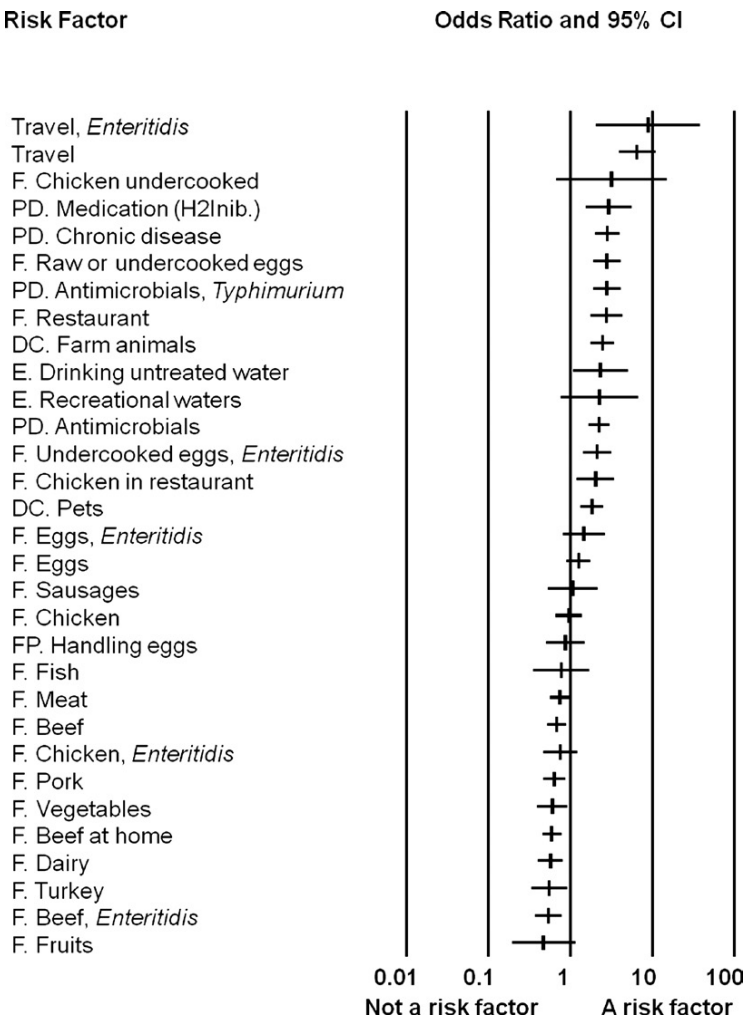


FIGURE 5.5

Relative importance of risk factors for sporadic salmonellosis (odds ratio and 95% CI). Notations: F: Food; DC: Direct contact with animals; E: Environmental transmission; PD: Predisposition factors; FP: Food preparation; H2Inib: Proton inhibitors medication.

From Domingues et al. [78]

given contact. Such data are currently only available to a limited extent, and the parameters are difficult to measure in practice. The current large number of data gaps results in large uncertainty intervals and makes uncertainty analysis an essential component of the results [81]. The data limitations are one of the reasons why other methods for source attribution of human salmonellosis are preferred.

Intervention studies can provide evidence of the burden of a foodborne disease attributable to specific sources through the measurement of the impact of interventions that are implemented in the food production chain on the number of human cases. Intervention studies can be designed as small or large-scale studies to control a specific pathogen, and the measure of the impact on specific sources of human disease is facilitated when interventions are conducted in a randomized design. Intervention studies conducted in a controlled environment have the advantage of allowing for a direct measure of the impact of a reservoir or source on the burden of human disease, excluding the interference of external sources or risk factors. However, for large-scale studies usually several interventions are implemented at the same time, and this hampers the interpretation of data on the public health impact of a specific intervention and food-animal source. An example of a large-scale intervention study is the reduction of the burden of human salmonellosis in Denmark following the implementation of several different control programs in the three most important food sources (Figure 5.3; [67]).

Expert elicitations are often used to address data gaps during source attribution exercises, but a structured approach to use expert judgments can also be used to assist attribution. These structured approaches require more resources and technical expertise than conventional, unstructured evaluations and need a multidisciplinary approach, which may compromise their acceptance in practice. Expert estimates typically combine information from different sources, which can be considered both a strength and a weakness. An important limitation is that conclusions are based on individual judgment, which may be misinformed or biased [81].

Expert elicitation studies may be particularly useful to estimate the fraction of the burden of a disease that can be attributable to foods in general, followed by the application of another source attribution approach to estimate the relative importance of different food sources. To date, a number of studies have been performed using expert elicitation to attribute foodborne disease, e.g., in the USA [2,82], in Australia [83], and in the Netherlands [84]. In 1999, Mead et al. used expert data to estimate that 95% of human salmonellosis in the USA was foodborne [2]. Recently, this estimate was updated by [6], who found a foodborne proportion of 94%. In Australia, an expert elicitation including experts from public health and food safety fields estimated that 87% of the *Salmonella* illnesses could be attributed to foodborne sources [83].

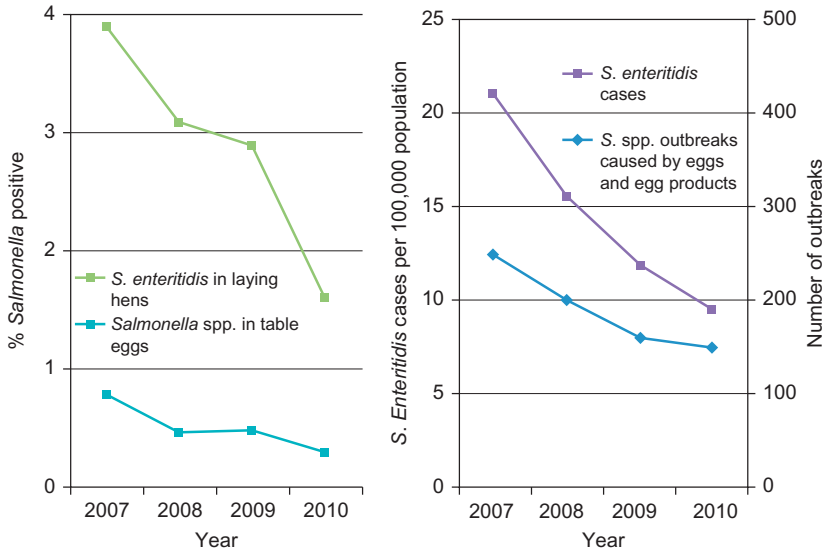
In contrast, estimates of foodborne transmission of human salmonellosis appeared considerably lower in New Zealand, where [85] report a most likely value of 60.7% based on expert elicitation. Also a study by Havelaar et al. [84] suggested a much lower overall foodborne proportion of 55%. The same study attributed the foodborne proportion into 11 food categories, where eggs were estimated to be the most important food source (22%), followed by poultry (15%), pork (14%), and beef and lamb (13%). Another expert elicitation reported by Hoffmann et al. [82] in the USA suggested that, among foodborne salmonellosis, 35.1% were attributed to poultry, 21.8% to eggs, 11.7% to produce, and 10.9% to beef. Finally, a Canadian source attribution study using expert elicitation showed that poultry (34.2–42.4% of foodborne cases),

eggs (18.6–20.7%), produce (8.4–17.8%), and pork (7.2–8.1%) were the main food sources responsible for foodborne salmonellosis [86].

Discussion of sources of human salmonellosis

Source attribution approaches are increasingly being applied to surveillance data in an effort to inform control and intervention. In this chapter, we have focused on studies based on microbial subtyping and outbreak data. Each approach has different data requirements, has method uncertainties, and attributes illnesses to different points in the farm-to-consumption continuum. Methodological differences and differences in data availability and quality contribute to the observed variability in source attribution across the studies. However, the variation in the relative importance of different sources between countries and regions undoubtedly also reflects differences in the epidemiology of salmonellosis, including differences in animal and food prevalences, food preferences and patterns of consumption, and animal and food production systems.

In the EU, the overall incidence of human salmonellosis decreased between 2006 and 2010, which is mainly explained by a decrease in the number of *S*. Enteritidis infections which, presumably, is a result of improved surveillance and control of *S*. Enteritidis in laying hens in EU MSs (Figure 5.6) [10,68]. In contrast, the incidence of *S*. Typhimurium infections has changed little during this period,



Note: Data for laying hens and table eggs are presented only for sample sizes ≥25. For laying hens only, data from sampling during the production period were included.

FIGURE 5.6

Salmonella in human cases, eggs, and laying hens and the number of *Salmonella* outbreaks caused by eggs within the EU, 2007–10 [10].

indicating the need for improved monitoring and control of *Salmonella* in the major sources of these infections, particular pigs and pig meat, which recently has been pointed out as the major source in many EU countries [87].

Despite the decreasing trend of *S.* Enteritidis cases, eggs from laying hens are still considered one of the most important sources of *S*. Enteritidis infections in many EU MSs. This is supported by the source attribution analysis based on subtyping data and outbreak data [73,75]. A certain proportion of human *S*. Enteritidis infections are also attributed to broilers, particularly in countries with a high *S*. Enteritidis prevalence in broiler flocks.

In the US model described by [11], chickens were found to be the most important source, whereas egg products were of less importance. This is in contrast to the EU study and may be explained by differences in consumption patterns (the average US citizen eats 2–3 times more chicken meat than the average EU citizen) and/or prevalences in the two regions. Also, in the US model, representative data on shell-egg contamination were not available and egg products, which presumably are associated with a lower foodborne risk, were included instead [11]. In New Zealand, eggs were also found to contribute only to a few percent of the human cases, but here the reason is probably due to the fact that *S.* Enteritidis has never been established in the national shell-egg production [71].

Human *S.* Typhimurium infections represent between ca. 10–20% of all cases in the EU and the majority of these cases are likely to be associated with pig meat consumption [73,87]. Certainly broilers and beef also contribute to these infections, but the contribution is assessed to be low due to low prevalences and/or lower impact through the food production chain. The latter is derived from the fact that some of the dominant *S.* Typhimurium phage types in broilers only occur in low frequencies in humans. In New Zealand, pork was found to be the major contributor, probably reflecting the fact that *S*. Typhimurium is the single most important serovar found in domestically acquired salmonellosis cases [13,21]. In the USA, the contribution from pork was very low, which cannot be readily explained by the data available.

Travel appears to be an important "source" of sporadic salmonellosis, particularly in Northern Europe. However, travel data are lacking from many countries, so the role may be underestimated in other parts of the EU. Attribution studies from the USA and Japan have not considered travel.

Only a few countries systematically collect data on *Salmonella* in imported food. Experience from Denmark indicates that the relative importance of this source increases when efforts to control domestic sources are successful. This will, however, depend on the amount and origin of the imported food.

Fresh produce is described as an increasing source of foodborne outbreaks and salmonellosis in the USA and Canada. In Europe, multistate outbreaks caused by fresh produce have also been observed (e.g., [47,93]) but, based on the attribution study using outbreak data, fresh produce does not seem to be a major source in the EU in general. Fresh produce has not been included in the attribution studies based on subtyping due to lack of appropriate surveillance data. Still, from a risk

management point of view, it can be argued that fresh produce is included indirectly in attribution estimates since food-producing animals constitute a reservoir for the contamination of fresh products. Reducing *Salmonella* occurrence in food-producing animals will, therefore, also to some extent reduce the contamination level of fresh produce produced in the same country/region [66].

Prevention and control

Salmonella can occur in a wide range of different food products. It is reasonable to assume that all products contaminated with *Salmonella* at the point of consumption have the potential to cause human disease. Control of salmonellosis should involve all stages in the "feed-to-consumption" continuum, and ideally be conducted as an integrated effort, where control measures in each stage of production are coordinated with efforts in other stages to optimize the effect.

In the EU the Zoonosis Directive (92/117/EEC) was an attempt to initiate an EU-wide control effort against *Salmonella*, mainly in broiler and layer breeders. As it turned out, only 7 of 15 countries implemented the Directive, whereas the majority of EU countries found that they either could not or would not implement the Directive. The Directive did not permit use of vaccines and/or antimicrobials as elements in the control program of *Salmonella* in broilers or layers. This was seen as a major obstacle by some countries. In 2003, a new Directive (2003/99/EF of November 17th 2003) and a new EU Regulation (No. 2160/2003 of November 17th 2003) were adopted. The Directive requires the EU MSs to monitor the occurrence of certain zoonoses including *Salmonella* and report the results to EFSA on an annual basis. According to the Regulation, EU MSs are obliged to implement national control programs for *Salmonella* serovars assessed to be of particular public health significance in animal species presenting a high potential risk of transmitting *Salmonella*, such as poultry and pigs.

EU targets for the reduction of *Salmonella* prevalence in food-animal populations have been set by the EC in consultation with MSs, and the MSs are obliged to fulfill the targets within a certain period of time. The target setting has been based on the results of a number of EU-wide baseline surveys with the main objective to establish an EU baseline and MS-specific prevalences, so that realistic targets could be set. The animal populations that are specifically targeted currently include breeding flocks of *Gallus gallus*, laying hens, broilers, and breeding and fattening turkeys. The national control programs are established to achieve the agreed targets to reduce *Salmonella* prevalence at primary production level for these poultry productions [10]. For breeder and finishing pigs, baseline surveys have been conducted along with a quantitative risk assessment [88] and a cost–benefit analysis, whereas the establishment of a harmonized monitoring and the setting of EU targets are still under negotiation.

Other countries, including the USA and Canada, have implemented national *Salmonella* monitoring and control programs to improve food safety for selected

meat and poultry products. Control efforts in North America have primarily been focused at the processing level through the implementation of HACCP.

Prevention and control at the farm level

The purpose of control strategies for *Salmonella* in food-producing animals is to reduce or eliminate *Salmonella* in animals entering the slaughter line or for laying hens to reduce or eliminate the occurrence in eggs. The need for pre-harvest control depends on the feasibility and effectiveness of subsequent post-harvest control. The focus of the pre-harvest control also depends on the animal species, but in general the following basic principles of pre-harvest control apply:

- prevent introduction into the herd or flock;
- prevent transmission within the herd or flock;
- reduce the occurrence and shedding in infected populations.

Control in poultry farms

In poultry production the very efficient vertical dissemination, particularly of *S.* Enteritidis, through the breeding system demands a top-down eradication control strategy. Successful reduction of *Salmonella* in the broiler and laying hen production has been achieved by several EU countries [32]. Strict batch production and biosecurity in confined production systems and heat treatment of commercial poultry feed have been important elements in the control. Thus, the flock prevalence in Danish broiler flocks was reduced from approximately 70% in 1990 to a few percent since 2001, and in 2011 Denmark obtained the same special guaranties as Sweden and Finland due to the very low occurrence of *Salmonella* in laying hens.

Control in pig farms

Eradication of *Salmonella* in pig farms has only taken place in a few low-prevalence North European countries, but monitoring and reduction in pig populations with an endemic occurrence of *Salmonella* have become more common. Risk factors for high occurrence of *Salmonella* in pig herds are well known, and it has proven possible to reduce high infection levels in pigs to moderate or low levels by purchase of non-infected pigs, strict all-in/all-out production, and strengthened hygiene levels and biosecurity. In addition, the use of certain feed and feeding practices has been shown to reduce proliferation of *Salmonella* in the gastrointestinal tract through microbiological and chemical changes.

Control in cattle farms

In the last decennium, evidence-based intervention tools to control *S. dublin* in cattle herds have been developed. Introduction of *Salmonella* from purchased animals must be avoided, and introduction from tools and the herd environment may be prevented by strict hygiene and biosecurity measures. In infected herds, the risk of triggering an outbreak of salmonellosis will be reduced by good hygiene in calving and calf units. Infection from cow to calf through contact after calving can be

avoided by supplementing calves with colostrum from a colostrum bank, and a one-way flow of animals in the farm should be established.

Vaccination against *Salmonella* is mainly used in poultry and may, under some circumstances and for some serovars, reduce carriage of *Salmonella* in the flocks. Serologic vaccine reactions in routine monitoring assays (i.e., false positive reactions) should be taken into account when vaccination is considered.

The use of antimicrobials should never be used to control subclinical infections with *Salmonella* due to the risk of development of antimicrobial resistance.

Control and prevention post harvest

Salmonella cannot be dealt with through classical meat inspection practices as production animals are mostly asymptomatic carriers [93,94]. In many countries physical (e.g., steam) or chemical (e.g., lactic acid or chlorine) decontamination of carcasses is used to reduce pathogen levels. Laboratory and in-line investigations of decontamination effects show that hot water and steam on average reduce *Salmonella* levels by 100–1000 times, while the effect of chemical decontamination often is within the range of 10–30 times. The combined use of two or more decontamination methods is common and can further improve efficacy. In Denmark, serologic classification of herds has been used to direct 1% of all pig carcasses to hot water decontamination. Individual control systems are often in place and allow current monitoring of slaughter hygiene. There may also be requirements for individual slaughterhouses in relation to export, e.g., demanded by the Food Safety Inspection Services in the USA. In the USA, *Salmonella* performance standards at different levels in the meat chain have been implemented, and in the EU process hygiene criteria have been instituted.

Conclusions

In conclusion, salmonellosis continues to represent a considerable foodborne disease burden in a majority of countries. There is considerable under-reporting, and the true number of cases of illness is likely to be much higher than what is reported.

The successful control of *Salmonella* (and other zoonotic pathogens) requires knowledge about the most important sources or reservoirs as well as the principal routes of transmission, and these are very likely to vary between countries and regions. The identification of sources should, whenever possible, be based on several source attribution approaches, as the combined results will increase confidence in the conclusions. In addition to evaluating the trends and dynamics of sources to human infections, the results will support risk managers in their resource allocation decisions in order to achieve the highest possible benefit. The fact that we are living in a "global village" furthermore calls for food safety surveillance and efforts to reduce transmission of *Salmonella* by food and other routes to be implemented on a global scale.

Finally, it is stressed that the successful surveillance and control of *Salmonella* (and other zoonoses) requires collaboration between all experts in the food production chain, i.e., between microbiologists and epidemiologists, across veterinary and public health borders, and between food safety authorities, scientists, and the food industry.

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CHAPTER

Clostridium perfringens Gastroenteritis

6

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Introduction

The classical work of [1] identified *Clostridium perfringens* as an important cause of foodborne illness in developed countries. In the USA, for example, it is second only to *Salmonella* in estimated annual number of cases due to bacterial agents [2]. Its involvement can be attributed to its wide distribution in the environment, its ability to form heat-resistant spores, its rapid growth rate at relatively high temperatures, and its ability to form an enterotoxin.

Clinical features

Symptoms associated with foodborne illness

C. perfringens is involved with a number of veterinary and human diseases apart from foodborne illness. The focus here is on the latter. The classic symptoms of *C. perfringens* type A food poisoning are diarrhea and lower abdominal cramps. Vomiting is not common, and fever is rare. Symptoms typically occur 8–24 hours after ingestion of temperature-abused foods containing large numbers of vegetative cells of the organism. Mortality is low and occurs primarily in elderly patients. Symptoms subside within 1–2 days, although cramps can continue a little longer. The brevity of the events is probably due to the normal turnover of intestinal cells to which the toxin is bound, as well as to removal of unbound CPE (*C. perfringens* enterotoxin) by the diarrheal process which it itself induced. Antibiotic therapy is not recommended because the symptoms are self-limited. Dehydration, especially in the elderly, is the most important concern.

Mode of action of *C. perfringens* enterotoxin

Foodborne illness due to *C. perfringens* is due to the production of an enterotoxin. Members of the claudin family of proteins have been identified as functional CPE

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receptors. The claudin family includes several transmembrane proteins that play important structural roles in epithelial tight junctions. CPE binds to these proteins resulting in desquamation of intestinal epithelium. This results in the loss in fluid and electrolytes into the intestinal lumen, which is expressed clinically as diarrhea. Details of the cellular and molecular mode of action of CPE have been described by [3].

A potential medical application of CPE for treating pancreatic cancer cells has been identified [4]. Such cells overexpress claudin-4, one of the transmembrane proteins mentioned above. *In vitro* and *in vivo* treatment of pancreatic cancer cell lines and tissues with CPE led to a dose-dependent cytotoxic effect, which suggests a promising new treatment modality for pancreatic cancer and other solid tumors [5].

Microbiology

Taxonomy

C. perfringens is a Gram-positive, spore-forming, rod-shaped bacterium that is encapsulated and non-motile. The G-C content is from 24–27 moles %. Based on analyses of 16S rRNA, its closest relative is *Clostridium pasteurianum* [6]. Spores, usually located subterminally, are formed only in specially formulated media. Important cultural characteristics (used in confirmation of isolates) include the reduction of nitrate, liquefaction of gelatin, and fermentation of lactose. The production of lecithinase (α-toxin) has also been used for classification purposes, and is demonstrated by a pearly opalescence surrounding colonies grown on egg yolk agar that can be inhibited by type A antitoxin (Nagler reaction). A detailed description of the cultural characteristics of *C. perfringens* was provided by [7]. To date, the complete genome sequence of three type A strains has been published [8,9].

Location of CPE

The gene responsible for CPE may be located on a plasmid or chromosome. Most food poisoning isolates have been shown to carry CPE on the chromosome, while most *C. perfringens* isolated from patients with other clinical syndromes show a plasmid location [10,11]. Vegetative cells of isolates that have chromosomally-located CPE survive heating better than cells of plasmid CPE isolates [12], show higher $D_{4\ ^\circ C}$ and $D_{-20\ ^\circ C}$ values (i.e., survive longer at these temperatures), and have a higher maximum growth temperature. Spores with chromosomally-located CPE are also more resistant (see below) [13]. These characteristics may explain the strong association of chromosomal CPE isolates with foodborne outbreaks.

Factors affecting growth

Under proper conditions, *C. perfringens* displays the shortest generation time of any bacterial pathogen and perhaps of any bacterium. Generation times of 7–8 minutes in ground beef have been reported [14–16]. Meat and poultry items subjected to improper holding times and temperatures easily fulfill these conditions. Spores that occur naturally in such products can survive cooking temperatures that provide the heat activation for germination. The upper and lower temperature limits of growth temperature are 50 °C and 15 °C, with occasional reports of temperatures slightly above and below [13].

C. perfringens is not particularly tolerant of low A_w (a measure of available water) or extremes of pH or curing salts, but is more tolerant of elevated E_h (reducing conditions) than most anaerobes, explaining the role of cooked meat and poultry as vehicles in outbreaks [17–18].

Growth during cooling

During refrigeration of large cuts of cooked meat, the internal temperatures can pass through the growth range of *C. perfringens* for sufficiently long to permit significant growth. This highlights the need to reduce the size of meat portions for cooking, or to reduce large volumes of meat and gravies after cooking.

The ability of *C. perfringens* to grow over a wide, relatively high temperature range has prompted additional studies on the kinetics of growth during cooling. In the case of *C. perfringens*, negligible growth of a three-strain composite of spores occurred in cooked beef cooled from 54.4 °C to 7.2 °C in 15 hours or less, although a several-log increase did occur if the cooling period was extended to 18 hours [19]. Similarly, no growth from spores occurred within 150 minutes in cooked ground beef inoculated at 60 °C and cooled to 15 °C at a falling rate of 15 °C/h [20]. Also, refrigerated, aerobically or anaerobically packaged cooked ground beef containing 10^3 *C. perfringens*/g and temperature-abused at 28 °C for 5 hours did not support the growth of *C. perfringens* [19]. It appears that short periods of temperature abuse of small portions of refrigerated meat containing *C. perfringens* do not necessarily result in a hazardous level of cells. Other factors affecting growth and survival, such as Eh, Aw pH, and curing salts, have been discussed elsewhere [21,22].

The potential for rapid growth of *C. perfringens* during cooling is reflected in the USDA-FSIS compliance guidelines for chilling thermally processed meat and poultry, which state 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 hours and cooling from 26.7 °C to 4.4 °C (40 °F) should take no longer than 5 hours [23]. Predictive models have been developed for growth during cooling of uncured meat [24–26], the latter accessible via www.combase.cc or http://ars.usda.gov/Services/docs.htm?docid=6786.

Sporulation

The ability to sporulate is an important property of *C. perfringens* because: (a) spores can survive cooking procedures and resume vegetative cell growth if proper conditions are present; (b) high levels of enterotoxin formation are associated with sporulation; and (c) it is a factor in classification.

The essential role of CPE in foodborne illness was shown unequivocally by the use of knockout mutants [27]. Because of its association with enterotoxin formation, a good deal of work has centered on the factors that affect sporulation, which is highly strain-dependent. Though sporulation does occur in the small intestine, as stated above, most strains sporulate only reluctantly in laboratory media, and even then only to moderate levels. Low levels of spores, sufficient for culture carriage, are usually produced in commercially available cooked-meat medium (CMM). However these levels of sporulation are insufficient for high CPE yields, or for obtaining adequate spore crops for additional studies.

Over a period of many years, several sporulation media have been devised based on the medium of ([28] summarized in [29]). Optimal conditions are often determined empirically, and a specific protocol and medium may not apply for all strains. Solid sporulation media have also been described [30,31] but not widely adopted.

Sacks and Thompson [32,33] developed a defined sporulation medium which has proven useful for metabolic studies of the sporulation process and for radioactive labeling of CPE [34,35]. As with other complex sporulation media, not all strains respond with a high percentage of sporulating cells.

Enterotoxin formation during sporulation

Clinical symptoms associated with foodborne illness due to *C. perfringens* are due to an intracellular enterotoxin (CPE) produced in the small intestine during sporulation of ingested vegetative cells. CPE and the mature spore are released from the mother cell together. These events can be reproduced in the laboratory, and the pattern of CPE formation in laboratory media is now well established [36,37]. The sequence of events is presumably similar during sporulation in the intestine. *In vitro*, heat-resistant spores can be detected 3–4 hours after inoculation of a sporulation medium with vegetative cells. Maximum spore levels are reached within 7–8 hours, with levels as high as 3–4 $\times$ 10^7/ml possible. After approximately 10 hours the toxin and mature spore are released from the sporangium, at which point the CPE is detectable in the culture fluid. The kinetics of these events generally corresponds to the time required for onset of symptoms in clinical cases. CPE is not secreted outside the cell, and is therefore not an exotoxin in the classical sense.

CPE can represent a significant portion (20% or more) of total cell lysate protein [38,39], perhaps due to the presence of multiple CPE promoters. The levels are distinctly strain- and nutrient-dependent [40]. Nevertheless, the function of CPE in the metabolism of *C. perfringens* remains unknown.

Spore heat resistance

The ability of spores of *C. perfringens* to survive heat-processing procedures commonly used when cooking meat contributes to its role in virtually all outbreaks of foodborne illness caused by this organism. Spores vary widely in their heat resistance, dividing *C. perfringens* into so-called heat-resistant (HR) and heat-sensitive (HS) strains [41,42]. One explanation is the location of the enterotoxin gene, CPE, as mentioned above. Spores from food poisoning isolates show approximately 60-fold higher $D_{100\ °C}$ values compared with spores from isolates with CPE on a plasmid or lacking CPE [12]. Another extrinsic factor in spore heat resistance is the temperature at which sporulation occurs; increased heat resistance is associated with higher sporulation temperatures [38]. Other factors affecting spore heat resistance include the menstruum in which inactivation was performed, the medium in which the spores were grown [42–45], and the presence and type of small acid-soluble spore proteins [46].

Spore germination

In contrast to the reluctance of *C. perfringens* to undergo sporulation, its heat-activated spores germinate readily in common, complex laboratory media, as well as in meat and poultry. In aqueous systems, the process can readily be monitored on the basis of loss of absorbance over time. In such systems a difference in germinants (amino acids) was noted for chromosomally-located versus plasmid-located CPE [47].

Another difference between HR and HS strains is their requirement for heat activation of spores [42,48]. For example, only 0.13–3.5% of HR spore strains grew without heat activation, compared with 30–50% of HS strains. A temperature/time of 75 °C/10 minutes effectively activates spores without injury. Heat activation not only promotes germination of spores, but also increases sporulation and enterotoxin formation of certain strains when applied following successive passages through a sporulation medium, presumably by selecting for a spore-forming population [49].

Detection of the organism and enterotoxin

Criteria for outbreaks

A number of epidemiologic criteria have been proposed for establishing an outbreak of *C. perfringens* type A food poisoning. These include: (a) $>10^6$ ent^+ spores/g feces from ill individuals; (b) $>10^5$ ent^+ cells/g in incriminated food; (c) the presence of the same serotype in all ill individuals in an outbreak; or (d) detection of enterotoxin in feces of ill individuals. However, the finding that healthy humans can harbor mean *C. perfringens* spore levels/g of $10^{5.5}$ [50] raises questions as to the accuracy of criterion (b). Likewise the usefulness of serotyping has been questioned (see below).

Enumeration

The procedure selected for enumeration of *C. perfringens* depends upon whether the sample is from routine sampling or from a suspected outbreak. In the former

case, low numbers would be expected and a most probable number (MPN) procedure is the most appropriate (see below).

The plating medium currently recommended in North America and elsewhere is tryptose-sulfite-cycloserine (TSC) agar, in which *C. perfringens* appears as black colonies. The egg-yolk-free variation is recommended, because it is just as effective and simpler. Incubation is at 37 °C in an anaerobic chamber. Detailed procedures for its use are available elsewhere [51,52]. An elevated fecal spore count (after heating at 75 °C for 20 min) is also a diagnostic criterion for implicating *C. perfringens* in foodborne outbreaks.

More recently [53] compared a number of media which have been proposed for recovery of *C. perfringens*. TSC (without egg yolk, TSC−) and reinforced clostridial agar (RCA) supported optimum growth of vegetative cells while RCA was superior for recovery of spores. However only three strains were evaluated and an expanded study to confirm these results would be of interest.

An MPN procedure is used for routine sampling where low numbers are expected. Iron milk medium (IMM), consisting of pasteurized whole milk with 2% iron powder, is simple to prepare, inexpensive, and relatively sensitive [54]. Selection is based on the rapid growth of *C. perfringens* at 45 °C, and the typical "stormy fermentation" reaction, during which acid from lactose fermentation rapidly coagulates the milk and is followed by fractionation of the curd into a spongy mass; if results are read after 18 hours of incubation, confirmatory tests (see below) are not necessary [55]. Similar counts of *C. perfringens* were obtained from the environment and food samples using TSC medium or the IMM method [56,57].

Confirmatory tests

None of the plating methods mentioned above is completely selective for *C. perfringens*. The possible (though unlikely) presence of other sulfite-reducing clostridia requires the confirmation of a representative number (about 10) of isolates. The recommended media for this purpose in North America and Australia are tubes of motility-nitrate (MN) and lactose-gelatin (LG) (US FDA). The use of TSC agar for enumeration together with MN and LG for confirmation has been adopted as official first action by AOAC International. The ISO methods recommend the use of lactose-sulfite medium alone as a confirmatory test [51]. However lactose-sulfite has been shown to be inferior to MN and LG media for confirmatory purposes [58].

In terms of identification, a commercial rapid miniaturization test, RAPID ID 32A, was reported to correctly identify all 49 *C. perfringens* strains tested [59].

Detection of the enterotoxin

The direct detection of CPE in outbreak stools is a useful technique for confirmation of a foodborne outbreak due to *C. perfringens*. Currently the most widely used assays are an ELISA and reverse passive latex agglutination (RPLA), which have sensitivities of 2–4 ng/g feces [41,60]. Stools from healthy individuals contain undetectable levels of CPE [60] compared with outbreak stool samples which contain >1 µg/g [60–62].

Two kits for serological detection of CPE are available commercially: an ELISA (Tech Lab, Inc.), and RPLA (Oxoid). When it is necessary to determine the enterotoxigenicity of isolates, the instructions included with commercial CPE detection kits may be misleading by implying that a single sporulation medium will induce sporulation in all isolates. As mentioned above, *in vitro* sporulation is highly strain- and medium-dependent, and diarrheal strains often fail to sporulate *in vitro* [63].

Molecular methods

Non-CPE toxin genes

In addition to CPE, *C. perfringens* produces more than a dozen extracellular toxins [3], although each isolate only produces a specific subset of these. A novel toxin, termed beta-2 (β-2) toxin, has been identified, and β-2-producing strains have been associated with intestinal disorders in animals [64,65]. Yet another toxin, NetB [66], has been putatively associated with necrotic enteritis in chickens, which has re-emerged as a major economic problem in the poultry industry (reviewed by [67]).

Strains of *C. perfringens* are divided into five types based on the production of four extracellular toxins: alpha, beta, episilon, and iota. In principle, all types produce α-toxin (lecithinase, phospholipase C), although occasionally a lecithinase-negative strain is isolated from outbreaks [68–69]. Neutralization of biological activity in the skin of mice or guinea pigs by specific antisera is the traditional method of toxin typing [70–72]. However the PCR has replaced seroneutralization assays in mice or guinea pigs for the principle *C. perfringens* toxins. ([73] [reviewed by [74]]). Frequently these assays are multiplexed together to detect several toxin genes of *C. perfringens*. Real-time multiplex PCR assays for toxin genes have been developed [75]. Determining the presence of the various toxin genes is typically performed for veterinary isolates. Since all foodborne isolates are type A, it is not necessary to determine the toxin type of isolates from outbreaks.

CPE Gene (CPE)

Even with the availability of commercial test kits for detecting *CPE*, the presence of enterotoxin often is not determined. The potential of isolates to produce enterotoxin can be determined with PCR procedures to detect the *CPE* gene (*C*PE); the assumption is that, if present, *CPE* will be expressed. Typically the detection of *CPE* by PCR is done together with the detection of the *C. perfringens* alpha toxin gene. Both conventional and real-time PCR formats have been developed and are widely used [76,77]. As mentioned above, *CPE* can be located on the chromosome or on a plasmid. A multiplex PCR to determine its location has been described [78,79].

Serological typing

It is unusual to find the same serotype of *C. perfringens* in a human population selected at random. However, after a common-source outbreak the same serotype can be recovered from the stools of most patients. Nevertheless, the development of an effective serologic typing scheme has been hindered by the antigenic

heterogeneity of strains of *C. perfringens*, so that isolated outbreak strains are often non-typeable. In addition, the necessary extensive collection of antisera is not generally available outside the UK. This has led to the use of other subtyping schemes. The procedure for serotyping has been described by [80].

Pulsed field gel electrophoresis (PFGE) is widely used in epidemiologic investigations of outbreaks, and procedures for *C. perfringens* have been described [81,82]. Several subtyping schemes have been proposed for *C. perfringens* including the use of bacteriocins, zymotyping, and plasmid profiling. These and more recent molecular methods of identification, such as ribotyping, variable number tandem repeat analysis (VNTR), random amplified polymorphic DNA (RAPD), and multiple-locus sequence typing (MLST), have been reviewed elsewhere [74,83].

Exposure pathways

Reservoirs

C. perfringens is widely distributed in the environment, including soils, the intestinal tract of animals, and retail foods. Generic *C. perfringens* are widely distributed in meat and poultry with a prevalence of 30–80%. However, surveys conducted in recent years have revealed that most isolates from retail foods are *ent*$^-$ [84–86], reducing the prevalence of *ent*$^+$ strains to 0–12%. Human feces commonly have higher rates of *ent*$^+$ isolates, with a prevalence of 6–31% (summarized by [84]), leading to the novel proposal that the human gastrointestinal tract may serve as a source of *ent*$^+$ strains [84,87–89]. Taken together, the evidence suggests that the mere presence of generic *C. perfringens* in retail foods does not indicate public health concern.

Points of entry

Food service establishments are the most likely settings for foodborne illness due to exposure to *C. perfringens*. Foods prepared in restaurants, in schools, and by caterers, for example, are often prepared well in advance and held for later serving, allowing for holding at improper temperatures for improper lengths of time. The case-to-outbreak ratios for this organism are higher than for other bacterial foodborne disease agents, due to its common occurrence in mass feeding settings. Typical vehicles involved in foodborne illness by *C. perfringens* are high-protein foods such as meat and poultry, as the organism lacks the genetic ability to produce a number of amino acids. Spores of the organism may survive cooking procedures and then germinate and grow during slow cooling or improper holding temperatures with rapid population increases possible between 37 and 50 °C. This has led to the regulatory requirements for cooling of uncured meat and poultry following cooking, as noted above.

Infectious dose

Foodborne illness due to *C. perfringens* is neither a true infection nor intoxication. Rather, large numbers of vegetative cells are ingested in temperature-abused foods.

Criteria for outbreaks included at least 10^5 ent$^+$ CFU/g in incriminated foods. Early human feeding studies involved the administration of whole cultures in food vehicles. In one study, cells were grown for 3 hours at 46 °C in beef stew before administration. Of volunteers given strains previously shown to produce enterotoxin in the rabbit ileum, 61% had diarrhea, whereas none given rabbit-negative strains became ill [90]. When purified CPE was administered, one of two volunteers given 8 mg and all of the five volunteers given 10 mg developed classical symptoms of food poisoning [91]. However preformed enterotoxin is not believed to be involved in foodborne illness due to *C. perfringens.*

Prevention and control

It is not practical to prevent clinically healthy carriers from handling food, because most people harbor *C. perfringens* (*ent*$^-$) in their intestinal tracts. Since the organism is present in animals, it is not surprising that *C. perfringens* is found in raw meat and poultry. The spores will also survive indefinitely in dust and in environmental nooks. Cooking at temperatures not exceeding 100 °C will allow the survival of spores of *C. perfringens*. The cooking process drives off oxygen, creating nearly anaerobic conditions in foods such as rolls of cooked meat, meat pies, stews, and gravies and in poultry carcasses. Therefore, prevention must be concerned not only with destruction but also with preventing multiplication of vegetative cells in cooked foods—this is the most practical way of preventing *C. perfringens* foodborne illness.

Every outbreak due to *C. perfringens* has involved temperature abuse, whether improper hot holding, improper cooling, or improper reheating of cooked, chilled foods. Proper reheating to an internal temperature of 75 °C before serving is important if the organism has had an opportunity to multiply because of previous temperature abuse. Public health agencies recommend holding cooked foods at or below 4 °C (40 °F) or above 60 °C (140 °F). Such temperatures will prevent the growth of *C. perfringens* in hazardous foods. Other recommended procedures include reducing the size of large portions of meat to hasten cooling and ensuring proper cooling capacity of refrigeration facilities. In the case of the food service industry, certain trade organizations offer short courses that describe recommended food-handling procedures in detail.

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CHAPTER

Vibrios

7

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Introduction

Vibrio species, principally *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, are the leading cause of human bacterial disease associated with the consumption of seafood, primarily raw oysters. Although *V. cholerae* was first described as the etiological agent of cholera epidemics more than 150 years ago [1], this ancient scourge remains a significant global threat to current public health. The seventh cholera pandemic has sustained for over 50 years, and disease incidence has been steadily increasing since 2000, with more than 300,000 cases and 7500 deaths reported in 2010 [2]. The spread of this pandemic progressed from Asia to South America in the 1990s, with more recent emergence in Africa and Haiti. Similar pandemic spread of milder diarrheal illnesses related to *V. parahaemolyticus* infections was also observed in the 1990s and still impacts the seafood industry on the Pacific coast from Alaska to South America via endemic disease associated with raw shellfish consumption. *V. vulnificus* is the leading cause of fatalities associated with seafood-borne infections in the USA [3], but cases are relatively rare and generally limited to oysters harvested from the Gulf of Mexico. Pre-harvest monitoring and post-harvest processing of oysters harvested from the USA have been implemented to reduce *Vibrio* disease, but efforts are complicated by the presence of both pathogenic and non-pathogenic strains in aquatic ecosystems and by the relative lack of information on the bacterial determinants for the survival and prevalence of potentially virulent types in seafood.

Clinical features

Disease manifestations

Disease symptoms commonly associated with the three primary pathogenic Vibrios are summarized in Table 7.1. The most severe *Vibrio*-associated disease is epidemic cholera, caused by cholera toxin-producing (toxigenic) strains of *V. cholerae* and

Foodborne Infections and Intoxications.

Table 7.1 Disease Characteristics of the Major Pathogenic Vibrios

Species	Symptoms[a]				Transmission Source	Virulence Markers (genes)
	Diarrhea		Sepsis	Wound		
	Severe	Mild				
V. cholerae						
(Toxigenic)	+++	–	–	–	Water, food, humans	Cholera toxin (*ctxA, ctxB*); TCP (*tcpA*)
(Non-toxigenic)	–	+++	+	+	Oysters	Variable
V. parahaemolyticus	–	+++	+	++	Sushi, oysters	Hemolysins (*tdh/trh*); type three secretion system
V. vulnificus	–	+	+++	+++	Oysters, seawater	Variable (*vcg, rrn*)[b]

[a]*The frequency of symptoms associated with individual* Vibrio *spp. are shown as rare or not associated (–); infrequent (+); intermediate association (++); and most common (+++).*
[b]*Different alleles of 16S genes (*rrn*) and the* vcg *gene with unknown function discriminate most clinical from environmental isolates of* V. vulnificus*, as described in the text.*

characterized by purging, life-threatening diarrhea. Disease can be rapidly fatal due to massive dehydration, but with timely treatment using oral or intravenous rehydration therapy recovery is rapid and does not generally involve any long-term consequences. This ancient disease still plagues public health efforts today, particularly during natural and military disasters that undermine sanitation infrastructure. Transmission models are complex and include the coincidence of environmental conditions that increase reservoirs of disease and the socioeconomic and other factors that promote human-to-human transmission and may facilitate a "hyperinfective" state [4].

V. parahaemolyticus was historically the most frequent cause of all bacterial foodborne disease in Japan, but has recently become a prominent cause of diarrheal disease in China [5] and in the Western Hemisphere [6]. A "pandemic" strain of *V. parahaemolyticus*, namely serotype O3:K6, began spreading globally from Asia in 1992, eventually producing outbreaks in New York, Texas, Alabama, California, Oregon, and Washington. Recently, about 10,000 persons were affected by an outbreak in Chile [7]. *V. parahaemolyticus* produces much milder symptoms than *V. cholerae*, and disease is typically characterized by rapid onset diarrhea, vomiting, and abdominal pain. Recovery without treatment generally occurs after several days without any long-term consequences.

V. vulnificus also causes mild diarrhea, but symptoms are more frequently associated with a severe septic shock that is rapidly fatal in >50% of infected persons (Blake, 1979). Although other *Vibrio* species are capable of producing septic shock, this single species produces 95% of all fatal bacterial infections from seafood consumption in the USA [3]. *V. vulnificus* is also a leading cause of wound infections related to handling of seafood or simply by exposure of lesions to seawater [8]. Wound infections associated with hurricane Katrina [9]. and a large outbreak (62 cases) in Israel [10] highlight the potential for this route of infection. Wound infections attributed to *V. parahaemolyticus* also appear to be increasing, and *V. alginolyticus* is a significant cause of ear infections in children [8,9].

Host susceptibility

While healthy persons are generally susceptible to *V. cholerae* and *V. parahaemolyticus* diarrheal disease, persons with blood group O are likely to have more severe symptoms from cholera than the general population [11,12]. Compared with *V. parahaemolyticus*, the incidence of *V. vulnificus* infections in the USA is relatively rare (about 20 to 30 cases/year), likely due to the limits of host susceptibility and the limited distribution of virulent strains. Sepsis caused by *Vibrio* spp. requires some type of underlying, predisposing condition, such as hemochromatosis (an iron overload condition), hepatic disease, diabetes, or immune system dysfunction (Blake, 1979). Clearly the host iron status contributes to these infections, and concurrent injections of iron during experimental *V. vulnificus* infection dramatically reduce the 50% lethal dose from approximately 100,000 to <10 bacteria [13]. Wound infections are reported in healthy persons, but fatalities by this route are also mostly limited to persons with altered host immune status.

Microbiology

Genetics and evolution of pathogenic *Vibrios*

Estuarine habitats are common reservoirs for Vibrios associated with human disease, but distribution of potentially pathogenic strains in this niche is extremely limited compared with strains lacking virulence factors. Human-to-human transmission of *Vibrio* diseases is generally limited to cholera epidemics; thus, human infection for non-cholera Vibrios is likely to be a dead end for evolution. Adaptations are more likely a response to the constantly changing estuarine conditions, and putative "virulence factors" may actually have evolved to enhance survival in this environment rather than in human hosts. For example, virulence determinants of *V. vulnificus* were recently shown to promote uptake and survival of the bacterium in the oyster host [14].

Manifestations of cholera epidemics result from strains that have acquired the genes encoding the toxin co-regulated pilus (TCP) and the cholera toxin (CT). TCP is required for intestinal colonization, and CT produces all symptoms of intoxication. Although non-toxigenic strains can be associated with seafood-borne illnesses, symptoms are much milder and do not produce epidemic disease. CT is encoded by *ctxA* and *ctxB* genes, which are expressed during colonization of the small intestine. As shown in Figure 7.1, the CtxA subunit is responsible for toxic activity, and the five CtxB subunits bind the toxin to intestinal epithelial cells via GM1 gangliosides [15]. The holotoxin is endocytosed by enterocytes and processed in the golgi, allowing release of CtxA1, which ribosylates G proteins and thereby permanently activates adenylate cyclase to catalyze cAMP formation. The subsequent increase in chloride ion efflux, followed by sodium ions and massive fluid loss, produces the characteristic diarrheal symptoms.

For reasons that are not completely understood, the ability of *V. cholerae* to produce epidemic disease is limited to serotypes O1 and O139 [16]. These serotypes appear to be genetically related, as the O139 serotype was derived from O1 by acquisition of novel genes encoding alternate polysaccharide synthesis pathways [17]. Interestingly, genes encoding CT are carried on a bacteriophage that transduces the *ctx* genes, thereby converting non-toxigenic strains into toxigenic *V. cholerae* [18]. The TCP serves an additional function as the receptor for the *ctx* phage, and genes for TCP (*tcpA*) and other factors are located on a pathogenicity island [19]. Most environmental isolates of *V. cholerae* are serotypes other than O1 or O39 and are negative for both *ctx* and *txpA* [20].

Clinical strains of *V. parahaemolytics* are characterized by the thermal stable direct hemolysin (TDH) or the genetically similar TDH-related hemolysin (TRH). Hemolytic strains are detected on human blood agar plates, known as the Kanagawa phenomenon [21]. Conversely, most environmental isolates lack TDH or TRH. However, the relationship of these hemolysins to disease is debatable, and recent data have implicated the type three secretion system as a more likely virulence factor [22]. Two TTSS (TTSS1 and TTSS2) are present in *V. parahaemolyticus*, but

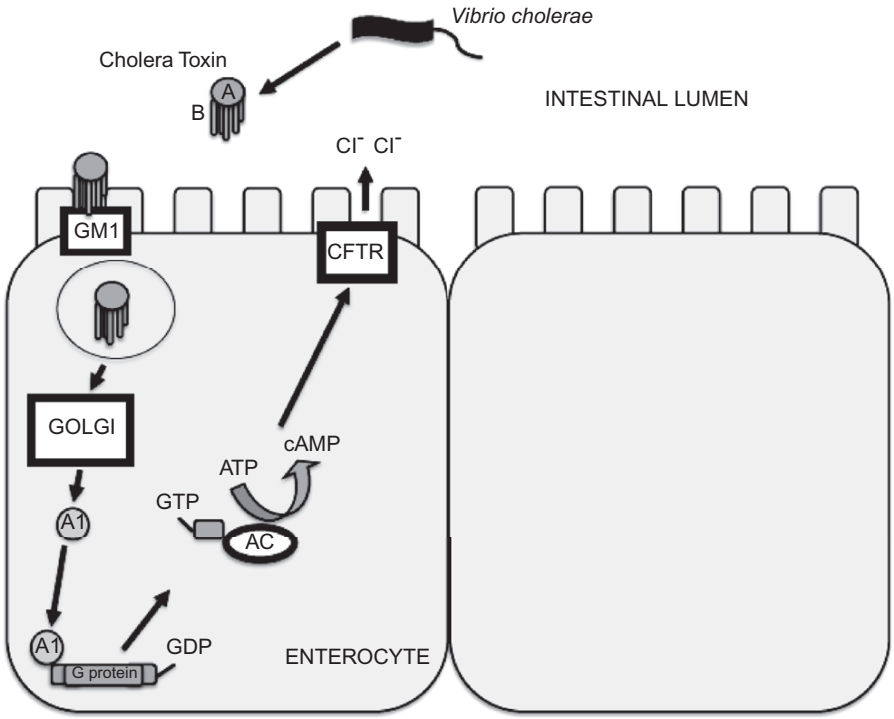

FIGURE 7.1

Cholera toxin (CT) consists of one A subunit and five B subunits. CT is secreted by *V. cholera* in the lumen of the small intestine and binds the GM1 gangliosides on enterocytes via the B subunits. CT is endocytosed and transported to the golgi, releasing the enzymatically active A1 subunit. Intoxication involves the ADP—ribosylation of the G protein component of adenylate cyclase (AC) by CTA1, resulting in AC that is locked into the GTP-bound state, thereby dramatically increasing activity. Corresponding increases in cAMP drive the massive extrusion of Cl ions via the cystic fibrosis transmembrane conductance regulator (CFTR), causing fluid loss and the associated diarrheal symptoms.

TTSS2 has wider representation in clinical strains and has been shown to be responsible for enterotoxicity in animal models (Hiyoshi et al., 2010).

V. vulnificus produces a hemolytic cytotoxin, but mutational analysis showed that non-toxigenic strains were as virulent as the wild-type strain in a mouse model of infection [23]. A polysaccharide capsule is required for systemic disease in mice [24], and other putative virulence factors were recently reviewed [25]. However, no single factor is likely to predict the virulence potential of environmental strains of this species, as these factors are generally present in all strains and genetic analyses have not demonstrated a profile that is fully associated with disease potential in mice [26].

Diagnosis and species identification

Pathogenic Vibrios show optimal growth between 30–37 °C, 1–3% NaCl, and prefer neutral to slightly alkaline pH. All species produce chitinase, and chitin degradation has been proposed as a key to their evolution via associations with chitinaceous algae and copepods [27]. All species also ferment glucose and produce oxidase, but subspecies variability in substrate utilization leads to ambiguous identification [28]. Thiosulfate citrate bile salts sucrose (TCBS) agar differentiates *V. cholerae* based on sucrose fermentation [29]. However, one study reported that 49% of the bacteria on TCBS were not Vibrios (Cleland, 1985). Modified cellobiose polymyxin B colistin (mCPC) [30], cellobiose colistin (CC) [31], and VVE [32] agars are used to isolate *V. vulnificus*, with improved recovery reported for mCPC+ agar [33]. A commercial chromogenic agar medium (CHROMagar *Vibrio* CV) increases recovery of *V. parahaemolyticus* [34]. Recovery of Vibrios may be inhibited on selective agars [35], and enrichment broth cultures, such as alkaline peptone water (APW), are often employed to enhance recovery. Overgrowth of swarming colonies on solid media also complicates identification and enumeration. Standard detection methods for detection of Vibrios are described in the FDA (Food and Drug Administration) Bacteria Analytical Manual (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070830.htm).

DNA probes or PCR have greatly improved species identification; however, direct detection from food samples (without prior isolation or enrichment) may require DNA purification for optimum sensitivity [36]. Species-specific assays are available for *V. vulnificus vvhA* (Brauns et al., 1991; [35]); *V. parahaemolyticus tlh* (McCarthy et al., 1999); and *V. cholerae ompW* (Baron et al., 2007). Recent methods also include real-time, quantitative PCR (qPCR) for quantitative detection of Vibrios (Blackstone et al., 2007; [37–40]; Panicker et al., 2004a). Multiplex PCR analyses for simultaneous identification of multiple *Vibrio* species have also been described (Blackstone et al., 2007; [41,42]; Panicker et al., 2004b; [43,44]). A commercial kit is now available for real-time PCR detection of all three major pathogens (DuPont Qualicon).

Additional methods for *Vibrio* detection include the use of species or toxin-specific antibodies such as commercial epifluorescent microscopy [45]. A flagella or "H" antigen has been used for species identification of *V. vulnificus* [46,47]. These methods are useful in detection of *V. cholerae* from water samples but may lack the sensitivity of PCR. Emerging technologies for *Vibrio* detection include DNA-dependent biosensors and DNA microarrays (Cariani, et al., 2011). Liquid microsphere arrays (Luminex) offer the advantage of detecting more targets than is possible by qPCR [48]. Biosensors that recognize specific cellular targets or products have been employed to detect *V. cholerae* without culturing [49]. However, recovery of cells is often preferable for additional assessment of strain characteristics [50]. Loop-mediated isothermal amplification PCR (LAMP) [51] offers the prospect of a simple, inexpensive, single tube technique that is performed at one temperature with positive reactions detected by the naked eye, eliminating the need for expensive thermal cyclers.

Detection of virulence factors

Serotyping for O1 and O139 is commonly used to discriminate more virulent *V. cholerae* strains. The recent *V. parahaemolyticus* pandemic was initially associated with a single O3:K6 serotype [52]. However, multiple serotypes have since emerged and now form the pandemic clonal complex [53,54]. *V. vulnificus* strains, except for serogroup E eel pathogens [46] are serologically diverse. Vibrios are also categorized by metabolic profiles called biotypes. For example, *V. cholerae* serogroup O1 is divided into "Classical" and "El Tor" biotypes based on antibiotic resistance and hemolysin expression [55]. Pandemic *V. parahaemolyticus* is urease-negative, while non-pandemic strains are generally urease-positive [52]. *V. vulnificus* has three biotypes correlating with the ability to cause human (biotype 1) vs. fish (biotypes 2 and 3) disease [10,56,57].

Molecular diagnostics are also available to discriminate virulence potential and include *V. cholerae ctx* gene probes and PCR [58]. The *tdh* and *trh* genes are virulence markers for *V. parahaemolyticus* and have been used to monitor shellfish [52]. Pandemic *V. parahaemolyticus* O3:K6 clonal complex strains are generally tdh^+ and trh^-, have a distinct *toxRS* sequence [59], are positive for *orf*8 from filamentous phage f237 [60], and have genes for type three secretion system 2 [61]. Single locus binary typing has been used to differentiate the virulence potential of *V. vulnificus*. For example, 16S rRNA alleles segregate strains that are more likely to be from environmental origin (16S types A or AB) from those typically associated with clinical origin (type B) [62,63]. PCR of a virulence correlated gene (*vcg*) with unknown function [64] also discriminates environmental (mostly type E) from clinical (mostly type C) strains. These binary typing strategies (A/B and E/C) are highly correlated with one another and other typing methods [65]. However, the discovery of virulence markers in *V. vulnificus* is complicated by the observation that most strains are equally virulent in animal models [66], and these typing systems correlate with but do not necessarily predict the ability of *V. vulnificus* to produce disease in animal models [26].

Molecular and genomic typing

Molecular typing methods include multilocus DNA sequencing typing (MLST) and restriction enzyme-based assays, such as pulsed field gel electrophoresis (PFGE) and ribotyping. MLST is more discriminatory than restriction enzyme-based systems for molecular typing of *Vibrio* species [67,68]. More rapid PCR-based techniques include randomly amplified polymorphic DNA (RAPD), repetitive extragenic palindromic PCR (rep-PCR), and PCR or sequence analysis of variable number of tandem repeats (VNTR). These assays produce patterns of multiple PCR amplicons by amplification of either random or repetitive sequences that are distributed throughout the genome. RAPDs discriminate *V. parahaemolyticus* O3:K6 and non-O3:K6 [52] and were used to assess temporal and spatial changes in *V. vulnificus* populations [69]. However, RAPDs are not always reproducible due to the relatively low stringency in this PCR reaction [70].

Repetitive element-based methods provide discrimination similar to PFGE but are more reproducible than "random priming" and have been used to type *V. cholerae* [71,72], *V. parahaemolyticus* [54], and *V. vulnificus* [73]. VNTR PCR typing techniques showed increased discrimination among O1 and O139 *V. cholerae* strains compared to PFGE (*Not*I) or phage typing [74]. A commercial rep-PCR typing system for *Vibrio* species is not available, but the DiversiLab rep-PCR-based typing system for *Salmonella* (BioMerieux) discriminated among *V. vulnificus* strains [65,75] and showed greater genetic diversity than multilocus sequence typing [26]. Whole genome sequence analysis brings the promise of more precise discrimination. The potential of this technology was exemplified by two-day acquisition of whole genomes during the recent Haitian cholera outbreak, which confirmed the accidental introduction of a *V. cholerae* El Tor into Haiti from South Asia [76].

Exposure pathways

Reservoirs and entry into the food supply

Vibrio diseases are commonly attributed to the consumption of raw or undercooked seafood, particularly oysters. However, the source of cholera epidemics is more likely contaminated water, followed by human-to-human transmission [4,16]. While most bacterial foodborne infections have declined in recent years, the estimated incidence of *Vibrio* disease in the USA actually increased by 115% in 2010 compared with 1996–98, primarily as a consequence of *V. parahaemolyticus* outbreaks [77]. Warmer seawater temperatures and post-harvest temperature abuse (e.g., maintaining seafood at room temperature) promote higher densities of *Vibrio* spp. [78]. A report of *V. parahaemolyticus* disease in Alaska was linked to unusual water temperatures exceeding 15 °C and supports climate change as a factor for increased disease risk [79]. Unusual outbreaks of *V. vulnificus* wound infections in Texas [80] and in Israel [10] may also be a function of rising seawater temperatures. Interestingly, *V. parahaemolyticus* shows a temperature response whereby expression of virulence factors is increased at 37 °C compared with 28 °C in clinical but not environmental strains [81]. The prevalence of more virulent genetic clades may also coincide with increased temperature [82], but more data are needed for meaningful predictions and risk assessment. Other risk factors may include lower salinity, algal blooms, increased nutrient availability, and aquaculture ([83]; Mahmud et al., 2011).

Risk determinants for Vibrios show interesting geographic separation. For example, disease risk for *V. vulnificus* is greatest by far from Gulf of Mexico oysters, while *V. parahaemolyticus* disease risk is much greater from Pacific compared with Gulf coast oysters, despite similar distribution of these species throughout the US coast. Global scale impact of the seventh pandemic of *V. cholerae* continues in Asia and Africa [16], and more recently in South and Central America [84] and Haiti [76]. However, transmission of cholera through contaminated seafood in the USA was demonstrated by a *V. cholerae* O75 outbreak (10 cases) in Florida [85].

Approaches to food attribution

The incidence of *Vibrio*-related diseases is at least partly dependent on preferences for raw shellfish consumption and varies with the distribution of the virulent variants in the environment. The environmental factors and processing practices that contribute to increased prevalence of more virulent *Vibrio* species are the topic of intensive investigations. Temperature is clearly a factor that influences pathogenic *Vibrio* levels, but reduced temperatures may induce conversion of Vibrios to a viable but non-culturable phase and result in underestimation of disease risk [86]. Surveillance strategies for seafood safety should consider molecular characterization of virulence potential in order to accurately assess public health risks associated with *Vibrio* disease.

Prevention and control

Hazard analysis of critical control points

Seafood safety is regulated by the US FDA and the Interstate Shellfish Sanitation Conference (http://www.issc.org/). Specific regulations for harvesting and processing of seafood are determined by individual state agencies. The most rigorous controls are directed at molluscan shellfish because filter-feeders concentrate pathogens and such products are frequently consumed raw without cooking. Therefore, regulations governing Vibrios in seafood focus on hazard analysis critical control points (HACCP) for time/temperature controls to minimize exposure of shellfish to elevated temperatures and include defined minimum time from harvest to refrigeration, rate of refrigeration, and allowed time for refrigerated storage. Additional controls have been recommended when water temperatures exceed 24 °C, including closure of shellfish harvesting areas and post-harvest processing protocols [87]. Oysters intended for raw consumption are tagged for date and location of harvest in order to track potentially contaminated shellfish.

Vibrio monitoring

Although shellfish safety is based primarily on monitoring fecal indicator bacteria (FIB) in shellfishing waters, FIBs rarely correlate with *Vibrio* presence in US seafood because *Vibrio* spp. are indigenous to seawater and are not generally derived from fecal input. Direct monitoring of *V. parahaemolyticus* levels in the US Pacific coast is related to seawater temperature and disease incidence. qPCR can be used to directly enumerate pathogens in seafood; however, growth in enrichment in media, such as APW, can be enumerated by most probable number (MPN). MPN using qPCR for species confirmation provides rapid analysis with increased sensitivity, and several studies have shown that qPCR-MPN reduces the limit of detection to about one bacterium/g [37,40,88].

Post-harvest processing

Sufficient cooking will eliminate *Vibrio* pathogens, but raw fish and shellfish remain a delicacy in many countries. The FDA and ISSC have recommended the post-harvest processing (PHP) of oysters harvested from the Gulf of Mexico that are intended for consumption as a raw product. Approved processing protocols include rapid ultra-low temperature freezing, high hydrostatic pressure, mild heat treatment, and ionizing irradiation (http://www.issc.org/php.aspx). These primary treatments are often followed by frozen storage to adequately reduce Vibrios. These methods do not completely eliminate the associated risks for highly susceptible persons whose infectious dose may be extremely low [89]. The expansion of global seafood markets, co-mingling of food resources, and aquaculture of fish and shellfish in cholera endemic regions may also increase disease risk [75]. PHP processes generally kill the oyster; therefore, non-lethal methods using "relay" or transport of shellfish from a location of high pathogen concentration to an environment without pathogenic Vibrios is under investigation [90]. However, caution needs to be exercised with this type of transport because dramatic shifts in salinity may kill oysters, and relocation could lead to increased distribution via introduction of pathogenic Vibrios to pristine sites.

Conclusions

Successful implementation of consumer education programs and PHP in strategic Gulf coast states has undoubtedly reduced human exposure to *Vibrio* disease, but these efforts may not completely eliminate the risk for highly susceptible individuals. Seafood monitoring is expanding from evaluation of fecal indicators to pathogens assessment. However, *Vibrio* monitoring is complicated by the fact that highly virulent strains are relatively uncommon and therefore difficult to detect in environmental samples. The close association of Vibrios with marine fauna is an inherent obstacle to improving seafood safety and quality, as effective processing methods generally kill the mollusc-an shellfish host as well. The development of more rapid and practical methods for detection and enumeration of Vibrios is crucial for safer seafood production and for development and validation of processing methods. Better understanding of the evolution of these species in the context of the environmental, bacterial, and host factors that underlie associated public health risks will provide the keys to improved monitoring and interventions strategies.

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CHAPTER

Escherichia coli

8

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Introduction

Escherichia coli are ubiquitous and almost always innocuous members of the colonic microbiota, and are a very diverse set of organisms. Some of the members of this species can cause diarrhea, in which case they become relevant to food safety. This chapter reviews major categories of *E. coli* that have reasonably strong associations with human disease: enterohemorrhagic, enterotoxigenic, enteropathogenic, enteroaggregative, enteroinvasive, and diffuse adhering *E. coli*, as well as the hybrid enterohemorrhagic/enteroaggregative *E. coli* O104:H4. In clinical reality, few of these agents can be identified using current technology, so outbreaks and sporadic infections are quite likely very much undetected.

Three major challenges are encountered when trying to detect diarrheagenic *E. coli*. First, diagnosis at presentation is rarely possible in anything approaching a CLIA-certified diagnostic environment, because, except for O157:H7 and non-O157:H7 Shiga toxin (Stx)-producing (STEC) *E. coli*, *E. coli* that cause diarrhea have no readily detectable phenotypes that set them apart from other *E. coli* in stool. Instead, the detection of diarrheagenic *E. coli* largely relies on cumbersome phenotypic determinations (cell invasion, patterns of adherence to cells, and toxicity assays) and/or DNA hybridization assays (colony hybridizations or the polymerase chain reaction (PCR)), which are not widely used. Identifying the O (somatic) antigen only partly suggests that diarrheagenic *E. coli* might be present. Also, most illnesses caused by diarrheagenic *E. coli* resolve spontaneously and without major injury to the host (except for STEC), so clinicians cannot make a compelling case for finding these agents in patients with diarrhea. These challenges have stalled attempts to study the extent of disease caused by these organisms. Moreover, without rapidly identifying such patients, and conducting treatment trials, it is impossible to know if antibiotics change the clinical course of such illnesses.

Second, diarrheagenic *E. coli* are quite difficult to precisely define as enteric pathogens. The ability to cause disease among *E. coli* relates to the presence

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or absence of multiple genes, which act in concert to avoid host defenses, and to enable the organism to injure host organs via a variety of mechanisms. *E. coli* use adhesins, sugars (capsular polysaccharides and lipopolysaccharides), protein toxins, invasion-promoting proteins (invasins), and iron acquisition systems (siderophores) to survive in and injure their hosts. Loci encoding these molecules are rarely restricted to pathogens, and are often on mobile genetic elements. Moreover, presence or absence of any given locus is usually not enough to cause disease: there are additional attributes of bacterial gene expression levels and allelic variation that need to be considered.

Third, healthy controls can harbor putatively diarrheagenic *E. coli* strains [1–3], and few case-control studies consider pathogen density. Outbreaks are often needed to confirm or refute pathogenicity.

The inability to attribute specific episodes of enteric infection to specific *E. coli* also complicates attempts to determine burden of disease on populations. The attributable etiologic percentage is the first step in determining the short- and long-term burden of any pathogen that underlies a particular syndrome, but as noted above and throughout this chapter, most *E. coli* diarrheas elude diagnosis. Furthermore, there are definable burdens of illness as measured by diarrhea-related mortality [4,5], but the widespread use of oral rehydration and the increasing use of the rotavirus vaccine are diminishing diarrhea-related mortality. Diarrhea-related morbidity, beyond the cost to individuals and families of the acute episode, is much more difficult to ascertain [6], especially as outcomes (stunting, cognitive deficits) can take years to be apparent. The agents in this chapter rarely are lethal. Hence, the burden to society in terms of disability-adjusted life years lost because of infection by any or all diarrheagenic *E. coli* remains incalculable.

Enterohemorrhagic *E. coli* (EHEC)

In 1977, Konowalchuk et al. identified toxin-producing *E. coli* in foods and human stools [7]. Three landmark papers in 1983 established the role of these organisms in human disease: [8] identified fecal *E. coli* that produced a toxin lethal to cultured eukaryotic (Vero) cells in children with the hemolytic uremic syndrome (HUS). HUS is an acute multisystem disorder consisting of hemolytic anemia, thrombocytopenia, and renal injury, and often follows bloody diarrhea. Riley et al. [9] described two clusters of bloody diarrhea that were caused by *E. coli* expressing O antigen 157 and H antigen 7. These outbreaks in Michigan and Oregon were linked to the consumption of undercooked hamburgers. O'Brien et al. [262] showed that *E. coli* O157:H7 produced a purified cytotoxin that could be neutralized by antibodies to Stx. In 1987, Calderwood et al. [10] cloned and sequenced Stx from *E. coli* O157:H7. STEC are also termed "verotoxin-producing or verocytotoxigenic (VTEC)." The subset of STEC that causes human disease is often termed "EHEC."

Clinical features of EHEC infections

E. coli O157:H7 infections display a spectrum of gastrointestinal severity, but most patients report bloody diarrhea [11–13]. *E. coli* O157:H7 is the predominant cause of HUS, which complicates ca. 15% of diagnosed *E. coli* O157:H7 infections in children under age 10. Fever is rarely documented in health care settings [13]. Non-O157:H7 EHEC infections are less well characterized, but are much less likely to cause HUS [14] or bloody diarrhea [15].

HUS is the most frequent and consequential complication of infections with EHEC, ensuing between 5 and 13 days after onset of diarrhea. Exceptional patients infected with *E. coli* O157:H7 will develop HUS without diarrhea [16]. Most *E. coli* O157:H7 are susceptible to antibiotics [217], so it would seem intuitive to use these agents to prevent HUS in infected patients. However, multiple studies of outbreaks and of sporadic infections in children and adults [13,17,218–221], and one randomized controlled study [222], have failed to demonstrate any benefit from antimicrobials given early in illness, and several large studies demonstrated increased risk from antibiotics [221]. We urge against their use in patients who are definitely, or even possibly, infected with *E. coli* O157:H7.

Volume expansion during the pre-HUS phase, and prevention of dehydration, are associated with milder courses of HUS [18–20]. The importance of this intervention cannot be understated. Patients who present to medical attention with diarrhea caused by *E. coli* O157:H7 already have evidence of vascular injury and profound prothrombotic abnormalities [223,224]. Intravenous volume expansion with normal saline quite likely provides substantial nephroprotection, and this support is associated with a clinically important outcome: aversion of anuria is HUS develops. HUS treatment is largely supportive. There is no credible evidence that plasmapheresis or eculizumab (a monoclonal antibody directed against the fifth component of complement) is helpful, and some evidence that these interventions are deleterious [176,225].

The approach to the patient with possible or definite infection with *E. coli* O157:H7 and with other EHEC has been reviewed in greater detail [22], and, as noted above, the mainstay of therapy is volume expansion. Anti-Shiga toxin monoclonal antibodies are under evaluation [23], and clinical trials will be informative.

Microbiology of EHEC infections

It is best to subject all stool cultures to diagnostic technology that detects O157:H7 as well as non-O157:H7 EHEC. However, it is most critical, given current options, that stools continue to be plated on receipt in the laboratory on sorbitol MacConkey agar. It is inappropriate for diagnostic microbiology laboratories to assume that toxin assays, which are generally enzyme immunoassays (EIAs) can serve as the only screen, with reflex testing only for positive specimens, because such strategies miss about 15% of *E. coli* O157:H7 [15]. This is the same position that has been

taken by the CDC [24]. Regrettably, laboratory practices often do not follow these clear recommendations, and we have seen many examples of delayed or compromised patient care from suboptimal protocols, i.e., protocols that do not perform plating and toxin assays in parallel.

The principal virulence phenotype of EHEC is the production of one or more variants of Stx (the structure and function of Stx are reviewed in [25]). Stxs consist of an A-B_5 structure, with the pentameric B subunit binding to a glycosphingolipid receptor on eukaryotic cell surfaces (globotriaosylceramide, or Gb_3) [26]. The toxin is then internalized, and the A subunit injures the host cell. The best known effect of Stxs is their role in ricin-like N-glycosidase inhibition of protein synthesis, but the B subunit itself can cause apoptosis [27,28]. EHEC probably do not actually kill very many host cells, but, instead, probably induce sublethal cellular injury [29,30]. Of the two Stxs, Stx 2 and its variants, especially Stx 2 and Stx 2c, have strong associations with human disease, while Stx 2d and Stx 2e have much weaker associations [31]. Stx 1 is considered less injurious than Stx 2 in animal and *in vitro* studies [32,33]. Interestingly, *E. coli* O157:H7 that produce both Stxs are less firmly associated with severe human disease (i.e., HUS) than those producing only Stx 2 [12].

EHEC also possess a diversity of proposed, or actually confirmed, virulence loci other than Stx. The most familiar and thoroughly studied of these non-Stx virulence loci is intimin, encoded by the *eae* gene. Human pathogenic EHEC usually express either intimin-γ (*E. coli* O157:H7) or intimin-β (many non-O157:H7 EHEC). EHEC intimins are allelic variants of enteropathogenic *E. coli* intimins. These surface expressed molecules mediate the intimate attachment of EHEC to cells that line the gut. Through an elaborate type three secretion system (T3SS), intimin and coordinated molecules subvert host cellular machinery to induce the attaching and effacing lesion [34]. With very few exceptions, human pathogenic EHEC all produce intimin variants [35]. Interestingly, eukaryotic nucleolin can serve as a receptor for intimin [36], but EHEC Tir (translocated intimin receptor), injected by EPEC into host cells, is also a ligand for intimin, though Tir, unlike its counterpart in EPEC, is not tyrosine phosphorylated [37].

Exposure pathways for EHEC infections

In the three decades since its first description, we have learned much about transmission pathways for *E. coli* O157:H7 [38]. Most of our data have been derived from outbreak analyses, which is facilitated by readily available microbiology (sorbitol MacConkey agar screening), a limited and easily recognized set of symptoms [11], definable outcomes, i.e., HUS, and the policy of reporting infections to disease control authorities. This accumulated knowledge demonstrates the value of a diagnostic for a diarrheagenic agent, and surveillance and reporting systems, because cases and risks can be easily defined, and data can then be systematically collected and analyzed.

From outbreaks of *E. coli* O157:H7 and occasionally of non-O157:H7 EHEC, we have learned of the transmission risk posed by a variety of vehicles and mechanisms, including poorly cooked ground beef, which has been a risk since the first description of this pathogen [9], prime cut beef [226], petting zoos [227], unpasteurized fruit juices [228,229], contaminated water [230], contaminated vegetables [231], mettwurst [41,42], salami [232], deer meat [233,234], fresh cookie dough [235], airborne transmission [236], raw milk [43,237], and person-to-person spread, especially in day care facilities [238] and the community [239,240]. The mechanism of acquisition of these infections outside of epidemics is less well defined, but includes domestic overnight travel, eating food from a food stand, table service restaurant, or self-serve buffet, having a septic system for home sanitation, recreational water exposure in Washington State children [39], and consuming undercooked beef, contact with farm animals, or other children with diarrhea in Argentina [241]. Residential proximity to cattle and their waste is an additional risk factor for acquiring *E. coli* O157:H7 infections [242–246], but environmental risk factor analyses are very complex [247].

Prevention and control of EHEC infections

With the above risks in mind, some prevention recommendations can be formulated, but none are specific for *E. coli* O157:H7 infections. To the extent possible, meat should be cooked, and contact with animals limited. Children who shed *E. coli* O157:H7 following symptomatic infection should be excluded from sensitive settings such as day care facilities [238]. Hospitalization of symptomatic patients is associated with diminished secondary cases in households [240]. An updated set of recommendations regarding petting zoos [227] emphasizes avoidance of food consumption in proximity to animals, access to hand washing facilities, and education.

Vaccination of children against *E. coli* O157:H7 in human populations is a possibility [44], as is vaccination of, or use of probiotics in, the bovine reservoir [45,46].

In the context of prevention, it is critical that government health authorities receive clinical isolates of *E. coli* O157:H7 from infected patients as quickly as possible. Molecular analysis can often suggest or refute common origins of isolates, because when widely disseminated contaminated vehicles cause disease, outbreaks are frequently not confined to one area, and microbial linkage by genetic analysis of strains is an important disease prevention tool. Of the many typing techniques available, pulsed field gel electrophoresis (PFGE) has been the most frequently used [248], and remains the basis for national and international pathogen control [249,250]. Recently, multilocus variable analysis of tandem repeats (MLVA) has been used to provide greater discriminatory power to PFGE [251–253]. Whole genome sequencing has also been proposed to differentiate strains [254,255], and we anticipate that this technology will eventually supplant non-sequence typing methodologies.

Enterotoxigenic *E. coli* (ETEC)

In 1956 in Calcutta, De et al. described that *E. coli* from patients with watery diarrhea caused fluid to be secreted in rabbit ileal loops, as does cholera toxin [47]. From these observations, a new conceptual class of diarrhea-causing *E. coli* emerged, termed "ETEC," but nearly two decades would pass before their role in travelers' diarrhea was established [48]. It is notable that recognition of the ability of antibiotics to prevent travelers' diarrhea (TD) presaged this etiologic discovery [49].

Clinical features of ETEC infections

ETEC are classically considered to cause non-inflammatory watery diarrhea, but intestinal inflammation is underappreciated. Leukocytes, lactoferrin, and IL-8 can be detected in ETEC-infected stools [50,51]. The severity of diarrhea extends from asymptomatic infection to shock from massive enteric fluid loss; fever is unusual, but vomiting (at least among children) is not [52].

As with any dehydrating illness, the principal component of treatment is fluid management. The role of antibiotics in ETEC infections in the developing world is not clearly delineated, but antibiotics do benefit ETEC-infected travelers to endemic areas. Treatments have evolved over the decades and included tetracyclines, trimethoprim-sulfamethoxazole, fluoroquinolones, and azithromycin, and, more recently, rifaximin [53].

Antibiotics are usually not recommended to prophylax against TD, because of potential side effects and a desire to avoid selective antimicrobial pressures on microbiota in the countries the traveler visits. Rifaximin appears safe, but more experience is needed to build confidence in its safety profile.

Repeated episodes of diarrhea in children in low income countries, of which ETEC are common causes, quite likely contribute to malnutrition and stunting [54]. ETEC probably cause the diarrhea that is associated with the onset of irritable bowel syndrome (IBS) that follows TD [55].

Microbiology of ETEC infections

ETEC diagnosis obligates nucleic acid hybridization or cell culture assays, and are limited to research laboratories. Diagnosis is usually based on clinical profiling, and also takes into account the likelihood that cholera could be a cause of the illness.

ETEC elaborate two classes of enterotoxins [52]. Heat-labile toxins (LTs) structurally and functionally resemble cholera toxin. LTs are AB_5 toxins. The B pentameric subunit binds GM1 (as does cholera toxin), but LT has a broader range of binding structures [56]. The A subunit of the LT holotoxin is, as in cholera toxin, the enzymatically active component. This subunit irreversibly inactivates (by ADP-ribosylation) the G (stimulatory) protein, which then activates adenylate cyclase,

which increases intracellular cAMP. These intracellular cAMP molecules activate the cAMP-dependent kinase (PKA), which, in turn, promote chloride secretion via the cystic fibrosis transmembrane conductance regulator (CFTR), also as in cholera [57]. This cascade produces net secretion of Cl^- from crypt cells of the gut; these changes also hinder sodium and chloride reabsorption in the villous tip cells. The B subunit of LT is, like the B subunit of cholera toxin, a mucosal immune adjuvant, when co-administered with other antigens. LT is delivered in a polar fashion by adherent ETEC [58]. Partial protection against symptomatic re-infection has been achieved by vaccination with LT, but there was discordance between circulating antibodies to this antigen and protection [59], possibly because the LT might be delivered to enterocytes in a manner that keeps this important secretory effector sequestered from immunoglubin [60].

ETEC also produce heat-stable toxins (ST). STa (or STI) has two variants: ST-P (or ST-Ia) and ST-H (or ST-1b). Both of the ST peptides are associated with human infection. Confusingly, another ST variant, designated STb, is associated chiefly with porcine infection, and has little or no linkage to human diseases.

STa molecules are 18 or 19 amino acid peptides that have structural similarity to guanylin, which is a host molecule that binds to epithelial guanylate cyclase. STa binds reversibly to guanylate cyclase, which then increases intracellular cyclic GMP [61]. Intracellular cGMP then activates the cGMP-dependent protein kinase II (PKGII). Activated PKGII inhibits the cAMP-dependent phophodiesterase PDE3, thereby enabling intracellular cAMP to activate protein kinase A. The dual intracellular effects of elevated PKGII and PKA increase chloride secretion via phosphorylation of the CFTR, leading to net chloride secretion into the lumen. cGMP also promotes small bowl bicarbonate secretion and blocks sodium reabsorption, via additional processes dependent on the CFTR.

ETEC employ many different adherence factors to colonize the gut. These factors were previously designated colonization factors (CF). More recently, these largely fibrillar molecules have been assigned the designation "coli surface antigens" (except for CFA/I, which retains its initial designation) [62]; they are parsed into sets that contain conserved epitopes. Some CF are disproportionately represented among human ETEC, but adherence mechanisms used by the many different serotypes of ETEC that infect humans warrant additional characterization [63]. Interestingly, ETEC secrete a molecule, EtpA, which binds to regions on flagellin to promote attachment to eukaryotic cells [64], in a novel mechanism of adherence.

Exposure pathways for ETEC infections

ETEC are probably the most common bacterial cause of acute diarrhea worldwide. In resource-poor countries, ETEC infections have their highest incidence in children under age 2. ETEC are spread via consumption of food or water that has not undergone reliable bactericidal processes. Endogenous North American ETEC infections have occurred, usually from contaminated food [65]. Malnourished

children are predisposed to infection [66,67]. Repeated attacks of diarrhea (caused by ETEC as well as by other agents) could underlie malnutrition [54,68]. The infectious dose is high, estimated at greater than 1 million organisms [69,70], so person-to-person spread is less likely.

Some data exist regarding modes of ETEC transmission in resource poor environments, but, overall, the vehicles of infection are poorly delineated. These infections are endemic and not epidemic, so opportunities to study highly credible routes of infection have not arisen. Nevertheless, in these regions ETEC have been identified in food items in quantities sufficient to cause disease [256–259]. They have also been received from surface water [260], and there is a solid association between ETEC infection and consumption of street-vended food [261].

Prevention and control of ETEC infections

Prevention is directed at avoiding food and water that has not been subjected to a reliable bactericidal process, and avoidance of street vended food in areas of the world where ETEC infections are endemic.

Vaccination is appealing, because of the high frequency of ETEC disease, and delineation of immunogenic virulence factors. However, this approach faces challenges from antigenic variation in the CF, and would be aided by use of a common antigenic target [63]. As noted above, LT vaccination offers partial protection against symptomatic re-infection [59]. Killed ETEC cocktails that include a range of colonization factors, combined with recombinant cholera toxin B subunit, elicit, after oral challenge, a systemic response. Live vaccines have consisted of ETEC from which ST and LT genes have been deleted. Efficacy studies demonstrate immune responses, but protection of populations of children in endemic areas has been more difficult to demonstrate. Another approach is to express ETEC virulence factors in an attenuated *Shigella* vector. These strategies have recently been summarized [71].

Enteropathogenic *E. coli* (EPEC)

In the early 1940s, Verela et al. [72] found specific *E. coli* associated with severe infant diarrhea, as did Bray and his co-workers, in England later that decade [73]. Outbreaks occurred in the United States as well [74,75]. These organisms were termed “enteropathogenic” *E. coli* in 1956 [76]. The signature phenotype of these organisms is the classical attaching and effacing (AE) lesion where bacteria tightly adhere to the intestinal epithelium resulting in adjacent loss of microvilli [77], at sites of microcolonies (Figure 8.1).

AE lesions were readily apparent in human biopsies viewed via transmission electron microscopy [75]. The underlying mechanism of formation of these AE lesions was determined to be a pairing between intimin on the bacterial surface and Tir, which is injected by the EPEC via a T3SS [79].

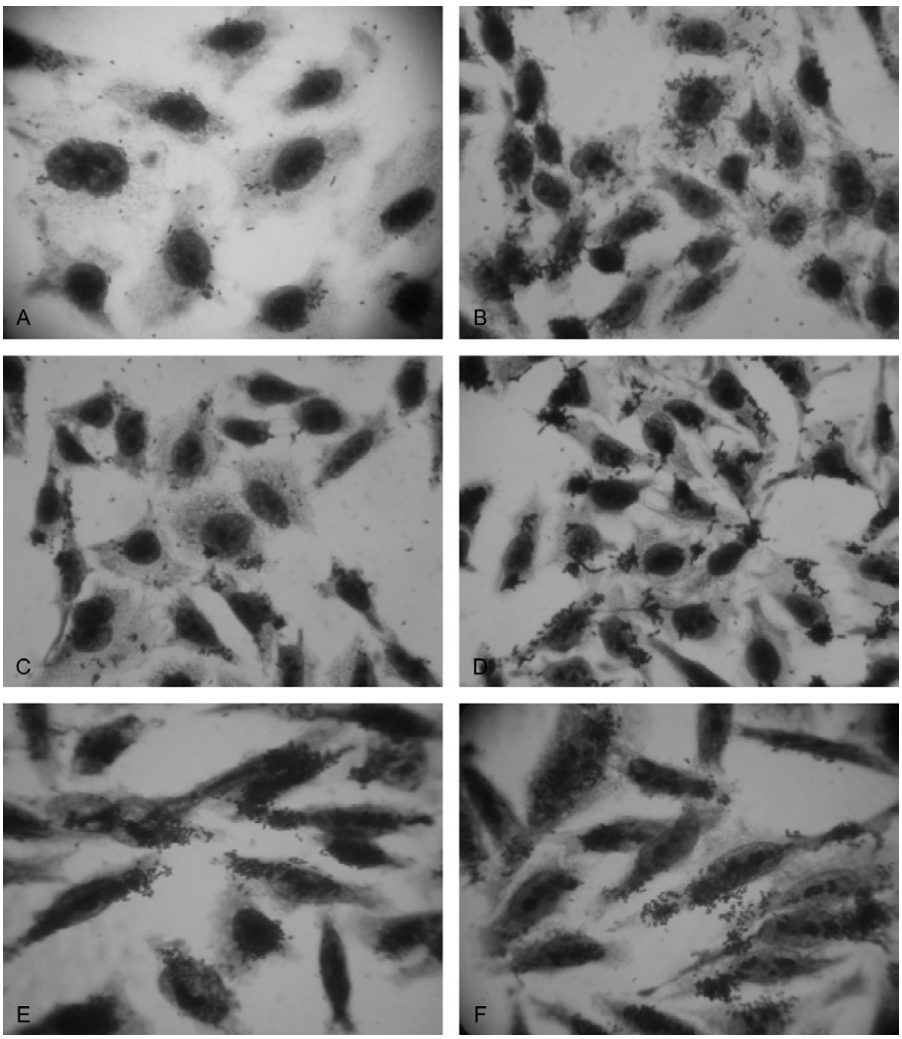

FIGURE 8.1

Variant adherence of phenotypes of EPEC. Sparse adherence (also termed "localized adherence-like") patterns in atypical EPEC strain HDV133 incubated with HEp-2 cells for 3 (Panel A) or 6 (Panel B) hours. Localized adherence-like patterns also seen with atypical EPEC AMB6-3-3 (Panel C), HSP23-5 (Panel D), and SC241-1 (Panel E) in the 6-hour assay. Classic localized adherence (LA) pattern of typical EPEC strain E2348/69 (3-hour assay) (Panel F) [78].

Clinical features of EPEC infections

EPEC are not routinely sought in stool cultures, so it has been difficult to accrue sufficient information with which to describe the illnesses associated with these agents. Infants and children are primarily affected with severe, often persistent, non-bloody diarrhea [1,80]. Oral challenge in adults produced non-bloody diarrhea [81]. Oral rehydration therapy can be used, but due to the inactivation of SGLT-1, sugar-based solutions are considered less effective than are intravenous fluids [82,83]. Resistance to antibiotics is common [84], and oral non-absorbable antibiotics do not appear to be very beneficial [85], though some small series suggest some value to this approach [86].

Intestinal failure along with various associated nutritional defects and growth stunting can be found in some cases [86]. In the developing world, persistent diarrhea can result in malnutrition and stunting, and persistent diarrhea is a consequence of EPEC infections, but the etiologic attribution of severe cases of persistent diarrhea to EPEC has not yet been made beyond the neonatal period.

Microbiology of EPEC infections

EPEC are typically detected through PCR for intimin encoded by *eae*, or *bfp*, or adherence assays [87]. These are not routinely employed.

EPEC form microcolonies or clusters of bacteria using type IV pili known as the bundle-forming pili [88,89]. Initial attachment may be due to these pili or other adhesins such as EspA, the core of the T3SS syringe [90], flagella [91], or LifA/Efa [92]. However, intimate attachment, which marks the characteristic AE lesions, is due to a paired Tir/intimin interaction and not to mono-functional adhesins.

While many pathogens use a T3SS, this mechanism is of critical importance to EPEC pathogenesis as EPEC do not produce classic toxins such as LT, ST_a, or Stx. The T3SS is encoded by a pathogenicity island known as the locus of enterocyte effacement (LEE). The EPEC LEE is 35 kb and inserted at the same chromosomal location as that of LEE in EHEC O157:H7 [93]. While the LEE is regulated via internal regulators (LEE-encoded regulator and global regulator of LEE activator), it is also regulated by Per, the plasmid-encoded positive regulator which is found on the same plasmid (EAF) as the bundle-forming pili. The LEE encodes for all structural components of the syringe-like T3SS as well as several key effectors including Tir, EspF, Map, and EspG. However, in the last 10 years, a number of non-LEE-encoded (Nle) effectors including NleA, NleB, NleC, NleD, NleH1, and NleH2 have been identified outside of the LEE region that contribute greatly to EPEC pathogenesis.

As EPEC come into contact with host cells the EspA filament of the T3SS acts as a channel for effectors which pass through the cap of EspB/D proteins that form pores in the host cell membrane. Tir, the prototypic effector protein, is injected. Tir also plays a key role in the formation of actin pedestals via phosphorylation by eukaryotic kinases, which leads to the recruitment of Nck, N-Wasp, and Arp 2/3 for actin polymerization [94]. Tir uses actin reorganization along with clustering provided by intimin to form the actin pedestal. Other LEE-encoded proteins such as

EspF, Map, EspG, and G2 disrupt tight junctions along with the more recently discovered NleA which acts by inhibiting COPII-dependent trafficking [95–98]. EspF, Map, EspG, and G2 also modulate sodium and chloride absorption. Specifically, EspG and G2 have been shown to decrease DRA/SLC26a3 Cl^-/OH^- exchange through disruption of microtubules needed for transporter recycling [99]. EspF and Map down-regulate the SGLT-1 Na^+/glucose co-transporter, while EspF alone causes inhibition of $Na^{+/}H^+$ exchanger 3 (NHE3) [82,100].

EPEC infections are not particularly inflammatory, and there are positive and negative effects of EPEC on host inflammation. In *in vitro* organ culture of duodenal biopsies, EPEC caused a flagellin-dependent activation of IL-8 through TLR-5 [101]. In contrast, EPEC have several effector proteins that block NF-κB activation. The first of these are the homologues NleH1 and NleH2 which prevent IκBα ubiquitination and degradation [102]. These proteins also bind to a subunit of the NF-κB complex called RPS3, with NleH1 blocking NF-κB-mediated transcription by keeping RPS3 sequestered in the cytoplasm [103]. NleE interferes with the recognition of ubiquitin by TAB2 and TAB3, by transferring a methyl group to a critical cysteine [104]. NleC is a protease that degrades the p65 subunit of NF-κB in the cytoplasm [105]. Finally NleB, through an unknown mechanism, inhibits TNF-dependent activation of NF-κB [106]. In addition, the Map kinase pathway is targeted by NleD, which has proteolytic activity toward JNK [107]. Somewhat surprisingly, Tir itself has been shown to be important in the very earliest phases of infection in blocking inflammation by interacting with and causing the degradation of TRAF proteins [108].

Typical EPEC can be distinguished from atypical EPEC (aEPEC) based on the presence of the EAF plasmid and expression of bundle-forming pili; they adhere less avidly than typical EPEC (see Figure 8.1). aEPEC infections are more abundant in developing and developed worlds [87], and the role of aEPEC in disease has probably been underappreciated [1,80,109,110].

Exposure pathways for EPEC infections

The most notable feature of EPEC epidemiology is the high frequency of infection in children, often under the age of 2, and frequently in infancy. Little is known about transmission methods, though contaminated formula has been suggested as a vehicle.

Prevention and control of EPEC infections

Good water sanitation is desirable. There are currently no vaccines available.

Enteroaggregative *E. coli* (EAEC)

History

EAEC's characteristic "stacked-brick" adherence pattern to epithelial cells (Figure 8.2) was first described after examination of *E. coli* isolates from Peruvian infants with acute

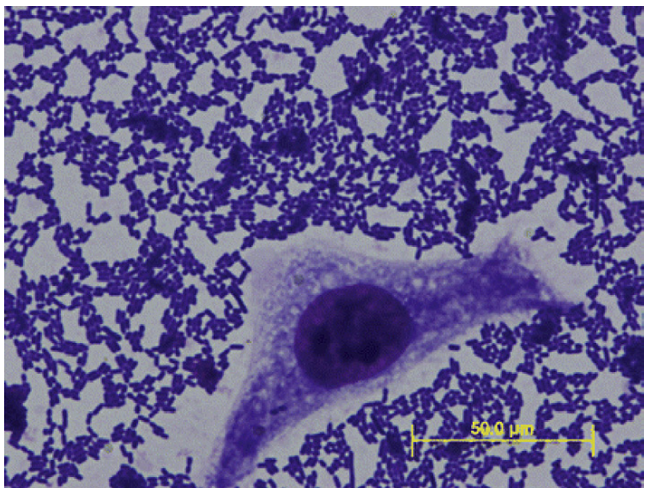

FIGURE 8.2

Classic stacked-brick appearance of EAEC [111].

diarrhea [112]. *E. coli* strains with similar patterns were previously isolated from patients with travelers' diarrhea after they visited Mexico but were classified as enteroadherent [113]. Soon after its first description EAEC infection was associated with persistent diarrhea in children from India [114], and later it was demostrated that EAEC infection even without clinical symptoms, including diarrhea, has a major effect on malnourished children [115]. EAEC strains have been suggested as causes of diarrhea in people infected with HIV [116] and have caused outbreaks [117].

Clinical features of EAEC infections

In view of the heterogeneity of EAEC strains, it is not surprising that EAEC infections have varied clinical features, which include acute and chronic watery diarrhea and occasionally bloody diarrhea with mucus, fever, nausea, vomiting, and abdominal pain [15,114,118–120]. Since their earliest reports from South America, EAEC have been recovered from patients worldwide (reviewed in [117]).

EAEC are notably associated with persistent childhood diarrhea [114]. Over the years studies have supported this association from poor [119,121,122] as well as industrialized countries [1,123,124]. Furthermore, Brazilian [115,122,125] and African [126] studies demonstrate EAEC-associated persistent diarrhea is linked to malnutrition. EAEC have been proposed as a cause of growth shortfalls and decreased intellectual development in children, and with intestinal inflammation, even in the absence of diarrhea [126,127]. These observations are in line with the fact that pathogenic EAEC induce release of cytokines [50,127] and lactoferrin [126] from the gut. Such continuing mucosal injury could increase gut permeability

and cause inadequate nutrient assimilation. In turn, malnutrition further facilitates infection and perpetuates the cycle of infection, malnutrition, and developmental deficits, increasing the burden of disease caused by EAEC [115].

EAEC are recognized important agents of acute and persistent diarrhea in adults [116,128–130] and children with HIV [131–134].

EAEC have also been associated with IBS and chronic gastrointestinal complaints. Among 60 US travelers who developed diarrhea while in Mexico, mostly due to ETEC and EAEC, who were surveyed 6 months after travel, many reported chronic gastrointestinal symptoms such as loose stools, abdominal pain, and fecal urgency, and 7 (11%) met the criteria for IBS, six of whom were newly diagnosed [135]. EAEC were isolated significantly more frequently from patients with IBS (81.8%) than healthy controls (32.3%) [136]. However, despite these associations, the precise role of EAEC in IBS and chronic gastrointestinal symptoms remains speculative.

EAEC are not sought in diagnostic microbiology laboratories. Hence, it is not known if antimicrobial agents are effective. When examining the sparse literature on treatment, it is important to keep in mind that it has been difficult to define EAEC using nucleic acid characterization, even with adherence studies. Nonetheless, fluoroquinolones have been used to treat human EAEC infections in people with HIV infections and symptomatic travelers [137,138]. Many EAEC are resistant to antibiotics [139–142], including fluoroquinolones [143]. Rifaximin might be helpful in treating EAEC infections in travelers [144–146]. Some enteric pathogens are moderately resistant to rifaximin, based on cut-points correlating to serum concentrations of rifampin, a related rifamycin. However, rifaximin is not absorbed, so the intestinal concentrations of this drug are very high, and well above the inhibitory concentrations of pathogens such as DAEC [147].

Microbiology of EAEC infections

The "stacked-brick" pattern with which EAEC adhere to epithelial cells was first described, as noted above, in isolates from Peruvian infants with diarrhea [112]. These isolates included prototypical EAEC strain 042 (serotype O44:H18). The ability of EAEC to mediate diarrhea was established through volunteer studies [120]; EAEC strain 042 elicited the most diarrhea [120]. EAEC's adherence phenotype is derived from the ability of EAEC bacteria to attach to each other, to epithelial cells, and to inert surfaces (e.g., tissue culture plates or glass). The AA pattern was also observed on intestinal mucosa infected with EAEC, characterized by a biofilm composed by bacteria aggregates in association with a thick mucus layer produced by intestinal cells that may explain why EAEC diarrhea illness is often associated with mucus. Data now suggest four major features of EAEC pathogenesis:

1. Abundant adherence to intestinal mucosa mediated by several adherence factors such as the aggregative adherence fimbriae (AAF) and others;
2. Induction of increased production of mucus from intestinal cells;

3. Elaboration of enterotoxins and cytotoxins (e.g., Pet and Shet); and
4. Induction of mucosal inflammation and secretion of inflammatory intestinal mediators by LPS and flagellin [84,148].

Prototype EAEC strain 042 has a genome consisting of a chromosome and one 60 MDa aggregative adherence plasmid (pAA) [149]. The aggregative adherence phenotypes are encoded by loci on the pAA plasmid. The *aggR* regulon (transcriptional activator factor) controls a number of plasmid virulence genes and at least two chromosomal pathogenicity islands involved in several steps of pathogenesis [150,151]. *AggR* is an AraC-like protein that positively regulates itself and several virulence-associated genes, including the ones for the AAF, dispersin, efflux pump TolC, and the type VI secretion system [150]. Therefore, EAEC strains carrying *aggR* have been designated as typical whereas *aggR*-negative strains are considered atypical [152].

Several fimbrial and non-fimbrial adherence factors enable EAEC to adhere to the intestinal mucosa. AAF I-II-III have been identified in typical EAEC strains and each EAEC carries only one AAF subtype [85]. An aggregative adhesin pilin Hda, regulated by *AggR*, was identified in EAEC strains lacking AAF [153]. A fimbrial structure has also been found in an atypical EAEC strain [150]. The *E. coli* common pilus (ECP), initially described in *E. coli* O157:H7, is also present in many EAEC with or without AAFs [154]. However, AAF genes are found in only a minority (<15%) of EAEC worldwide [84,155–158]. Furthermore, adherence phenotypes can be quite variable, even among putatively isogenic strains recovered from the same patients [15], and studies on human intestinal specimens demonstrate that EAEC strains can bind to jejunal, ileal, and colonic epithelium [117].

The efflux pump TolC promotes aggregation and adhesion of EAEC [159]. EAEC dispersin is an antigenic anti-aggregative protein that modulates fimbrial adherence and facilitates penetration of the microorganism through intestinal mucus by binding to lipopolysaccharide and altering the electrostatic properties of the outer membrane surface [160].

EAEC colonization of the intestinal mucosa is followed by elaboration of cytotoxins, the effects of which include microvillus vesiculation, enlarged crypt openings, and increased epithelial cell extrusion [155]. These toxins include EAEC heat-stable toxin (EAST-1), the plasmid-encoded toxin (Pet), the chromosomally encoded *Shigella* enterotoxin 1 (ShET1) toxin, hemolysin E (HlyE), and Pic, a mucinase associated with pathogenic *E. coli* and *Shigella* spp. Pet and Pic belong to the serine protease autotransporter of enterobacteriaceae (SPATE) family [85]. Pet cytotoxicity and enterotoxicity depend on its serine protease activity; Pet binds to and cleaves epithelial fodrin, resulting in cell elongation and exfoliation [161]. Pic is a mucinase with a lectin-like activity, which promotes serum resistance, hemagglutination, intestinal mucus hypersecretion, intestinal colonization, and growth in the presence of mucin [85]. Additionally, Pic also targets a broad range of human leukocyte adhesion molecules [162]. ShET is an oligomeric toxin that induces intestinal secretion via intracellular increase of cAMP and cGMP [163]. HlyE is predominantly an α-helical protein,

which oligomerizes into higher order structures to form a pore-forming toxin mediating cytolytic and cytopathic effects on cultured human cells [164].

Host intestinal inflammatory mediators elicited after EAEC infection include IL-1β, IL-1 receptor antagonist, IL-8, interferon-γ, lactoferrin, fecal leukocytes, and occult blood [165,166]. *In vitro* studies suggest that many of these inflammatory responses are stimulated by EAEC flagellin, which binds to toll-like receptor 5 and induces transcription of IL-8 [167]. Also, AAF/II fimbriae upregulate eukaryotic proinflammatory genes [168].

EAEC are challenging to identify. They are traditionally identified with epithelial cell adherence assay (typically Hep-2 cells), or DNA hybridizations and PCR (reviewed in [117]). In view of the heterogeneity of strains, there has been difficulty finding a gene or set of genes that is capable of distinguishing between pathogenic and nonpathogenic EAEC strains and the same challenge occurs with the Hep-2 cell adherence assay, the gold standard for EAEC identification. Nevertheless, several studies have shown that most typical EAEC isolates that are identified by their possession of the *aggR* gene (from subjects with diarrhea) harbor several other virulence genes [128,169].

Exposure pathways for EAEC infections

Foodborne outbreaks of EAEC infection have occurred in industrialized countries (reviewed in [117]). Cheese [170], food handlers [171], vegetables [172], and sprouts [40] have caused these outbreaks; nosocomial transmission has occurred in a neonatal unit in Serbia [173]. In a massive epidemic outbreak of EAEC infections in Japan, 2697 children and adolescents, from two kindergartens, eight elementary schools, and six junior high schools, developed gastroenteritis [174].

There is evidence of genetically ordained host susceptibility to EAEC infections. Polymorphisms in the promoters of lactoferrin, osteoprotegerin, and CD14 genes, all important elements of the intestinal immune system, have been associated with increased susceptibility to diarrhea in US travelers to Mexico [175,176]. A single nucleotide polymorphism in the -251 position of the IL-8 gene promoter has been associated with EAEC diarrhea and greater levels of fecal IL-8 in visitors from developed countries to EAEC endemic areas [175].

Prevention and control of EAEC infections

Prevention measures beyond good food and water hygiene cannot be recommended based on current knowledge [128].

Enteroinvasive *E. coli* (EIEC)

EIEC are very similar to *Shigella*. In fact, they were originally thought to be *Shigella*. EIEC first were introduced as possible pathogens by Allied soldiers in

World War II who were treated for what was considered to have been bacillary dysentery [177]. Some strains had biochemical differences from *Shigella* though unambiguous differentiation between *E. coli* and *Shigella* is not realistic [178]. Similar *E. coli* recovered from British schoolchildren in the 1940s [179] caused diarrhea in human volunteers [180].

Clinical features of EIEC infections

EIEC infections are poorly profiled. At least some EIEC cases present to medical attention with dysentery [177]. EIEC cause truly bloody, inflammatory dysentery only in a small subset of patients in outbreaks [179,181] and in experimentally challenged subjects [180]. In low income countries, EIEC are most frequently recovered from children under age 5 [181]. Human EIEC infections are rarely diagnosed, so antibiotic efficacy is unknown.

Microbiology of EIEC infections

The cardinal virulence trait of EIEC is their ability to gain entry into epithelial cells, a trait shared with *Shigella*. After cell entry, EIEC lyse endocytic vacuoles, replicate, and extend into contiguous cells [182]. Actin propels their intracytoplasmic movement. The invasion phenotype is encoded by T3SS genes on a 140 MDa plasmid present in EIEC and *Shigella* [183]. Most notably, IpaB and IpaC are injected into host cells, where they punch pores in membranes and initiate cytoskeletal rearrangements. Apoptosis and inflammatory cytokine release also ensue. IpaC polymerizes actin via IpaC and translocated IpgD, an inositol 4-phosphatase that dephosphorylates phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 5-monophosphate, which then accumulates intracellularly. This accumulation separates the eukaryotic plasma membrane from the cells' actin cytoskeleton, and produces extrusions, also termed "blebs" [184]. Vinculin is also bound by IpaA, leading to actin fiber polymerization. After this event, the IpaC-induced extensions transition into structures that permit bacteria to enter the host cells. EIEC also possess iron acquisition systems [185].

EIEC rarely ferment lactose on standard MacConkey agar, so the lactose fermentation phenotype is one way to make a preliminary identification. Optimal detection of EIEC in stool specimens would include PCR detection using primers specific for invasion loci, and/or phenotypic assays to assess invasiveness. In reality, such testing is not performed in clinical laboratories.

Exposure pathways for EIEC infections

The modes of disease transmission include food [186], water [187], and person-to-person spread [188].

Prevention and control of EIEC infections

Neither vaccines nor specific prevention measures can be recommended.

Diffusely adhering *E. coli* (DAEC)

DAEC, which adhere to epithelial cells in a diffuse pattern, were isolated from children with diarrhea [189]. There have been subsequent reports associating organisms of this phenotype with diarrhea in case-controls studies.

Clinical features of DAEC infections

DAEC tend to be isolated from non-bloody diarrhea; details such as fever and duration are not well described. In France [190] and Cincinnati [1], DAEC were statistically associated with diarrhea. In the most recent North American study [1], there was an appreciable carriage of DAEC in control children, but these organisms were more often excreted by ill children. The risk of DAEC diarrhea in children appears to increase with age, with stronger associations seen in children aged more than two years old [191]. DAEC have been suggested to cause diarrhea in high [1,190] and low [192] income countries. It is not known if antibiotics improve illnesses caused by DAEC, or if these infections have long-term consequences.

Microbiology of DAEC infections

DAEC are quite heterogeneous. The Dr family of (fimbrial) adhesins are the best characterized of the DAEC virulence factors. These adhesins are also expressed by some uropathogenic *E. coli*. Dr adhesins are fimbrial structures and permit *E. coli* to bind to diverse cell surface proteins, including decay-accelerating factor, carcinoembryonic antigen, and β1 integrins [193–195]. Interestingly, the DraE adhesin is internalized into the eukaryotic cell by these host cell surface structures, and not by bacterial invasion [196]. After DAEC bind to eukaryotic cells, the epithelial cell cytoskeleton is altered by signal transduction [197], following which there are alterations in the structures of epithelial brush borders and tight junctions [195,198]. An inflammatory response is also induced [199,200].

DAEC diagnosis largely relies on phenotype (adherence to epithelial cells) and genotyping. Detection methodologies have been formulated in research studies, and useful diagnostic strategies have not emerged.

Exposure pathways for DAEC infections

There are insufficient data with which to propose acquisition routes.

Prevention and control of DAEC infections

Other than standard food and water hygiene, specific prevention measures cannot be recommended.

The 2011 *Escherichia coli* O104:H4 outbreak

As we finalized this chapter, new data emerged on the epic foodborne outbreak that swept through northern Germany in 2011. Because of its importance to many realms of food safety, we devote a special section to this tragedy, which was caused by a hybrid pathogen with EHEC and EAEC traits.

On May 19, 2011, Hamburg pediatricians notified the Robert Koch Institute of a cluster of children with HUS [201]. Their stools did not contain *E. coli* O157:H7 and it soon became apparent that these were sentinel cases in a massive epidemic caused by *E. coli* O104:H4. One false epidemiologic start was based on a regional report that incriminated Spanish cucumbers (http://www.reuters.com/article/2011/05/26/us-germany-ecoli-idUSTRE74P3ND20110526, accessed on July 24, 2012). Most patients were adult women. There were unexpectedly high HUS and mortality rates. There was a second smaller outbreak in France [202]. By July 21, 2011, more than 4075 persons in 16 countries became ill, 908 developed HUS, and 50 died [203].

Matched case-control and a recipe-based restaurant cohort studies in Germany [40] found that the outbreak was associated with sprout consumption. Furthermore, trace-back investigation in Germany of sprouts from the distributor that supplied restaurant K led to producer A. All 41 case clusters with known trading connections could be linked to producer A, but the outbreak strain could not be identified in the implicated seed lots. French [204] and German STEC O104:H4 outbreak investigations enabled identification of a common food vehicle, fenugreek sprouts, and resulted in implementation of Europe-wide control measures in July 2011.

The several-week delay in unequivocally identifying the sprouts as the vehicle is not uncommon in vegetable-associated outbreaks. Sprouts are not easily recalled as having been eaten (they are often used as inconspicuous garnishes) and are not sold in brand-name emblazoned, easily remembered containers. They are, however, a perfect vehicle for infection: They are not cooked or irradiated and are sprouted in large facilities in which contamination and spread is not difficult, especially as potentially contaminated water is in continuity with many different lots. They remain for prolonged periods at temperatures conducive to the proliferation of human pathogens, and these pathogens often adhere quite avidly to the vegetable.

There were few secondary cases [205–208], as the predominance of primary cases were adults. Except for the cluster in France [204], additional outbreaks of infection with this pathogen have not occurred in the subsequent year. This attests to the fact that EHEC tend not to persist in human populations. This outbreak offered no new affirmative infection control lessons, as the microbial hazards of sprouts, a food that is prone to contamination but which undergoes no sterilization processes before consumption, are amply documented. This outbreak was a sad

reminder that without pasteurization, appropriate thermal or chemical microbicidal measures, or radiation, no food can be guaranteed to be free of bacterial hazards.

This episode illustrated challenges that disease prevention authorities face. The Spanish cucumber misattribution was based on an incomplete small area assessment of the epidemiologic data. Also, there are various and intricate disease reporting mandates between jurisdictions in Germany (and elsewhere). Such lines of investigative authority still work for church picnic outbreaks, but for pathogens that do not respect jurisdictional boundaries, the whole of the epidemiologic data are often much more informative (and defensible when action is needed) than any of its parts. Also, for many outbreaks, a critical early step to detection begins in the clinical laboratory, and there is considerable inter-laboratory variation in the speed and thoroughness with which stool specimens are evaluated for etiologic agents. Also, in Germany, it is the responsibility of the physician who requests stool cultures from adults to specify the agents to be sought. Hence, it cannot be assumed that an evaluation is uniform or complete. As the outbreak evolved, there was an acceleration in the interval between symptom onset and reporting [209], demonstrating baseline opportunities for improvement in the information chain.

The human illnesses caused by *E. coli* O104:H4 closely resembled those caused by *E. coli* O157:H7 [210], except for a possibly longer incubation period [201]. Therapies that are not standard in pediatric HUS were frequently employed, chiefly plasmapheresis and eculizumab, but their benefit was not confirmed [21].

In less than a week after the causative agent was isolated, University of Muenster investigators adroitly provided a thorough and exceptionally useful characterization of the agent as an *E. coli* O104:H4 that produced Stx 2, shared virulence profiles combining typical STEC (Stx 2, *teed, iha, lpf* [O26], lpf [O113]), and EAEC (*aggA*, *aggR*, *set1*, *Pic*, and *aap*) loci, and displayed an extended-spectrum β-lactamase phenotype [211,212]. The French and German outbreak strains were genetically closely related [202], though not identical [213].

E. coli O104:H4, like other Shiga toxin-producing *E. coli*, is best detected using a toxin EIA on a broth culture of stool. Its beta lactamase-resistance phenotype might be useful to exploit in selection during primary isolation, but it is not known if strains isolated in the future will be similarly resistant.

Some data suggest a role for azithromycin late in illness (i.e., after HUS is resolving) to hasten post-symptomatic clearance [214], but we caution against the use of this antibiotic for what is almost certainly a time-limited colonization of no clinical consequence [215].

The long-term sequelae of HUS following this particular EHEC infection are not yet compiled, but in the intermediate term, most patients have made apparently complete recoveries [21,216].

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CHAPTER

Campylobacter

9

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Introduction

Campylobacter species are pathogens of both humans and animals and show a worldwide distribution. *Campylobacter jejuni* and *C. coli* are recognized to be common human enteric pathogens [1]. In 2011, the Centers for Disease Control and Prevention (CDC) reported that there were approximately 845,000 cases of infections in the United States per year, representing 9% of all foodborne diseases caused by bacteria, viruses, and parasites. Globally, incidence rates up to 73 cases per 100,000 people have been reported [2] and it is reasonable to assume that even higher rates occurred in developing countries. Despite the fact that there was a 23% reduction in incidence of *C. jejuni/coli* infections in the United States in 2010 compared with 1996–8, the economic loss associated with these infections remains considerable. In 2004, the calculated cost-of-illness for *C. jejuni*-induced Guillain-Barré syndrome (GBS) was $1.7 billion, only in the United States [3]. Currently, there are 25 species and 8 subspecies of *Campylobacter*; however, *C. jejuni* accounts for approximately 90% of human *Campylobacter* infections [4] and is the main focus of this chapter (Figure 9.1). Despite the prevalence of *C. jejuni*, several other *Campylobacter* species are increasingly recognized as emerging human pathogens, including *C. lari*, *C. fetus*, *C. concisus*, *C. ureolyticus*, and *C. upsaliensis.*

The acute clinical illness

While *C. jejuni* and *C. coli* can exist as commensal organisms of domestic poultry and livestock, they are considered human pathogens (Figure 9.2). The clinical spectrum of human *C. jejuni/coli* enteritis ranges from loose stools to dysentery. In developed countries individuals experience inflammatory diarrhea characterized by abdominal pain, fever, nausea, fecal leukocytes, and sometimes grossly bloody stools [5]. In developing countries, infection by *C. jejuni/coli* generally is associated with a much milder illness, mainly watery diarrhea, without fever or blood and leukocytes

Foodborne Infections and Intoxications.

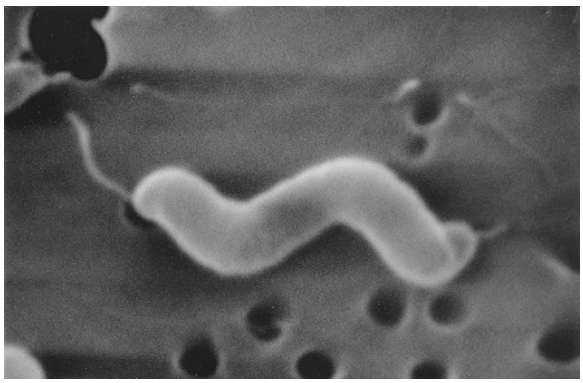

FIGURE 9.1

Scanning electron microscope image of *C. jejuni*. Note the corkscrew shape and the bipolar flagella [134].

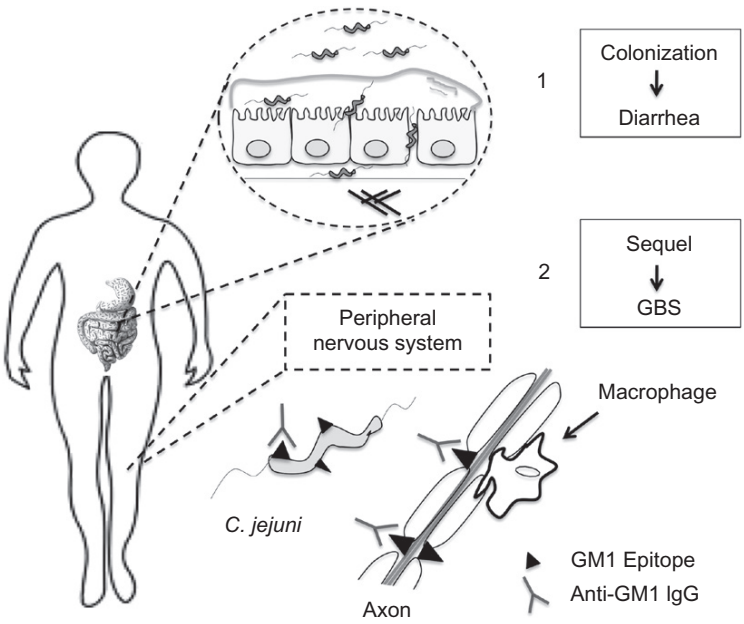

FIGURE 9.2

Disease caused by *C. jejuni*. The pathogen colonizes the human intestine and leads to diarrhea (1); in suceptible hosts *C. jejuni* colonization can lead to Guillain-Barré syndrome (GBS) (2). Antibodies against the GM1 epitope are produced and bind to the axon, leading to the blockage of the motor nerve.

in the feces; self-limiting acute enteritis is the most common syndrome. Prodromal symptoms are common and include headache, low fever, and myalgia lasting from a few hours to a few days. Symptoms of acute infection often begin with abdominal cramps followed by diarrhea and high fever, peaking during the first days of illness [6]. *C. jejuni*-specific serum antibodies confer immunity to symptomatic infection; however, the duration of protective immunity is not known [7,8].

In general, children >5 years of age and young adults (15–29 years) are at higher risk for *Campylobacter* infections, probably as result of poor immunity [9]. An estimated 100 fatal *C. jejuni* infections occur each year in the United States. These fatal infections occur often in infants, in the elderly, and in immune-suppressed individuals [10]. Bacteremia is most often seen in patients with certain underlying diseases [11] and is a potentially fatal complication of HIV/AIDS [12]. Chronic diarrhea is also a complication of HIV-associated campylobacteriosis. HIV-positive individuals who develop campylobacteriosis have shorter survival times and higher rates of bacteremia and hospitalization than HIV-positive individuals without campylobacteriosis [13]. This aspect of campylobacteriosis has major public health significance in developing nations [14].

Sequels of infection

Major complications associated with *C. jejuni/coli* infections include GBS and reactive arthritis. With several thousand cases each year, GBS is the most common presentation of acute flaccid paralysis in the United States [15]. GBS is an acute immune-mediated disorder of the peripheral nervous system. Leg weakness is often the presenting sign, followed by ascendant paralysis. After one year, 70% of patients make a complete neurological recovery, 22% partially recover, 8% remain unable to walk, and 2% remain bedridden or require ventilation. Most cases of GBS are believed to follow an infectious disease episode. Approximately 40% of GBS cases are thought to follow *C. jejuni/coli* infection, and GBS is estimated to occur in 1 in 1000 patients infected with *C. jejuni/coli*.

Although a diverse group of *C. jejuni/coli* serogroups is associated with GBS [16], this syndrome is strongly linked to a few strains of *C. jejuni*, such as Penner serotypes HS:19 [17] and HS:41 [18]. *Campylobacter* strains contain sialic acid linkages to lipo-oligosaccharides resembling sialic acid moieties on the gangliosides of peripheral nerve tissues [19]. New typing methods, high throughput sequencing, and whole genome comparisons could not identify any markers that might be involved in GBS other than the genes for lipooligosaccaride (LOS) synthesis. However, LOS involvement was clearly established, and in one study [20], two capsular biosynthesis genes (Cj1421c and Cj1428c) were significantly more prevalent in GBS strains than in non-GBS strains but have not yet been linked to pathogenesis in GBS. Patients with GBS develop antibodies against gangliosides that have antigenic epitopes similar to LOS, and that may result in autoimmune disease targeting peripheral nerve sites (see Figure 9.2).

Complement-mediated damage [21] and blockage of neurotransmission [22] are also suspected to play a role in GBS pathogenesis. Since many individuals are exposed to *C. jejuni* strains that mimic gangliosides and only a few develop GBS, it is suspected that host factors also contribute to GBS. In one study [23], *Campylobacter*-related GBS was associated with a major histo-compatibility antigen, HLA-DQB1*03; however, this association was not replicated in another well-designed study [17]. Proposed treatments for GBS have not been fully evaluated in clinical trials but include immunoglobulin therapy and plasmapheresis for depletion of anti-GM1 antibodies [24,25].

Reactive arthritis, or Reiter's syndrome, is another sterile post-infectious sequel to acute gastrointestinal campylobacteriosis. Presentation of reactive arthritis occurs 7 to 10 days after onset of diarrheal illness. The frequency of reactive arthritis as a sequel of campylobacteriosis has not been well described in the USA. In Finland, 45 of 870 (7%) patients with laboratory-confirmed campylobacteriosis developed reactive arthritis [26]. Arthritis was oligo- or poly-articular, and in most cases mild. In this Finnish study, 37 of the 45 patients (82%) had *C. jejuni* and 8 (18%) had *C. coli* infections. No cases of reactive arthritis occurred in children. In a Danish study, patients with joint pain had more severe gastrointestinal symptoms and longer duration of diarrhea than those without joint pain. Anti-*Campylobacter* antibody levels were similar in both patient groups. Antibiotic treatment did not prevent reactive arthritis [27].

Genetics, characteristics and evolution

The sequencing of the whole *C. jejuni* genome [28] was an important milestone for understanding *Campylobacter* pathogenesis. At present, more than 70 other *C. jejuni* genome projects, and projects for 19 other "non-*C. jejuni*" strains, can be found in the database of NCBI (January 2012). In general, *C. jejuni* has a small genome (1.6 kb) with a low G + C content of ~30%. The genome is unusual due to the lack of virtually any insertion sequences, phage-related sequences, or repeats, but it shows hypervariable sequences. These polymeric tracts allow *C. jejuni* to generate variations, which are important for host adaption and virulence [29]. Virulence determinants of *C. jejuni* including motility, adherence, enterotoxin and cytotoxin production, iron regulation, and cell invasion have been reported, and the majority of such genes have been experimentally linked to functionality *in vitro* and *in vivo* [30,31].

C. jejuni does not normally replicate outside the intestinal tract of warm-blooded animals [32]. The infectious dose is reported to be less than 1000 organisms [33]. Other adaptations to an intestinal niche include a single polar flagellum and the cell's corkscrew shape. These traits facilitate motility in the viscous intestinal mucus. Requirements for growth in the laboratory [32] also reflect its narrow ecologic niche—a micro-aerobic nitrogen-enriched atmosphere with low oxygen (5–7%) and high carbon dioxide tension (7–13%). *C. jejuni* is unable to replicate at temperatures below the body temperature of warm-blooded animals (approximately 30 °C), or at a pH <4.9. The organism is also sensitive to desiccation and osmotic

stress. *C. jejuni* gradually die outside the host intestinal tract. In one study, 58 of 85 (68%) *C. jejuni* strains could not be cultured from water after 3 weeks; however, a few strains were detected in unstirred water after 60 days [34]. Environmental factors may facilitate *Campylobacter* survival under adverse conditions. Survival times are longer in nutrient-rich water than in de-ionized water [35]. Some researchers postulate that campylobacters can survive in water in a viable but non-cultivable form [36,37]; however, the role of this dormant stage in the *Campylobacter* life cycle is controversial [38].

Similarly, biofilms are reported to facilitate the survival of *C. jejuni* in broiler houses [39]. *C. jejuni* produces biofilms only under certain growth conditions [40] and therefore it cannot be considered as a primary colonizing factor. However, *C. jejuni* can take advantage of already existing biofilms and can be considered as a secondary colonizer of biofilms [41]. Interestingly, *C. jejuni* has an enhanced ability to form biofilms with *Enterococcus faecalis* and *Staphylococcus simulans*—both bacteria that can be found in a poultry environment [42,43]. Hyper-variable sequences, biofilm formation with other species, natural competence to take up foreign DNA, and the ability for intra- and interspecies recombination drives this pathogen's evolution and adaption remarkably. This is displayed in a weakly clonal population structure.

Diagnosis and identification

Historically, classification of *Campylobacter* was not a trivial task, and advances in 16s rRNA sequencing extensively rearranged the taxonomy of *Campylobacter*. First, the genus *Helicobacter* was established as a separate lineage [44]. An extensive taxonomic study of the *Campylobacter* complexes finally determined its phylogenetic position [45]. The genus *Campylobacter* belongs to the rRNA superfamily VI, which are epsilon-Proteobacteria, and within this class they belong to homology rRNA cluster I. *C. jejuni* and *C. coli* are closely related to one another and cannot be easily distinguished by biochemical tests. Like most of the characterized species in this taxonomic family, they are recognized to be human or animal pathogens and have fastidious growth requirements reflecting dependence on a warm-blooded host for replication [46].

Diagnosis

Culture

Numerous procedures are available for recovering *C. jejuni* from clinical specimens. Direct plating is cost-effective for testing large numbers of specimens; however, testing sensitivity may be reduced. Pre-enrichment (raising the temperature from 36 °C to 42 °C over several hours) and/or filtration are used in some laboratories to improve recovery of stressed bacteria from some specimens (e.g., stored foods or swabs exposed to oxygen). Isolation can be facilitated by use of a selective

medium containing a combination of antimicrobial agents such as cephalothin, oxygen quenching agents, and a low oxygen atmosphere [32].

Polymerase chain reaction (PCR)

The PCR provides an important alternative to traditional microbiological culture techniques for detection and characterization [47,48] of *Campylobacter* strains. PCR diagnostic tests for *Campylobacter* are generally very sensitive and specific. Multiplex PCR assays can also be used to confirm the identity of a *Campylobacter* isolate among the six clinically most important species: *C. jejuni, C. coli, C. lari, C. upsaliensis, C. fetus* subspecies *fetus* [48], and *C. hyointestinalis* [49]. Advantages of multiplex PCR over traditional biochemical tests for characterization of *Campylobacter* strains include speed, ease of use, and high sensitivity and specificity. Conversely, selective culture is less expensive than PCR and provides an isolate for additional typing [50]. Al Amri et al. [51] described a multiplex PCR for differentiation for *C. jejuni* and *C. coli* with a turnaround time of about 6 hours from DNA extraction to gel elelectrophoresis, which has an estimated cost of US $ 3.7 per test. Conventional culturing and biochemical tests take 2–5 days with an estimated cost of US $ 0.92 per test.

Identification

Besides identifying *Campylobacter* in clinical samples, it is important to have sensitive and specific methods for identification of *Campylobacter* in environmental samples. An interesting approach is a PCR ELISA performed on samples of 48-hour enrichment cultures of foods that has a 99% sensitive and 96% specific for the detection of *C. jejuni* and *C. coli* compared to selective culture [52]. Alternatively, rapid identification and quantification of bacterial load is possible by using real-time PCR assays [53,54]. Real-time PCR is more expensive and the quantification of *Campylobacter* is less important for diagnostics, but the method will be of some help in epidemiologic studies.

Typing schemes

The two most accepted *Campylobacter* serotyping schemes are the Penner scheme [55], based on heat-stable antigens, and the Lior scheme, based on heat-labile antigens [56]. Both techniques yield a high proportion of non-typable strains and are technically demanding and expensive. These limitations have led to a development of alternative subtyping schemes, and genotypic approaches are increasingly used to characterize *Campylobacter* isolates [57]. Options include pulsed field gel electrophoresis (PFGE), flagella (*fla*) typing, and amplified restriction fragment length polymorphism (AFLP) analysis. Some schemes (e.g., *fla* typing) have advantages for use in limited situations related to ease and adequacy of discriminatory power; others (e.g., AFLP) provide the reproducibility and stability needed for large

epidemiologic and taxonomic studies. For a more accurate typing, the most efficient and cost-effective method is multilocus sequence typing (MLST) [58]. MLST is based on sequence variation analysis of seven or more housekeeping genes. MLST schemes are available for several *Campylobacter* species and can be accessed at the pubMLST website (http://pubmlst.org/campylobacter/). The website allows online analysis and comparisons [59]. To date, the database contains 7896 sequences and 5717 profiles for *C. jejuni/coli*.

Exposure pathways—risk factors for human illness

Poultry consumption

The initial epidemiologic studies of sporadic campylobacteriosis conducted in the United States [60–62] and Western Europe [63–65] revealed robust associations with handling [62,64] or eating [60,63,65] poultry, particularly undercooked poultry [61,66] (Figure 9.3). Other epidemiologic studies in the United States [67], the United Kingdom [68], and New Zealand [69] confirmed the association between

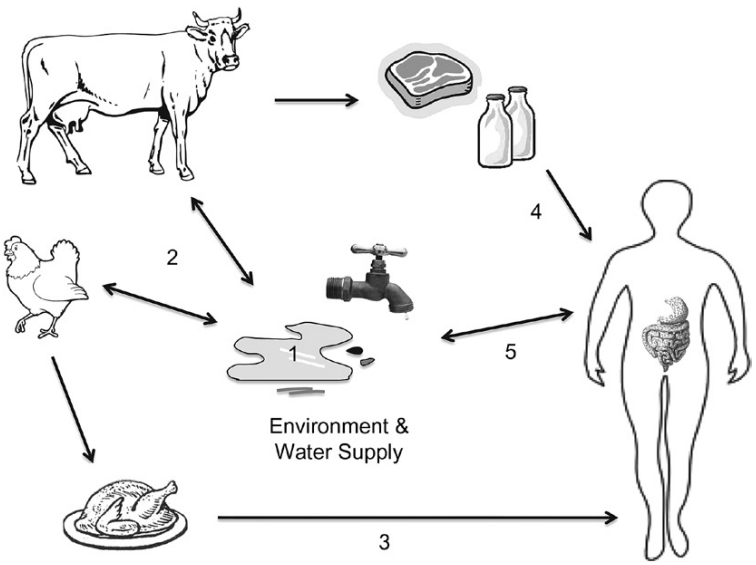

FIGURE 9.3

Route of transmission: (1) *C. jejuni* is found in environmental samples and water supply pipes (i.e., as a secondary colonizer of biofilms); (2) transmission to poultry and livestock; (3) undercooked poultry is the main source for human infections; (4) infections can be acquired through raw milk or meat; and (5) fecal runoff.

human campylobacteriosis and poultry consumption, and added an additional nuance—an association between *Campylobacter* infection and eating commercially prepared poultry [67,70]. These associations are not unexpected, given that the majority of chickens commercially available in stores are contaminated with *C. jejuni* [71]. Molecular subtyping studies demonstrate partial correspondence between poultry and human isolates [72,73]. In Quebec, 20% of genotypes from humans and poultry had matching PFGE patterns [74]. For children >3years, close proximity to raw poultry or meat (i.e., in shopping carts) is a risk factor for infections [75,76].

Commercially prepared foods

Case-control studies in the United States and other developed nations indicate that eating chicken in restaurants is associated with increased risk of *C. jejuni/coli* infection [67–69]. On occasions, other foods prepared in restaurants or commercial kitchens have been implicated in outbreaks of campylobacteriosis, including tuna salad [77], sweet potatoes [78], and lettuce [79]. Cross-contamination during food preparation is suspected to be a major contributory factor in such outbreaks, and it has been clearly shown that *C. jejuni* can survive on food contact surfaces and thereby cross-contaminate other foods [80].

Other food items

In addition to poultry, several types of meat have been epidemiologically implicated as sources of human campylobacteriosis in developed nations. Some of these implicated food items include pork loins, barbequed foods [81], and liver pâté [82]. Another important risk factor for *C. jejuni/coli* infections in infants is eating vegetables and fruits prepared at home [75].

Unpasteurized milk

While consuming chicken causes the most sporadic human *infections* with *Campylobacter*, drinking unpasteurized milk is the principal risk factor for *outbreaks* of campylobacteriosis. Between 1981 and 1990, 20 outbreaks of *Campylobacter* enteritis were reported in the United States [83]. Of these 20 outbreaks, 14 (70%) occurred among children who drank unpasteurized milk on school field trips or other youth activities. Unlike sporadic *Campylobacter* infections, which peak during the summer and are also associated with activities such as eating chicken, eating at restaurants, and international travel, milk-associated outbreaks have a bimodal seasonality, with peaks during the spring and fall corresponding with the peak seasons for youth activities such as school field trips. Despite regulatory efforts to address the hazard, unpasteurized milk-associated outbreaks continue to occur [84]. Recently, molecular typing studies have linked outbreak-associated infectious strains with unpasteurized milk from implicated dairies [84,85].

Water

One of the first case-control studies of campylobacteriosis, conducted in Colorado [66], found an association with consumption of untreated surface water. Waterborne outbreaks of campylobacteriosis typically involve lapses in community water sanitation [86,87]. The proportion of *Campylobacter* infections caused by contaminated water is likely to vary by region and with economic development. In a US study published in 2007, infants under 6 months of age who were drinking well water were at higher risk for infections [75].

Zoonotic transmission

Case-control studies identify contact with pet dogs and cats, and especially juvenile or diarrheic pets, as risk factors for *Campylobacter* infection, accounting for perhaps 5% of human campylobacteriosis [60,63,64,66,88]. The hazard of zoonotic campylobacteriosis may be greatest for young children, an age group with elevated rates of campylobacteriosis [89]. In one case report in which a 3-week-old girl in a household with a recently acquired puppy developed bloodstream *C. jejuni* infection, AFLP analysis confirmed that the human and canine isolates were genetically similar [90]. In an Australian case-control study, children less than 3 years of age who lived in a home with a pet puppy had a 17-fold increase in risk of campylobacteriosis compared with children with no puppy. Elevated risk of pediatric campylobacteriosis was also associated with pet chicken ownership [91]. Occupational risk factors for campylobacteriosis include farm residence, poultry-related occupations, and daily contact with chickens [90].

Foreign travel

Foreign travel is a commonly reported risk factor for campylobacteriosis [68,69,73]. In nations where campylobacters are uncommon in chicken (i.e., some Scandinavian nations), international travel is the dominant source of human *Campylobacter* infections [64]. In the United States it is estimated that between 20 and 25% of *Campylobacter* infections are acquired during international travel [92]. Campylobacteriosis was the most frequently reported enteric bacterial infection in Austrian tourists returning from Southern Europe and Asia [93]. In England, travel to South Africa was associated with *C. coli* infection [82]. Causal exposures (e.g., food, beverage, dining venue, antimicrobial usage, animal contact) for travel-associated infections remain to be determined.

Treatment indication and antimicrobial usage

Data suggest that antimicrobial therapy of campylobacteriosis generally is not necessary and may be harmful. For mild infections, antimicrobial treatment is not recommended. However, more severe infections require administration of electrolyte

solutions for rehydration, and if antibiotics are administered, they should be prescribed early in the infection [94]. Antibiotic use in the month before onset of illness was associated with a higher risk of campylobacteriosis, so it appears that prior antimicrobial use is a risk factor for *Campylobacter* infections [67]. Infection with ciprofloxacin-resistant *C. jejuni* is more likely in patients who used antibiotics in the month before onset [95]. One hypothesis for these observations is that antimicrobial usage lowers the infectious dose of drug resistant *C. jejuni* strains. Another potential explanation for the above findings is that the use of antibiotics may alter colonic flora, resulting in decreased resistance to infection even with antimicrobial susceptible *C. jejuni* strains. Inasmuch as infections generally are self-limiting and not life-threatening in otherwise healthy people, and because using antibiotics may lead to drug resistance in *C. jejuni/coli* infections, antibiotic usage for treatment of *C. jejuni/coli* infections in uncomplicated cases or non-high risk patients is not recommended.

Point of entry into the food supply and prevention

Studies confirm that the main route for *C. jejuni/coli* infections is consumption of poultry (see Figure 9.3). A detailed population genetic approach was performed in Lancaster, UK, where they investigated 1231 human isolates. They estimated that 57% of all *C. jejuni* infections could be connected to chicken consumption. In 35% of cases the source for infection could be traced back to cattle, and in 4% of cases infection is attributable to sheep. Only 3% of all cases were attributed to environmental sources or wild animals [96,97]. A study performed in Switzerland gave population attributable fractions (PAF) of 27% for poultry consumption, 27% for traveling abroad, and 47% for other risk factors [98]. The most effective prevention measurement for *C. jejuni* infections would be the reduction of bacteria in poultry carcasses, which could be achieved by having *Campylobacter*-free chicken flocks.

Processing controls

Carcass processing is a promising site for pathogen reduction efforts. The microbial quality of broiler carcasses has been associated with practices in the abattoir where processing occurred [99]. Treatment of wash water is a potential processing control to reduce contamination, and the poor microbial quality of poultry wash water is thought to contribute to higher contamination rates of poultry than in the case of red meat. The use of electrolyzed water for washing poultry carcasses reduced *C. jejuni* counts on chicken by $3 \log_{10}$ units [100]. Washing in 10% oleic acid significantly reduced the number of *Campylobacter* that remained attached to poultry skin [101]. Campylobacters are also very sensitive to active chlorine [102]. The chlorination of carcass wash water, an important component of the hazard analysis and critical control point (HACCP) programs in many processing plants [103], may have contributed to the decline in human campylobacteriosis in the United States since the mid-1990s.

Post-processing interventions have also been investigated. Freezing poultry carcasses to −20 °C resulted in an up to 2.8-$\log_{10}$ reduction in *Campylobacter* counts [104,105]. Electron beam irradiation of poultry would virtually eliminate *Campylobacter* from poultry products; however, some consumers report that the color and texture of chicken fillets are altered by irradiation [106]. If these food quality considerations are successfully resolved, irradiation of poultry products could become one of the most important technologies for the prevention of foodborne campylobacteriosis in the United States.

Food handling

Epidemiologic studies indicate that food preparation and service in restaurants [67] and home kitchens [62] are important factors in *C. jejuni* infection [68,69]. Surveys indicate that safe food-handling skills need improving in various demographic groups of the US population, including males and young adults [107]. Kitchen sanitation guidelines should emphasize the cleaning and disinfection of food contact services, hands, and utensils following contact with raw meat and poultry. In addition, raw meat and poultry should be stored separately from foods that are served without subsequent cooking. Meat thermometers are recommended to measure the internal temperature of meat and poultry when it is cooking; poultry should be heated to an internal temperature of 82 °C (180 °F) to kill *Campylobacter*.

Zoonosis prevention

Hand washing after animal contact is a sensible step to minimize zoonotic campylobacteriosis in both household and occupational settings. Additional sanitary precautions are recommended with juvenile or diarrheic pets. It is particularly important to ensure that children wash their hands after animal contact. If sufficient attention is given to hygiene, many immunocompromised patients can safely enjoy animal companionship [108].

Food

Retail food surveillance programs in developed nations provide valuable data on foodborne hazards by type of retail meat and poultry product. For example, in an English study of nearly 500 retail specimens, chicken meat had the highest contamination rate (83%), followed by lamb liver (73%), pork liver (72%), and beef liver (54%). *C. jejuni* predominated in chicken meat (77%), while *C. coli* predominated in pork liver (42%) [4]. In metropolitan Washington, DC, 130 of 184 (70%) packages of chicken sold at retail outlets contained *C. jejuni* or *C. coli*, followed by 4% of 172 samples of turkey, and less than 2% of pork and beef [71]. In a study of more than 2000 lamb carcasses from six large processing plants, less than 1% of carcasses were contaminated with *C. jejuni* or *C. coli* [71].

Milk and water

Surveys of bulk tank milk indicate that unpasteurized milk is a source of *C. jejuni*. In one study, approximately 10% of unpasteurized milk specimens from dairy bulk tanks were contaminated with *C. jejuni* [109]. Surface waters are often contaminated with campylobacters. In a Norwegian study, 32 of 60 water specimens from the Bo River contained campylobacters, and *C. coli* was detected more often than *C. jejuni* [110]. In that study, fecal coliform counts were not a reliable indicator of low-level *Campylobacter* contamination.

Other *Campylobacter* species

Campylobacter species other than *C. jejuni* and *C. coli* include *C. cervus*, *C. concisus*, *C. fetus* subspecies *fetus*, *C. hyointestinalis*, *C. lari*, *C. mucosalis*, *C. gracilis*, *C. rectus*, *C. showae*, *C. sputorum*, and *C. upsaliensis*. Many are suspected to be human or animal pathogens. Many of these related species are also inhibited by the antibiotics in selective media used for routine isolation of *C. jejuni* and *C. coli*, and several species (e.g., *C. concisus, C. sputorum, C. cervus, C. rectus*, and strains of *C. hyointestinalis*) require different micro-aerophilic incubation conditions than *C. jejuni* for growth. Furthermore, procedures for the accurate identification of *Campylobacter* organisms to the species level are time-consuming and difficult. Thus, the true prevalence of human infections with these other *Campylobacter* species is unknown.

C. lari

C. lari was first isolated from mammalian and avian species [111]. In 1984, the first case of human disease related to *C. lari* was reported—fatal bacteremia in an immunocompromised patient with multiple myeloma [112]. Soon after, sporadic cases of enteric infection were also described [113]. Although *C. lari* bacteremia is most often reported in patients with underlying disease [114], cases of *C. lari* bacteremia in immune-competent individuals have also been described [115,116].

C. fetus subspecies *fetus*

Until recently *C. fetus* subspecies *fetus*, which causes bovine and ovine abortion and sterility, was not regarded as a human pathogen. However, between 1980 and 1995, *C. fetus* was implicated in at least four reported outbreaks of human disease in North America; three were associated with foods—raw milk, a supplement containing raw calf liver, and cottage cheese [117]. In addition to being isolated from stools of patients with gastroenteritis, it is recognized to cause invasive infections and has been isolated from human blood, spinal fluid, abscesses, and cellulitis associated with bacteremia [118]. Bacteremia is usually seen in patients with underlying disease, such as metastatic malignancy or HIV infection [117].

C. hyointestinalis

Between 1979 and 1985, two of four laboratory-confirmed cases of *C. hyointestinalis* that were reported to the CDC were from stools of homosexual men [119]. Stool isolates were also obtained from an 8-month-old girl who lived on a farm with livestock, and a 79-year-old woman who had traveled to Egypt. A small outbreak among family members in Canada may have been associated with drinking raw milk [120]. *C. hyointestinalis*, with or without *C. mucosalis*, has been implicated as a causative agent of proliferative ileitis in swine [121] and diarrhea of calves [122].

C. upsaliensis

Since the first isolation of *C. upsaliensis* was reported in 1983 from the stools of healthy and diarrheic dogs [123], pet animals have continued to be suspected as a principal source of human infection [124]. In Los Angeles, for example, *C. upsaliensis* was isolated from pet dogs in the households of two of six patients with *C. upsaliensis* infection [125]. Initially, *C. upsaliensis* infections were associated with extremes of age or with underlying disease. Of 11 human isolates reported by the CDC between 1980 and 1986, 8 originated from blood [126]. Blood isolates originated from two infants with fever and respiratory symptoms, a woman with an ectopic pregnancy, three elderly men with underlying diseases, and two immunocompromised adults. Of the three stool isolates, one originated from an immunocompromised patient with persistent diarrhea.

It is suspected that there is under-reporting of enteric *C. upsaliensis* infections because the antibiotics that are used in selective media for isolation of *C. jejuni* (e.g., cephalothin) inhibit the growth of *C. upsaliensis* [127]. In a Swedish study, *C. upsaliensis* was the second most common *Campylobacter* species in children with diarrhea, after *C. jejuni* [128]; and in Los Angeles County in 1998, *C. upsaliensis* accounted for 4% of fluoroquinolone-resistant *Campylobacter* isolates from human patients [125]. Regional differences in the prevalence of *C. upsaliensis* infection are suspected, with low prevalence of human infection reported in the United Kingdom [129] and Denmark [130] compared with Sweden and Los Angeles.

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CHAPTER

Yersinia 10

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Introduction

Since the 1960s, *Y. enterocolitica* has been identified as a frequent and important cause of enteric yersiniosis in developed countries. As the consequences of yersiniosis are severe, and might include prolonged acute infections, pseudoappendicitis, and long-term sequelae such as reactive arthritis, the financial and public health effects of yersiniosis are of greater magnitude than the actual number of cases would suggest. Therefore, implementation of preventive measures in the food chain is important, in order to protect consumers against *Y. enterocolitica* infection.

Disease due to *Y. pseudotuberculosis* has been recognized since the beginning of the twentieth century. The view that *Y. pseudotuberculosis* might be a cause of foodborne disease has been encouraged by reports of isolation from vegetables and by implication in some foodborne outbreaks [1].

Clinical features of illness associated with infection in humans

Gastroenteritis is by far the most common symptom of *Y. enterocolitica* infection (yersiniosis) in humans [2]. The clinical picture is usually one of a self-limiting diarrhea associated with mild fever and abdominal pain [1]. Nausea and vomiting occur, but less frequently. The portion of the gastrointestinal tract usually involved is the ileocaecal region [1]. The colon may also be affected and the infection may simulate Crohn's disease, which has a different prognosis. Occasionally the infection is limited to the right iliac fossa in the form of terminal ileitis or mesenterial lymphadenitis, with symptoms that can be confused with those of acute appendicitis. In several studies of patients with the appendicitis-like syndrome, *Y. enterocolitica* has been found in up to 9% of patients [1].

Foodborne Infections and Intoxications.

Impact of host susceptibility on occurrence of illness

Infections with biotypes/serotypes 4/O:3 or 2/O:9 are, in some patients, followed by reactive arthritis [1], which is most common in patients possessing the tissue type HLA-B27. Other complications seen with *Y. enterocolitica* infection are reactive skin complaints, with erythema nodosum being the most common. Septicemia due to *Y. enterocolitica* is seen almost exclusively in individuals with underlying disease [2], while those with cirrhosis and disorders associated with excess iron are particularly predisposed to infection and increased mortality. Gastroenteritis dominates in children and young people, while various forms of reactive arthritis are most common in young adults, and most patients with skin manifestations are adult females [1].

Microbiology

Y. enterocolitica is a Gram-negative, oxidase-negative, catalase-positive, nitrate reductase-positive, facultative anaerobic rod (occasionally coccoid), and 0.5–0.8 × 1–3 µm in size [3]. It does not form a capsule or spores. It is non-motile at 35–37 °C, but motile at 22–25 °C with relatively few peritrichous flagellae. Some human pathogenic strains of biotype/serotype 4/O:3 are, however, non-motile at both temperatures. In addition, the bacterium is urease-positive and H_2S-negative, ferments mannitol, and produces acid, but not gas, from glucose.

Phenotypic characterization

Biotyping

The biotyping scheme which differentiates between pathogenic (biotypes 1B, 2, 3, 4, 5) and non-pathogenic (only biotype 1A) variants is recommended [4].

Serotyping by using O-antigens

Y. enterocolitica can be divided into serotypes using O-antigens. About 76 different O-factors have been described in both *Y. enterocolitica* and *Y. enterocolitica*-like bacteria [1].

Correlation between biotypes and serotypes and pathogenicity

Strains of biotype 1B belong to a small number of pathogenic serotypes, the most frequent being O:8. Biotype 2 only includes two serotypes, O:9 and O:5,27, which are pathogenic for man. Biotype 4/serotype O:3 is the main pathogenic bio/serotype for man.

Yersinia pseudotuberculosis

Y. pseudotuberculosis is classically subdivided into five serotypes (I to V). Additional serotypes and subserotypes have been identified by Japanese scientists but are quite uncommon in Europe [1]. Serotype I is by far the most common serotype associated with human and animal infections in Europe, followed by serotype III [1].

Characteristics of *Y. enterocolitica* and *Y. pseudotuberculosis* including genetics and evolutionary considerations

Y. pestis and *Y. pseudotuberculosis* are closely related, with a gene homology of nearly 97% and with a largely co-linear gene organization. In contrast, *Y. enterocolitica* is more distantly related, with about the same evolutionary distance away from *Y. pseudotuberculosis/Y. pestis* as *Escherichia coli* is from *Salmonella* species [5]. But the least related species, *Y. pseudotuberculosis* and *Y. enterocolitica*, are foodborne pathogens causing similar diseases far from the diseases caused by *Y. pestis*.

Based on DNA-DNA hybridization, determination of G + C mol% content, and sequence alignment studies [6], *Y. enterocolitica* is divided into:

- subspecies *enterocolitica* (the "American bio/serotypes") and
- subspecies *palearctica* (the "European bio/serotypes").

The high-virulent "American strains" (biotype 1B including serotype O:8) have a "high-pathogenicity island" [5]:

- with a huge chromosomal region,
- which contains virulence genes,
- which is stable, and
- which is involved in iron uptake.

The low-virulent "European strains" (biotypes 2–5 including bio/serotypes 4/O:3 and 2/O:9) do not have this "island." According to Tauxe [7] bio/serotypes 4/O:3 and 2/O:9 are examples of pathogens causing a global pandemic due to export of pigs and pork from Europe to America and Asia.

Approaches to diagnosis and identification

Selective enrichment

One of two approaches in the International Organization for Standardization (2003) method is based on a 2-day selective enrichment at room temperature in irgasan-ticarcillin-potassium chlorate (ITC) enrichment broth while the other approach is based on 5 days in phosphate-buffered saline, sorbitol, and bile salts. In general the conventional culture methods underestimate the occurrence of human pathogenic *Y. enterocolitica* in pork products [8].

Isolation

Due to low selectivity of the culture media available and the fact that non-pathogenic *Y. enterocolitica* have more or less the same appearance as human pathogenic strains on agar media such as cefsulodin-irgasan-novobiocin (CIN) agar, selection of the relevant colonies for further confirmation can be difficult [8], and therefore culturing methods are considered relatively insensitive. However, a new chromogenic agar medium for detection of potentially virulent *Y. enterocolitica* has been developed by Weagant [9].

Identification of human pathogenic Y. enterocolitica

Since the majority of strains capable of causing disease belong to only a few serotype/biotype combinations, for practical purposes serotyping and biotyping are usually sufficient to differentiate pathogenic strains from non-pathogenic ones.

Y. enterocolitica might also be identified by 16S rRNA gene sequencing, testing for homology by comparison with the sequence of a type strain of *Y. enterocolitica* [10]. Multiplex polymerase chain reaction (PCR) assays for identification of *Y. enterocolitica* cultures have also been described and might replace biochemical and serological tests for identification of *Y. enterocolitica* [11].

Detection methods

PCR methods, single or multiplex, using primers from virulence-associated genes, have been shown to increase the sensitivity in detecting virulent *Y. enterocolitica* in foods compared with the traditional culture methods [8]. Since the virulence plasmid might be lost, PCR assays using chromosomal primers from virulence-associated genes such as *ail*, *inv*, *yst*, and *virF* should be used alone or in combination with primers based on the virulence plasmid to avoid false negative results [8].

Epidemiological typing of Y. enterocolitica

Methods that have been used to differentiate pathogenic isolates of *Y. enterocolitica* include biotyping, antibiogram typing, phage typing, and multi-locus enzyme electrophoresis analysis [1]. Methods based on the characterization of the genotype include restriction enzyme analysis of plasmid DNA or chromosomal DNA, the use of DNA or RNA probes, and pulsed field gel electrophoresis (PFGE). PFGE has identified several pulsotypes among *Y. enterocolitica* biotype 4/serotype O:3, but most often certain pulsotypes dominated and most of the strains belong to one or two dominant pulsotypes [1,12]. Also, amplified fragment length polymorphism (AFLP) analysis confirmed a high degree of clonality among *Y. enterocolitica* biotype 4/serotype O:3 strains [13].

Multiple-locus variable number tandem repeat analysis (MLVA) was developed to improve the discriminatory power of classical genotyping methods [14]. MLVA based on six loci was able to distinguish 76 genotypes among 91 *Y. enterocolitica* isolates of worldwide origin and 41 genotypes among 51 non-epidemiologically linked bio/serotype 4/O3 isolates, proving that it has a high discriminatory power [15]. Due to the high discriminatory power of MLVA, it might be easier to detect outbreaks and sources of infection, and trace the pathways of different epitypes of *Y. enterocolitica* biotype 4/serotype O:3 in the production chain.

Exposure pathways

Reservoirs

Pigs and pork

There is a strong correlation between the biotype and serotype combinations (i.e., bio/serotype) isolated from humans and pigs in the same geographical area [7,16].

Pigs are often healthy carriers of pathogenic strains of *Y. enterocolitica*, in particular strains of bio/serotypes 4/O:3 and 2/O:9 [16]. The organisms are present in the oral cavity, especially the tongue and tonsils, submaxillar lymph nodes, and the intestine and feces [17]. As a result of slaughter and dressing procedures, *Y. enterocolitica* 4/O:3 is also isolated from freshly slaughtered pig carcasses [18].

Cattle, milk, and dairy products

In some cases, positive tests in serological control programs for brucellosis in cattle have proved to be cross-reactions with *Y. enterocolitica* 2/O:9 antigens [1]. There are examples of outbreaks caused by contaminated milk [1], reconstituted powdered milk [1], and contaminated chocolate milk [1] where pathogenic strains of *Y. enterocolitica* have been isolated. In the USA, chocolate milk [1] and pasteurized milk [1] have been implicated as sources in outbreaks of *Y. enterocolitica* infection. These outbreaks, all of which occurred before 1983, were caused by *Y. enterocolitica* biotype/serotype combinations which have been infrequently associated with human disease (such as biotype 1B combined with serotypes O:13 and O:18) or which no longer predominate in the USA (bio/serotype 1B/O:8). Some of the milk-associated *Y. enterocolitica* outbreaks have been linked to the addition of ingredients after pasteurization [1].

Sheep and goats

In Norway, outbreaks of *Y. enterocolitica* infection in goat herds were caused by bio/serotype 5/O:2 strains [19]. An animal attendant was infected by a strain of this bio/serotype. There are no publications from recent years describing potential pathogenic variants of *Y. enterocolitica* from sheep and goats.

Poultry

In Germany, *Y. enterocolitica* 4/O:3 and 2/O:9 were isolated from poultry [1]. This is probably the first and only time these virulent strains have been isolated from these animals, and there was no obvious opportunity for cross-contamination from pigs or other animals.

Pets

Raw pork might be an important source of *Y. enterocolitica* 4/O:3 infection in dogs and cats, and these animals may occasionally be healthy carriers. These pets might be vehicles or even infection sources for humans [1].

Other animals

Rodents are a reservoir of bio/serotype 1B/O:8 and 1B/O:21 strains in Japan [20], and probably also in North America [16].

Water and vegetables

Shallow wells, rivers, and lakes are exposed to fecal contamination from wild or domestic animals, or by leakage from septic tanks or open latrines in the surrounding areas. Thus water is a significant reservoir of *Y. enterocolitica*. However, most

Yersinia isolates obtained from water belong to non-pathogenic *Y. enterocolitica* biotype 1A or to *Y. enterocolitica*-like bacteria. But in 1981, an outbreak of infection due to *Y. enterocolitica* 1B/O:8 in Washington state occurred in association with the consumption of tofu packed in untreated spring water [21]. The outbreak strain was isolated from the spring water samples. Another outbreak caused by a bio/serotype 1B/O:8 strain was traced to ingestion of contaminated water used in manufacturing or preparation of food [16]. Two other *Yersinia* outbreaks have been associated with well water. One occurred among members of a Pennsylvania Girl Scout troop after they ate bean sprouts grown in contaminated well water [22]; the other was a familial outbreak of yersiniosis in Canada [23]. *Y. enterocolitica* 4/O:3 was isolated from members of the family, as well as from the well used as a source of their drinking water. During late winter and spring 2011, an outbreak involving 21 cases of *Y. enterocolitica* bio/serotype 2/O:9 infection was identified in Norway. Results of epidemiological and microbiological investigations indicated bagged salad mix from Italy as the possible source [24]. The salad, "Radiccho rosso," was stored at 1 °C before it was supplied to the market.

Infectious dose, pathogenesis, and immunity

Human infection due to *Y. enterocolitica* is most often acquired by the oral route. The minimal infectious dose required to cause disease is unknown. In one volunteer, ingestion of 3.5×10^9 organisms was sufficient to produce illness [25]. The incubation period is uncertain but has been estimated as being between 2 and 11 days [25].

Approaches to food attribution

Pork products are considered the main source of yersiniosis in humans. The preparation of raw pork intestines (chitterlings) was linked to an outbreak of *Y. enterocolitica* 4/O:3 infections among African-American US infants in Georgia [26]; the organism was isolated from samples of the pork intestines. Chitterlings were also the vehicle in outbreaks in Buffalo, New York, between 1994 and 1996 [27]. Case-control studies of sporadic cases of yersiniosis conducted in Belgium [28] and in Norway [29] have identified consumption of pork as an important risk factor for infection in humans. During an outbreak in 2006, 11 cases of *Y. enterocolitica* 2/O:9 infection were identified in Norway indicating a processed pork product ("julesylte": Christmas brawn) as the probable source [30]. One smaller family outbreak of yersiniosis caused by *Y. enterocolitica* 4/O:3 occurred in brawn in 2006 [30].

Y. pseudotuberculosis

Because *Y. pseudotuberculosis* is so common in animals, their feces are quite likely to contaminate soil, water, and vegetables and thus contribute to transmission to humans [31].

Occurrence in animals

Y. pseudotuberculosis mainly causes epizootic disease, especially in rodents, with necrotizing granulomatous lesions in liver, spleen, and lymph nodes; infections in animals are often fatal [31]. *Y. pseudotuberculosis* has been isolated from both wild and domestic mammals as well as birds [31]. Rodents kept as fur animals, laboratory animals, and zoo animals and birds kept in zoos and bird shops (and as farm animals or pets) are often infected [31]. *Y. pseudotuberculosis* infection (particularly by serotypes I and II) in hares has been reported in Germany [1]. Serotype III has frequently been isolated from asymptomatic pigs in Finland [1]. However, the principal reservoir hosts are believed to be rodents and wild birds. Most animals are asymptomatic carriers, but they may become ill and excrete the bacteria after a stress, such as cold and humid weather or starvation.

Water and vegetables

Water and soil in Japan and the far eastern regions of Russia are contaminated with *Y. pseudotuberculosis* [1]. Transmission of *Y. pseudotuberculosis* to humans through water contaminated by feces from wildlife and domestic animals has been reported in Japan and Korea [1]. This pathogen has sporadically been isolated from fresh produce in Finland and Russia, and in Japan from fresh produce and pork [1]. Iceberg lettuce and raw carrots have been implicated in some foodborne outbreaks in Finland [1].

Prevention and control

Yersinia enterocolitica is a zoonotic bacterium that has its main reservoir within the domestic pig population. Accordingly, interventions in the meat chain are essential for protection of consumers against infection with pathogenic *Y. enterocolitica.*

General control aspects connected to survival and growth of *Y. enterocolitica*

Y. enterocolitica is a facultative organism able to multiply in both aerobic and anaerobic conditions.

pH and water activity

Y. enterocolitica is not able to grow at pH <4.2 or >9.0 or at salt concentrations greater than 7% (a_w <0.945) [32].

Temperature

The organism does not survive pasteurization or normal cooking (boiling, baking, frying) temperatures. Heat treatment of milk and meat products at 60 °C for 1–3 minutes effectively inactivates *Y. enterocolitica* [33]. The ability of *Y. enterocolitica*

to multiply at low temperatures is of considerable concern to food producers. The reported growth range is −2 to 42 °C [3]. Optimum temperature is 28–29 °C [3].

Packaging

The ability of pathogenic *Y. enterocolitica* to propagate at refrigeration temperature in vacuum-packaged foods is of considerable significance to food hygiene [3]. As a facultative anaerobic organism, the gaseous composition of the surrounding atmosphere can have an important impact on the growth of pathogenic *Y. enterocolitica*. In Nissen et al. [34], growth of *Y. enterocolitica* was nearly totally inhibited in ground beef packaged in a high CO_2/low CO mixture (60% CO_2/40% N_2/0.4% CO) at both 4 °C and 10 °C.

The fact that recontamination after heat treatment may result in relatively unhindered growth of *Y. enterocolitica* during refrigerated storage due to the absence of microbial competition emphasizes the need for more effective interventions during processing of this type of product. Appropriate measures, targeted at prevention of recontamination after heat treatment, should be implemented.

Control in the meat chain

Control at farm level

Occurrence of *Y. enterocolitica* is less frequent in mixed breeding-finishing herds than in fattening herds. Accordingly, purchase of animals from other herds, with an unknown carrier state of *Y. enterocolitica,* should be avoided [35].

The results presented in Nesbakken et al. [36] indicated that it is possible to establish clusters of pig herds (closed health and breeding pyramids) free from *Y. enterocolitica*, and to keep the herds free from this agent for many years. Important prerequisites for such a strategy are health and breeding pyramids of pigs free from pathogenic agents. In this context, some important factors include:

- well-organized trade with animals,
- fast and reliable diagnostics,
- efficient preventive measures at herd level,
- methods for elimination of human pathogenic agents within infected herds, and
- cost–benefit analyses.

Control in the abattoir

Because of the high prevalence of *Y. enterocolitica* in pig herds, strict slaughter hygiene is important to limit carcass contamination with *Y. enterocolitica* [18]. The results presented in Nesbakken et al. [18] indicate that it is important to modify procedures for removal of the guts in order to avoid contamination of the carcass by intestinal contents from the rectum. According to data from the Norwegian Surveillance System for Communicable Diseases (MSIS) (www.msis.no), the occurrence of human cases of yersiniosis in Norway decreased after the plastic bag technique was introduced into the abattoirs in Norway slaughtering pigs.

When pigs are slaughtered when they are 150–80 days of age, the tonsils constitute an important source of pathogenic *Y. enterocolitica* [37]. A preventive measure which is not implemented today is removal of the head as early as possible in the carcass dressing procedure. During such a procedure the head, including tongue and tonsils, should be removed and put onto a separate line.

Meat inspection

Compulsory meat inspection procedures for pigs, which involve incisions in the submaxillary lymph nodes in order to detect tuberculosis, represent a cross-contamination risk [17]. According to a report from EFSA (2011) visual inspection (with no palpations or incisions) is highly recommended.

Decontamination

Treatments of carcasses with hot water (>70 °C) or steam techniques to remove surface contamination may be an option for post-harvest interventions [38]. In general, such generic options can reduce the spread of enteropathogens and do not need expensive procedures such as testing of herds and categorization of animals. Today, the use of these decontamination techniques is implemented in the USA but seldom established in pig abattoirs in the rest of the world, but might significantly reduce or even eliminate the occurrence of pathogenic *Y. enterocolitica* on pig carcasses.

Control of milk and dairy products

Well-controlled pasteurization of milk is necessary to safeguard against *Y. enterocolitica* as well as many other pathogens. Measurement of time/temperature during pasteurization is important. But even handling and addition of ingredients after pasteurization might cause recontamination with *Y. enterocolitica* and other bacteria that have a growth potential at refrigeration temperature. This aspect should be focused on the Hazard Analysis Critical Control Point (HACCP) system of the dairies.

Drinking water and vegetables

Drinking water that hasn't been disinfected is an important risk factor for human yersiniosis [29]. It is therefore important: (i) not to drink from raw water supplies that are liable to contamination by animals; (ii) not to allow such water supplies to be used in food production; and (iii) to ensure that drinking water supplies are treated effectively so that *Y. enterocolitica*, and the multitude of other pathogens, are inactivated or eliminated.

A direct transmission route from manure-amended soil to plants and vegetables is through splashes of soil either from heavy rainfall or water spreaders. Application of manure after plants have come up, or after transplanting of seedlings, may also lead to contamination of the plants and is not recommended.

Animal contact

Avoidance of contact with excreta from pigs or domestic pets may reduce transmission. Domestic animals should be kept away from food preparation areas. Disposal of animal feces should be carried out in a sanitary manner.

Y. pseudotuberculosis

Some of the preventive measures against *Y. enterocolitica* are also valid against *Y. pseudotuberculosis*, in particular measures regarding vegetables, water, personal and kitchen hygiene, and animal contact.

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CHAPTER

Listeria 11

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Introduction

Listeria monocytogenes is a Gram-positive bacterium first identified by Murray et al. [1] in rabbits and guinea pigs. Following its early discovery, disease caused by this organism was rarely reported. Only after a Canadian outbreak in the early 1980s did the organism become widely recognized in the food industry [2].

Listeriosis is a generic term for a variety of syndromes caused by infection with *L. monocytogenes.* Invasive listeriosis has a high case-fatality rate (20–30%) among immunocompromised individuals, neonates, the elderly, and pregnant women [3]. The majority of human listeriosis infections arise via the consumption of contaminated food [4] and *L. monocytogenes* is a problem to the food industry due to its capability to survive and/or proliferate in diverse environmental conditions, including low pH, high salt, and low temperatures [5], which makes it difficult to control in food-associated environments. It has been estimated that the costs due to medical treatment and production losses associated with *L. monocytogenes* total $2.3 billion annually in the United States [6].

While listeriosis represents a rare foodborne disease with 3–5 cases of listeriosis per million population per year for most developed countries [7], many countries have developed policies that range from "zero tolerance" (no detectable level of viable pathogens in 25 g of foods) to specific limits, depending on the food item and the associated risk [8,9]. Listeriosis is likely to have a greater impact on society in the future due to the change in social and economic patterns, such as increasing numbers of susceptible individuals, especially the elderly, as well as increasing consumption of extended shelf-life and refrigerated foods. In this chapter, we summarize current knowledge of this important foodborne pathogen with a focus on exposure pathways of *L. monocytogenes* and prevention and control of listeriosis.

Microbiological characteristics of *Listeria* spp.

Listeria species are short, Gram-positive, non-spore-forming, facultative anaerobic rods [5]. They vary in size (0.4–0.5 in diameter by 1–2 μm long), have rounded ends, and are

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not encapsulated. They are motile by means of a few peritrichous flagella, with motility typically manifesting itself at ≤30 °C but not at 37 °C. *Listeria* species are able to grow at temperatures ranging from 0–45 °C [5]. Growth can also occur between pH 6 and pH 9, or in nutrient broth supplemented with up to 10% (w/v) NaCl [5].

At present there are eight recognized species that belong to the genus *Listeria*, namely *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L. marthii*, and *L. rocourtiae* [10]. Among them, *L. monocytogenes* and *L. ivanovii* have been identified to be pathogens of warm-blooded hosts. Although 13 *L. monocytogenes* serotypes have been recognized, most cases of human disease involve strains of three serotypes, i.e., serotypes 1/2a, 1/2b, and 4b [11]. DNA sequencing and enzyme electrophoresis studies have elucidated at least four evolutionary lines for *L. monocytogenes*, namely lineages I, II, III, and IV (Table 11.1).

Table 11.1 Summary of *L. monocytogenes* Lineages

Lineage	Initial Identification	Serotypes	Genetic Characteristics	Distribution
I	First described in an MLEE study by Piffaretti et al. [13]	1/2b, 3b, 3c, 4b	Lowest diversity among the lineages; lowest levels of recombination among the lineages	Commonly isolated from various sources; over-represented among human isolates
II	First described in an MLEE study by Piffaretti et al. [13]	1/2a, 1/2c, 3a	Most diverse, highest recombination levels	Commonly isolated from various sources; over-represented among food and food-related as well as natural environments
III	First described using partial sequence data analyses by Rasmussen et al. [14]	4a, 4b, 4c	Very diverse; recombination levels between those for lineage I and lineage II	Most isolates obtained from ruminants
IV	First described as IIIB using partial sequence data analyses by Roberts et al. [15]; first reported as lineage IV by Ward et al. [16]	4a, 4b, 4c	Few isolates analyzed to date	Most isolates obtained from ruminants

Adapted from Orsi et al. [12].

Lineages I and II represent the majority of isolates involved in human clinical cases (Figure 11.1) whereas lineage III and IV strains have been found less frequently among humans and are more common among animals [17]. This observation suggests a putative difference in the ecological distribution of lineage III and IV strains compared with lineage I and II strains. It is possible that lineages III and IV are best adapted to non-primate mammal hosts. An alternative explanation is that the low frequency of lineage III and IV isolates among humans is due to low prevalence of these isolates in food [16,18], and thus a low exposure of humans to these isolates. This low prevalence of lineage III and IV isolates in foods may be related to the fact that these isolates appear to be more susceptible than lineage I and II isolates to at least some of the stresses experienced in foods and food processing environments [19].

In most cases, infection by *L. monocytogenes* appears to be self-limiting, with clinical symptoms of invasive listeriosis appearing predominantly in the immunocompromised, the elderly, pregnant women, and neonates. However,

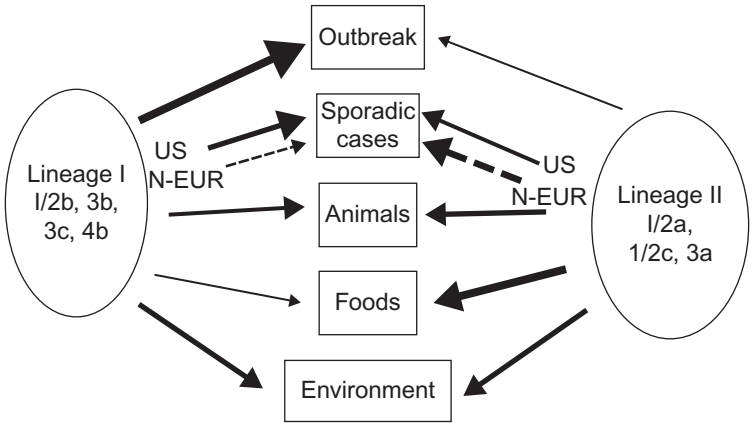

FIGURE 11.1 Distribution of *L. monocytogenes* lineages among different ecological compartments.

The thickness of each arrow represents the proportion of isolates belonging to each lineage among isolates obtained from a given ecological compartment (i.e., outbreak, sporadic cases, animals, foods, and environment). For sporadic cases, broken arrows indicate the proportion of isolates associated with cases in Northern Europe (N-EUR), where lineage II strains seem to be more common among isolates from sporadic cases than lineage I strains, while solid arrows indicate the proportion of isolates associated with cases in the USA (where lineage I strains seem to be more common among isolates from sporadic cases than lineage II strains). Lineage III and IV (previously designated IIIA/C and IIIB, respectively) are not represented in this figure, as isolates from these lineages are very rare among all ecological compartments. Lineages III and IV show the highest prevalence among animal isolates, but also have been isolated from human cases.

Adapted from Orsi et al. [84].

listeriosis has one of the highest case-fatality ratios of all foodborne bacterial infections. The pathogenesis of *L. monocytogenes* is well studied and key aspects of this research have been summarized in a number of reviews [20–25]. Importantly, *L. monocytogenes* is able to cross the gastrointestinal, placental, and blood–brain protective barriers. The development of listeriosis is typically initiated by ingestion of the organism, followed by its survival against the non-specific immune system defenses of the gastrointestinal tract. The organism is known to invade the intestinal epithelium or Peyer's patches allowing for crossing of the intestinal barrier. From there, the bacteria can enter the draining lymph nodes and disseminate via the bloodstream to the liver and spleen.

The intracellular cycle of *L. monocytogenes* infection is governed by multiple virulence factors such as internalin A and internalin B (encoded by inlA and inlB; facilitate host cell invasion), hemolysin (encoded by hly; facilitates phagosome lysis), phosphatidylinositol-specific phospholipase C (PI-PLC, encoded by plcA), phosphatidylcholine-specific phospholipase C (PC-PLC, encoded by plcB), and hexose-6-phosphate transporter (Hpt, facilitates intracellular growth), as well as actin polymerization protein encoded by actA, which facilitates cell-to-cell spread. The central transcriptional activator PrfA regulates the expression of many gene products that are required for bacterial virulence. Outside the host, PrfA exists in a low-activity state, with correspondingly low levels of virulence gene expression. Once inside the host, PrfA becomes activated and induces the expression of gene products that are required for infection [26]. Furthermore, there are overlapping and complementary interactions between the transcriptional regulations of PrfA and the alternative general stress response sigma factor, σ^B. One of the three promoters upstream of *prfA* (P2prfA) is σ^B dependent, indicating a direct regulatory link between σ^B and PrfA [27]. Some virulence genes (e.g., *lapB*, *bsh*, *inlA*, and *inlB*) are preceded by both PrfA boxes and σ^B promoters and appear to be co-regulated by PrfA and σ^B [27–29].

In the past few years, the genome sequences of more than 50 *Listeria* strains have become publicly available and at least 33 of them belong to *L. monocytogenes* (http://www.genomesonline.org and http://www.ncbi.nlm.nih.gov). The analyses of completely sequenced genomes should help resolve aspects of the pathogenesis of *L. monocytogenes*, i.e., shed further light on the set of genes required for the intracellular replication and cell-to-cell spread inside the host.

Nature of infection in man and animals

The majority (99%) of human listeriosis infections are foodborne [4]. Because initial contamination of food products usually occurs at low levels and the human infectious dose is high [25], post-contamination multiplication of *L. monocytogenes* (for example, in refrigerated foods) is usually required for the pathogen to reach levels that are high enough to cause human disease. The infectious dose of the

pathogen is also dependent on the type of food product, strain virulence, and host susceptibility [30].

Besides humans, *L. monocytogenes* can also naturally infect a large number of other vertebrate hosts. For example, it is responsible for a variety of clinical manifestations in ruminants, such as septicemia, meningitis, abortions, and mastitis [31]. Animal listeriosis is associated with the consumption of contaminated feedstuffs, mainly poor-quality silage [32]. Direct transmission from infected animals to humans is extremly rare.

L. monocytogenes can lead to mild illness (e.g., diarrhea and flu-like symptoms), usually in population groups that are not at increased risk, as well as severe invasive infections, chiefly in the elderly, immunocompromised persons, and pregnant women. Manifestations of invasive listeriosis in humans include meningitis, encephalitis and septicemia, spontaneous abortions and stillbirths, and infant septicemia and meningitis after the infection of pregnant women [33].

Risks of *Listeria monocytogenes* contamination

Listeria monocytogenes as a high risk in ready-to-eat (RTE) foods

Epidemiologic investigations of large outbreaks that have occurred since 1981 demonstrate that *L. monocytogenes* outbreaks are mainly associated with RTE foods which support growth of *Listeria* and are able to develop a high concentration of *Listeria* along the production and distribution chain [34–37]. Listeriosis cases are especially associated with processed RTE foods with a long shelf life that are kept at low temperatures because *L. monocytogenes* can grow at refrigeration temperatures [38]. The regulatory agencies have defined three conditions under which RTE foods do not support the growth of *L. monocytogenes*: (i) $pH \leq 4.4$; (ii) $a_w \leq 0.92$; and (iii) $pH \leq 5.0$ and $a_w \leq 0.94$. For RTE foods that fall into these categories, a microbiological limit of 100 colony-forming units (CFU) of *L. monocytogenes* per gram of RTE food throughout the shelf life has been proposed [8,35]. In the United States, RTE foods such as deli meats, frankfurters, unpasteurized fluid milk, smoked seafood, and cooked crustaceans have been identified as foods that have a high risk per serving for causing listeriosis [35].

Prevalence of *Listeria monocytogenes* in foods

Foods play a major role in *Listeria* infection and illness. Items from most food categories (i.e., meat products, dairy products, seafood, and produce) have been implicated in outbreaks of listeriosis.

RTE poultry and meat products

The contamination of RTE poultry and meat products with *L. monocytogenes* is a major concern for the food industry. To date, there have been multiple reported

outbreaks in North America and Europe of RTE meat products containing *L. monocytogenes* [39–41]. While contamination of raw poultry and meat products can originate from agricultural ecosystems [42,43] and abattoirs [44,45], these sources appear to have virtually no importance in transmission of human listeriosis, as human listeriosis cases are typically associated with RTE meat and poultry products that receive heat treatment during their production that effectively inactivates *L. monocytogenes* present on raw materials.

The *L. monocytogenes* contamination of RTE meat and poultry products that contributes to human listeriosis cases typically occurs post-processing in the processing plant or retail store [46,47]. Introduction of *L. monocytogenes* in RTE products is typically due to cross-contamination from environmental sites or, less commonly, cross-contamination between raw and RTE foods [45,48]. Persistent *L. monocytogenes* contamination of food contact surfaces or surfaces that facilitate effective transfer to RTE products appears to be the most important source of contamination of RTE meat and poultry products [49]. Efforts to control *L. monocytogenes* contamination in RTE meat and poultry product processing in the United States appear to have achieved considerable success, as the *L. monocytogenes* prevalence reported for these products by USDA-FSIS was approximately 0.3% in the United States in 2010, as compared with 2–5% in 1999 [50].

Seafoods

Outbreaks of listeriosis associated with RTE seafoods, such as smoked mussels, smoked trout, and smoked salmon, have been reported [51,52]. Additionally, the seafood category had the highest proportion of recalls of all the FDA-regulated foods that were recalled due to *L. monocytogenes* contamination from 1986 to 2006 [53]. In a study conducted by the European Food Safety Authority (EFSA) and European Center for Disease Prevention and Control (ECDC), the highest proportions of samples exceeding the legal safety limit of 100 CFU per gram in products on the market were observed in RTE fishery products, mainly in smoked fish in 2009 [103].

A range of seafood, such as smoked fish products (hot and cold smoked), lightly salted products (caviar and brined cooked shrimp), and marinated products, have a high risk of *L. monocytogenes* contamination [38]. These products have an extended shelf life at refrigeration temperatures, are capable of supporting the growth of *L. monocytogenes*, and are consumed without further cooking. As *Listeria* spp. are present in most aqueous ecosystems [54,55], water may be a source of contamination for fish and seafood. Nevertheless, since most RTE seafoods that have been linked to listeriosis outbreaks and cases have undergone listeriocidal heat treatment, contamination of raw materials has very limited public health relevance. Rather, cross-contamination of the RTE products, typically from environmental sources, represents the most important transmission pathway. Incoming *Listeria* on seafoods that are cold smoked and do not receive a listeriacidal heat treatment may represent a public health risk. However, while some studies have found evidence that linked contamination of cold-smoked salmon to contaminated raw materials [56,57], even

for this product most *L. monocytogenes* contamination appears to represent cross-contamination from environmental sources [58].

Milk and milk products

It is widely accepted that unpasteurized dairy products may carry a markedly elevated risk of causing listeriosis. Outbreaks in 2000 in North Carolina, in 2002 in Canada, and in 2003 in Texas highlight the risks posed by the use of raw milk in the manufacture of soft, unripened cheese [11]. However, products made from pasteurized milk that are contaminated after pasteurization represent a concern and have been involved in several outbreaks [59,60].

The occurrence of *L. monocytogenes* in raw milk has been linked with a number of on-farm factors [61]. Cows that consume contaminated feedstuffs can be infected by *L. monocytogenes*. Jensen et al. [62] proposed, as a conservative estimate, that infected cows could shed as many as 10,000 CFU per ml of *L. monocytogenes* in the milk. Such shedding would contribute to the contamination of the entire milk lot pooled in a tank.

The presence of *L. monocytogenes* in raw milk can lead to the contamination of cheeses made from unpasteurized milk. For example, Loncarevic et al. [63] in Sweden found that 42% of the cheeses made from raw milk were contaminated with *L. monocytogenes*, as compared with 2% of the cheeses made from heat-treated milk. *L. monocytogenes* can survive during the manufacture and ripening of certain cheeses and, if present initially, is likely to grow in the high pH and water activity of some soft cheeses such as Camembert [64]. Pasteurization is an efficient method to reduce or eliminate *L. monocytogenes* in milk. However, *L. monocytogenes* often occupies environmental niches in the dairy processing plant, and from there can contaminate the final product [65–67].

Produce

Fruit and vegetables initially were placed in a low-risk category with regard to their potential to cause foodborne listeriosis [35]. However, a number of recent outbreaks in the United States highlight the risk of listeriosis associated with fruit and vegetables, including an outbreak in 2011 associated with cantaloupe, an outbreak in 2010 associated with celery, and an outbreak in 2009 associated with sprouts [68].

Sources of contamination of produce include soil, water, inadequately composted animal manure, decomposing flora, and sewage effluents [69]. RTE vegetables may also be contaminated with *L. monocytogenes* from processing and handling procedures. *L. monocytogenes* may be as prevalent in precut prepackaged lettuce as in RTE turkey deli meat, a food categorized in the highest level of risk for acquiring foodborne listeriosis [70,71].

Quantitative microbiological risk assessment (QMRA) of *L. monocytogenes* in RTE foods

With regard to listeriosis, the overall goal of QMRA is to estimate the relative risks of serious illness and death associated with consumption of different types of RTE

foods that may be contaminated with *L. monocytogenes*, so that proper risk management strategies can be conducted to lower the risk of *L. monocytogenes* contamination. QMRA can be used to: (i) predict outcomes (i.e., effectiveness of an intervention in reducing listeriosis) to inform decision-making in public health affairs [3,72]; (ii) estimate the risk of certain foods in causing listeriosis [73,74]; and (iii) help processing plants to develop control strategies that can reduce the risk of listerial contamination in products [47,75].

Generally, the steps for QMRA are: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization (also see Chapter 3). As the first two steps of QMRA have been discussed earlier, this section will focus on steps (iii) and (iv). The relationship between risk management and these two steps is presented in Figure 11.2. The model includes an overview of factors that affect the risk of listeriosis to consumers from consumption of RTE meats.

The outcomes of QMRA are based on the risk assessment models developed, including the sources of data used, underlying assumptions, model equations, and techniques applied. QMRA of *L. monocytogenes* in RTE foods has revealed that the consumer risk of exposure to *L. monocytogenes* at the time of food consumption is dependent on: (i) amounts and frequency of consumption of RTE foods; (ii) frequency and levels of *L. monocytogenes* in RTE foods; (iii) potential of the food to support growth of *L. monocytogenes* during refrigerated storage; (iv) refrigerated storage temperature; and (v) duration of refrigerated storage before consumption [35,76,77].

Persistence of *Listeria monocytogenes* in processing environments

The common presence of *L. monocytogenes* in a variety of environments, combined with its tenacity, has made the control of this bacterium a vexing problem for the food industry and food safety authorities. Despite the implementation of intensive control measures, eradication and control of *L. monocytogenes* in food processing environments remains a considerable challenge. *L. monocytogenes* can be introduced into facilities from a variety of sources, e.g., when a small number of healthy carriers (beef, pork, or poultry) are introduced. *L. monocytogenes* strains can also colonize food processing niches such as rollers on conveyors, cracked tubular support rods, the space between close-fitting metal-to-metal or metal-to-plastic parts, worn or cracked rubber seals around doors, and on-off valves and switches [78], which represent an important source of contamination in the finished product [79]. Following introduction, *L. monocytogenes* has been shown to persist in manufacturing and processing plants from months to years [80,81] and this can become an important source of cross-contamination [46,82]. Lundén et al. [83] reported the transfer of a persistent strain of *L. monocytogenes* contamination from one plant to two others after the relocation of a dicing machine. Using full genome analyses, Orsi et al. [84] demonstrated that a specific *L. monocytogenes* strain persisted in a single processing facility over at least 12 years.

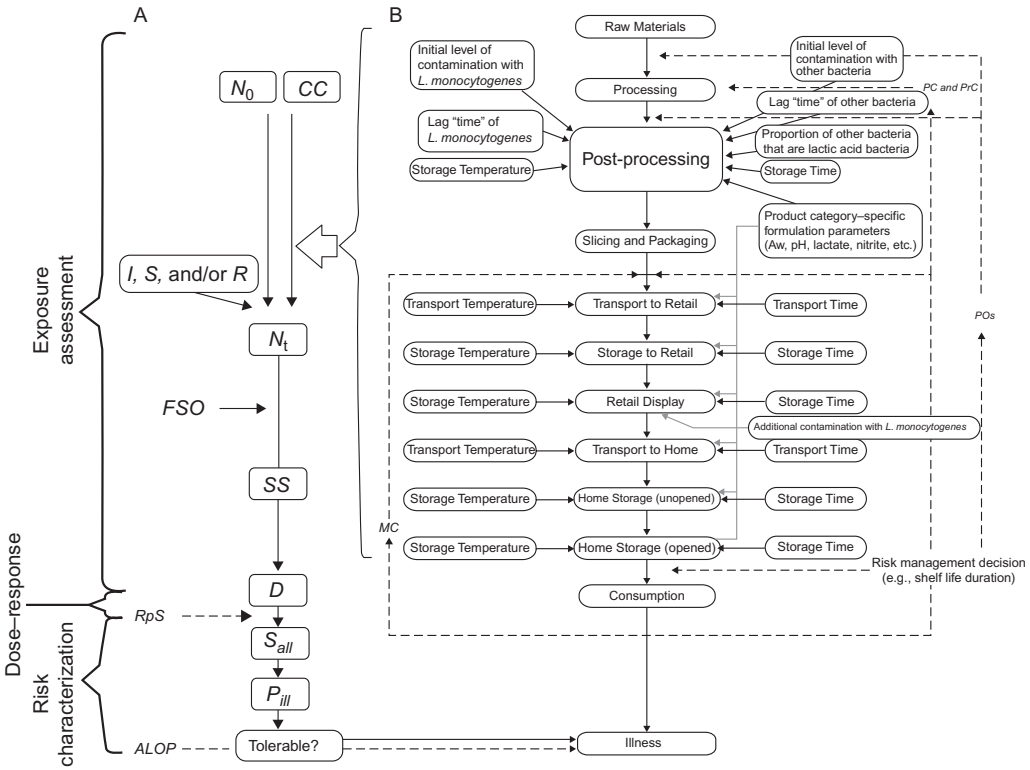

FIGURE 11.2 An influence diagram showing the overall structure of exposure assessment, dose–response, and risk characterization and their relationships with risk management.

In panel A, pathogen final concentration (N_t) is determined by initial contamination of products (N_0), potential cross-contamination during and/or after processing (*CC*), increase (*I*), survival (*S*), and/or reduction due to inactivation (*R*) during processing. Ideally, N_t should be no greater than the Food Safety Objective (FSO). Dose (*D*) consumed at the time of consumption, which is the final population (N_t) multiplied by the serving size (*SS*) consumed, combined with a dose–response model provides the risk per serving (*RpS*). The risk is converted into probability of illness (P_{ill}) or number of cases based on the total number of servings consumed in a year (S_{all}). The final risk is compared to the appropriate level of protection (*ALOP*). To meet the FSO, it is necessary to establish performance objectives (POs) [maximum frequency of occurrence (%) and/or concentration (cfu/g) of a pathogen], performance criteria (*PC*) [change (i.e., reduction or tolerated increase) in frequency of occurrence and/or concentration of a pathogen that should be achieved during processing or implementation of control measures], and process and product criteria (*PrC*) (conditions required to achieve the desired *PO*/*PC*, e.g., time–temperature combination) prior to consumption. Finally, compliance with *PO*/*PC*, and consequently with *FSO*, is verified by the application of microbiological criteria (*MC*) (level and/or frequency of occurrence of a pathogen detected by the implementation of specific analytical method and sampling plan). In panel B, bolded boxes represent the process that may exist to get the raw material to human consumption. Light-colored boxes represent factors associated with each process.

Adapted from Ross et al. [76] and Mataragas et al. [75].

Prevention and control of listeriosis

Detection of *Listeria* spp. and *L. monocytogenes*

Traditionally, food laboratories have relied on conventional culture-based microbiological methods for detection of *Listeria* spp. and *L. monocytogenes*. Chromogenic media such as "Agar *Listeria* according to Ottaviani and Agosti" (ALOA) [85,86] allow for the direct differentiation of *L. monocytogenes* from other *Listeria* spp. Standard methods of isolation of *Listeria* spp. from food and environmental samples used by regulatory agencies have been reviewed by Gasanov et al. [87]. The major disadvantage of conventional culture-based methods is that they are laborious and time-consuming. In addition, differentiation of *L. monocytogenes* from other *Listeria* species can be challenging.

Alternative methods, including immunoassay-based—e.g., enzyme-linked immunosorbent assays (ELISA)—and nucleic acid-based rapid detection methods, are now being widely used by the food industry. These tests are relatively fast, high-throughput, simple and easy to interpret, and require minimal treatment of the sample. The disadvantage of immunoassays is that they are dependent on antigens which vary in expression with changing environmental conditions, making them less reliable compared with nucleic acid-based detection methods [87,88]. Cross reactivity can also occur if antigens are shared between closely related species, e.g., *L. monocytogenes* and *L. innocua*, which leads to false positives [88]; this is one of the reasons why antibody-based assays are less commonly used for the detection of *L. monocytogenes*.

Compared with conventional and immuno-based methods, nucleic acid-based methods are less time-consuming and more sensitive. Nucleic acid-based methods, especially PCR-based assays, are being adopted in more and more commercially available detection systems. Various genes such as *hly* (the most common target), *inlA*, *inlB*, *iap*, intergenic spacer regions, genes encoding invasion-associated protein p60, aminopeptidase C, and phospholipase C protein have been targeted for detection of *L. monocytogenes* [88]. Real-time (quantitative) PCR allows the amplicon to be observed as it accumulates, in contrast to conventional PCR where the resulting product is observed at the end of the reaction. The progress of a real-time PCR is measured by monitoring the change in fluorescence levels, which in turn depends on the amount of the accumulated PCR product. Real-time PCR has advantages such as increased sensitivity, the ability to detect trace amounts of target DNA, possible automation, and the ability to quantify bacterial load without any post-PCR handling. Currently, a number of PCR-based detection systems for *Listeria* spp. and *L. monocytogenes* are available, including real-time systems such as the TaqMan real-time PCR, molecular beacons, and scorpions [89]. For example, a TaqMan real-time quantitative PCR assay targeting *iap* was able to distinguish *L. monocytogenes* from other bacterial species in five food matrices, including whole milk, soft cheese, turkey deli meat, smoked salmon, and alfalfa sprouts [90].

Subtyping of *L. monocytogenes*

Subtyping and rapid identification of *L. monocytogenes* are important for detecting outbreaks, identifying outbreak sources, and identifying contamination sources throughout the food chain. Multistate foodborne outbreaks create a complex situation for epidemiologists as the cases occur in different areas and may be separated over a considerable time interval. Molecular subtyping helps in timely tracing of the source of the original contamination and its elimination, which can prevent additional cases of listeriosis [91]. Molecular subtyping methods for *L. monocytogenes* can be differentiated into: (i) band-based methods, based on fragment pattern data or DNA fingerprints, and (ii) methods that generate DNA sequence data.

Pulsed field gel electrophoresis (PFGE) is a commonly used band-based method for routine molecular subtyping of *L. monocytogenes*. Since 1999, exchange of listerial PFGE patterns through PulseNet, a network coordinated by the CDC, has been an important tool in *L. monocytogenes* surveillance and listeriosis outbreak identification. For example, PulseNet was used to investigate an outbreak associated with pasteurized milk in 2007 [92].

Subtyping is also used in source tracking of contamination in food processing environments, which is very important for developing control strategies [93]. DNA sequencing-based methods such as multilocus sequence typing (MLST) have lower discriminatory power compared with PFGE in some cases. For example, MLST has limited discrimination power on differentiating serotype 4b isolates and epidemic clones, which can be differentiated by PFGE [94]. However, MLST provides excellent assessment of genetic relatedness between strains and therefore is useful for studies in phylogenetic analysis, evolution, and population biology. For example, a study conducted by den Bakker et al. [95] focused on studying the occurrence, ecology, and phylogenetic relationships of outbreak-related strains including previously described epidemic clones obtained from 15 listeriosis outbreaks in North America and Europe. Using housekeeping genes (*gap*, *prs*, *purM*, and *ribC*), virulence genes (*inlA* and *actA*), and a stress response gene (*sigB*), MLST yielded eight sequence types (STs) for the outbreak strains. Information obtained from this type of study is helpful in identifying *L. monocytogenes* subtypes that have been closely associated with human listeriosis outbreaks worldwide. MLST is also one of the most accurate and simplest procedures for inter-laboratory comparison. As a DNA sequencing-based approach, MLST results can be efficiently reproduced among different laboratories and yield reliable and unambiguous data [96].

Recently, the whole-genome data provided by completed and ongoing DNA sequencing projects have stimulated efforts to develop highly discriminatory and high-throughput DNA sequence-based subtyping methods for *L. monocytogenes* [97]. Further efforts are needed to better understand how this approach can be applied in a routine setting.

Prevention and treatment of listeriosis

Guidance for controlling *L. monocytogenes* in food processing environments has been reviewed by Tompkin [78]. In food processing environments, combinations of

interventions appear to be more effective than any single intervention in mitigating the potential contamination of RTE products with *L. monocytogenes* and reducing the subsequent risk of listeriosis. Interventions may include several aspects such as: (i) eliminating post-processing contamination (PPC) by using lethal or post-lethality treatments and/or growth inhibitors to control contamination by *L. monocytogenes* after processing [49]; (ii) preventing in-plant contamination, e.g., by performing good hygiene and manufacturing practices, testing and sanitation of food contact surfaces, and pre- and post-packaging interventions; and (iii) instituting controls on storage temperatures and periods. Because foodborne listeriosis outbreaks are often associated with high case-fatality rates, it is imperative that effective procedures are used for prevention in high-risk populations. Currently, no vaccines are available for humans. Science-based education messages targeted to susceptible populations and their caregivers are helpful in reducing foodborne listeriosis [98].

Current treatments for humans with invasive listeriosis involve a combination of ampicillin or penicillin G with gentamicin or another aminoglycoside [99]. Two to three weeks of therapy appear to be sufficient to prevent relapses. Most β-lactam antibiotics may be used. The first multi-resistant strain of *L. monocytogenes* was isolated in France in 1988 [100]. Since then, other strains of *L. monocytogenes* isolated from food or the environment, or in sporadic cases of human listeriosis that were resistant to one or several antibiotics, have been reported [101]. A study of *L. monocytogenes* isolates collected between 1955 and 1997 in the USA revealed that susceptibilities of clinical isolates to penicillin, ampicillin, erythromycin, tetracycline, and gentamicin remained unchanged while a high prevalence of clindamycin resistance was observed [102].

Concluding remarks

It is critical to develop knowledge-based intervention strategies to control *L. monocytogenes* in the RTE food chain to reduce the risk of causing foodborne listeriosis. In the past decade, control efforts have led to a decreased annual number of hospitalizations and deaths associated with *L. monocytogenes* and a reduced prevalence of *L. monocytogenes* in the food chain [4,50]. A better understanding of the ecology and biology of *L. monocytogenes*, with a focus on virulence and stress response systems that facilitate transmission through diverse environments encountered by *L. monocytogenes*, will further facilitate the control of this important foodborne pathogen.

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CHAPTER

Shigella 12

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Introduction

Shigella is estimated to cause 165 million illnesses annually worldwide and is the third most commonly isolated bacterial enteric pathogen in the United States [1,2]. Illness can range from mild diarrhea to potentially fatal dysentery, depending on *Shigella* species and host factors. Secondary infections are common due to the low infectious dose. Because humans and other primates are the only natural reservoirs for *Shigella* and a shigellosis vaccine is not available, rigorous human hygiene practices are the cornerstone of prevention of foodborne transmission.

Clinical features of illness associated with infection

The clinical features of shigellosis vary from mild diarrhea lasting a few days to an acute febrile illness that may include nausea, vomiting, tenesmus, and bloody stools. Symptoms begin 1–4 days after infection and typically last 4–7 days; they are usually self-limited and infrequently require hospitalization. The very young, elderly, and immunocompromised are at higher risk of severe illness, which can include toxemia and neurotoxicity. Seizures may occur, particularly in children under 2 years of age. Mild cases of shigellosis are often undiagnosed and not treated; asymptomatic infection is also possible. Once infected, individuals are not likely to get infected again with the same species for several years.

Disease severity also varies by *Shigella* species. *S. sonnei* infections often produce a short and uncomplicated clinical course. *S. dysenteriae* type 1 produces Shiga toxin and infections can result in hemolytic-uremic syndrome, toxic megacolon, and intestinal perforation; it can cause deadly epidemics where sanitation is poor. Persons infected with *S. boydii* or *S. flexneri* often experience bloody diarrhea

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and fever; those with *S. flexneri* infection, especially individuals with the HLA-B27 antigen, can also develop post-infection arthritis.

Shigella is highly infectious, with 10–200 cells sufficient to cause illness [3]. Because shigellosis is widespread and the infectious dose is so small, much of the population is at risk of illness if good hygiene practices are not strictly observed. In the United States and other industrialized settings, illness is most common among children in daycare or elementary school and their caretakers. Children aged 1–4 years have the highest rates of infection, with a range of 12.5–28.3 cases per 100,000 population per year [4]. Caretakers of young children are therefore at increased risk. Surveillance data indicate that more cases occur among women than men overall, with a range of 51–53% of cases among women. The female predominance is most apparent among young women. Among persons 20–29 years of age with infections due to *S. sonnei*, 70–75% are female [5].

Secondary transmission has been estimated at 30% in households with small children [4]. Others at increased risk of shigellosis include people with recreational water contact, international travelers (who are most likely to be infected with non-*S. sonnei* species), close-knit religious populations in which hygiene may be compromised, and men who have sex with men [4,6,7]. The number of reported outbreaks and overall case counts in the United States increases in the late summer months [5]. The number of reported foodborne shigellosis outbreaks does not appear to have similar seasonality [17].

Microbiology

Shigella is named after Dr. Kiyoshi Shiga, a Japanese scientist who discovered the bacteria in 1897 during a severe seasonal dysentery epidemic in which more than 90,000 cases were estimated, with a mortality rate >20%. Shigellae are Gram-negative, facultatively anaerobic, non-spore-forming, non-motile bacilli related to *Escherichia coli*. There are four species of *Shigella*: *S. dysenteriae* (group A, 15 serotypes); *S. flexneri* (group B, 8 serotypes), *S. boydii* (group C, 19 serotypes), and *S. sonnei* (group D, 1 serotype). Virulence is determined by a large plasmid essential for invasion of the colonic epithelium. Shigellae enter epithelial cells, multiply within the cytoplasm, and spread to other cells, resulting in tissue death, bleeding, and an intense inflammatory response. *S. dysenteriae* type 1 and some *E. coli* strains produce Shiga toxin which is associated with hemolytic-uremic syndrome and other severe, sometimes fatal, clinical manifestations.

Isolation of *Shigella* from patients is generally through culturing stool samples or rectal swabs. Chances of successful isolation are improved by prompt culturing and careful handling of samples, since *Shigella* has limited ability to survive outside the human body. It is optimal to use two different selective media such as low-sensitivity MacConkey (MAC) agar with high-selectivity xylose lysine desoxycholate (XLD) agar or S/S agar [8]. However, S/S agar is not appropriate for isolation

of *S. dysenteriae* type 1, as it inhibits growth of the organism. A rapid test for Shiga toxin is useful specifically for *S. dysenteriae* type 1. Food samples to be cultured for *Shigella* are usually enriched in GN broth and plated onto selective media such as XLD agar or Hektoen enteric agar or an agar medium containing antimicrobial agents if the resistance pattern of the outbreak strain is known. PCR may be used to screen enrichment broths for *Shigella* virulence genes such as *ipaH*. Although studies have documented that *S. flexneri* and *S. sonnei* are able to survive in certain foods for several days, the agent is rarely isolated from the incriminated food vehicle in outbreaks, possibly because it is often present in low numbers [9].

Exposure pathways

Humans and higher primates are the only known hosts of *Shigella*. Transmission occurs by the fecal-oral route. The organism does not persist long-term in the environment, but it can survive in foods at ambient or refrigerated temperatures in sufficient quantities to cause illness for the duration of the shelf life of some foods [10–12]. Chronic carriers are rare.

Shigellosis is common and most cases likely result from person-to-person transmission. However, approximately 130,000 cases are estimated to result from foodborne transmission in the USA each year [2]. Although surveillance data in the developing world are limited, outbreaks of foodborne shigellosis have been reported in India and South Africa, and in developed countries, due to food imported from less industrialized countries [13–16]. Investigations of shigellosis have implicated food handlers in many settings and circumstances, and transmission via a range of food vehicles. Reported foodborne outbreak settings include restaurants, private homes, institutions such as prisons and schools, and festivals. Widely geographically distributed outbreaks with large case counts have also been associated with centralized processing of commercial foods. Attributing shigellosis transmission to food or other vehicles can be challenging, and mixed transmission is likely in most outbreaks.

Secondary infections are quite common with shigellosis, so discerning foodborne transmission from a larger community outbreak can be difficult unless the epidemiological link to food is strong (such as a large number of people exposed to a point source). For example, caretakers of children in daycare centers may be at increased risk for exposure to *Shigella*, and these same caretakers also serve as food handlers in the home or commercial settings. Concurrent community propagation is supported by a geographic focus of cases and extended duration of occurrence. This was likely the case in a foodborne outbreak reported in a US state in 2003 with almost 1000 estimated cases in several adjacent counties [17]. Cases of shigellosis that are not associated with a known outbreak (often referred to as "sporadic") account for the majority of the disease burden. Haley et al. [18] estimated that one-third of sporadic cases could be attributed to foodborne exposure after eliminating exposure to other select risk factors.

Shigella is introduced into food by infected food handlers or other contamination from human activities, which may occur at many stages of food production.

Factors that frequently contribute to transmission include inadequate hygiene by infected food handlers, improper food storage temperatures, and unsanitary conditions at food production facilities. An example of contamination early in the production process was seen in a multinational outbreak associated with parsley [19]. The outbreak was traced to a farm in Mexico that used unchlorinated water, which is easily contaminated, in a recirculating hydrocooler and in ice used to keep produce cool during shipping. This practice permitted contamination of many batches of product. Similar environmental factors were noted in investigations of *S. sonnei* infections associated with baby corn processed in Thailand and transported to Denmark and Australia for consumption [15]. Transmission through commercially prepared, centrally distributed foods is infrequently reported, but such vehicles have been associated with some of the largest numbers of cases and most widespread geographic distribution. For example, in a nationwide outbreak of 406 *S. sonnei* illnesses associated with ready-to-eat five-layer dip, an infected food worker processed cheese using a machine that was difficult to clean [20]. This illustrates the importance of a single vulnerable point in production.

Contamination close to the time of serving is more common. Restaurants are a frequently reported setting in investigations of shigellosis outbreaks, and infected food handlers are often identified. Risk increases when infected employees handle large volumes of ready-to-eat foods or foods eaten raw, as occurred during the largest reported foodborne outbreak of *S. flexneri* infections in the United States. In this outbreak, "special grade" tomatoes were shared among several related restaurants with inadequate hygiene practices (e.g., there was bare-handed contact with food, lack of proper hand washing facilities) [21]. Additionally, damaged produce, such as these tomatoes, may permit increased survival of the pathogen.

Transmission through foods prepared in private settings such as homes and church events is less likely to be detected, reported, or investigated than in regulated commercial settings but is likely to result from similar risk factors. Foodborne shigellosis outbreaks have also been reported in institutional settings such as prisons and schools, on airplanes and cruise ships, and at festivals; attack rates can be high during outbreaks in these settings [17,22,23,24]. *Shigella* survives well in refrigerated foods, such as salads and parsley, but a wide variety of foods have been implicated in the transmission of *Shigella*. As described in the outbreaks above, circumstantial factors can amplify spread, disperse cases geographically, or complicate attribution to specific foods, but since there is not a food-animal reservoir, the human point of introduction remains central in the transmission of foodborne shigellosis.

Prevention and control

Impeccable hand washing and hygiene practices are the surest way to prevent shigellosis, because many foods are susceptible to contamination with *Shigella* and no food-animal reservoirs exist. Policies and procedures to ensure proper hygiene and

sanitation during the harvesting, production, distribution, and preparation of food will reduce foodborne transmission of *Shigella* and many other enteric pathogens. Paid sick leave, adherence to ill-worker exclusion policies, and other means of discouraging food workers from working while ill will reduce the chances that infected food handlers will expose others to foodborne pathogens, including *Shigella*.

Methods used to prevent *Shigella* in community settings are an important way to reduce the chances that others (e.g., caretakers of children) will become infected and subsequently contaminate food. Established methods of preventing transmission of shigellosis in daycare settings include: providing easy access to fully stocked and operational hand washing stations for children and child care staff; supervising and assisting young children during hand washing; scheduling hand washing at key times throughout the day; separating diapered children from those who are not; eliminating water play areas such as sensory tables and kiddie pools; and ensuring that child care workers who change diapers are not also preparing or serving food in the child care center or in restaurants.

Additionally, high-quality disease surveillance systems are essential for assessing the impact of any control measure. Several national surveillance systems provide valuable data on the incidence of *Shigella* infection in the United States; some of these systems also describe foodborne exposures (http://www.cdc.gov/ncezid/dfwed/PDFs/Shigella-Overview-508.pdf). Trends in shigellosis in the United States can be followed through reports that are routinely published using data from these systems. Efforts to build surveillance capacity in developing countries and support foodborne disease surveillance and epidemiology programs in developed countries will improve our understanding of foodborne transmission of *Shigella* and assist prevention efforts.

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CHAPTER

Streptococcal Disease 13

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Introduction

Streptococcal species, particularly those in Lancefield group A (*Streptococcus pyogenes*), are widely recognized as a major bacterial cause of pharyngitis and tonsillar infections. While most transmission is via a respiratory route (or via fomites contaminated with respiratory droplets or through infected skin lesions), foodborne transmission of pharyngitis is well recognized [1–7]. Until the 1940s, foodborne transmission was most commonly attributed to milk and milk products. Since that time, with the advent of near universal pasteurization and better handling procedures, transmission via milk has become rare, with foodborne outbreaks now linked primarily to contamination of food by infected food handlers. Estimating total disease burden in the United States is difficult, as foodborne cases are seldom recognized unless they are part of a large outbreak. Scallan and colleagues at CDC, in their estimates of numbers of cases of foodborne illness due to specific pathogens [8], reported an estimated mean of 11,217 cases of foodborne streptococcal disease in the United States annually; however, this was also accompanied by a very large 90% creditable interval of 15–77,875 cases annually, reflecting the substantial uncertainty inherent in this estimate. In a recent tabulation of food handler-associated outbreaks [9], there is a suggestion that the number of foodborne outbreaks associated with streptococci has been decreasing over time.

Clinical presentation

Streptococcal pharyngitis

Pharyngeal infections due to group A streptococci generally have rapid onset, with sore throat, tonsillar exudate, tender cervical adenitis, and fever; cough and runny nose are usually absent [10,11]. However, only a relatively small proportion (probably 10% or less) of adult cases of pharyngitis are due to group A streptococci; most cases can be attributed to viral etiologies, with a few representing infection with other bacterial species. Diagnosis requires a throat culture, with the likelihood of a

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positive culture exceeding 50% among adult patients with the combination of fever, tonsillar exudate, tender cervical lymph nodes, and no cough ("Centor criteria" [10]).

Illness is usually self-limited, lasting 2–5 days in untreated patients. However, a small percentage of patients develop potentially serious post-infection sequelae, including rheumatic fever and glomerulonephritis. Occurrence of sequelae tends to be linked with specific strain subsets, which have been linked with certain M types in rheumatic fever, and with production of key nephritogenic antigens in glomerulonephritis. Available data suggest that incidence of these complications is decreasing in the United States; this may relate to increased use of antibiotics in acute cases, and/or shifts in strain populations.

Treatment of group A streptococcal infections is recommended: (i) to reduce severity and duration of clinical signs and symptoms (although antibiotics probably only reduce duration of symptoms by no more than 1–2 days in uncomplicated cases); (ii) reduce the risk of transmission to close contacts (which has been estimated to occur in 35% of close family or school contacts); and (iii) reduce the risk of post-infectious sequelae. Penicillins, cephalosporins, and macrolides are effective in treatment of streptococcal pharyngitis; 10 days of therapy is recommended [12].

Strains in streptococcal Lancefield groups C and G have also been implicated as a cause of pharyngitis, but do not appear to trigger rheumatic fever or other post-infectious sequelae. Because of the lack of post-infectious complications, indications for treatment are not as strong as those for group A streptococci. In general, however, treatment is undertaken to reduce severity and duration of clinical signs and symptoms.

Foodborne streptococcal disease

Persons infected with group A streptococcal strains acquired from foodborne sources present with symptoms of pharyngitis that are virtually identical to those reported among persons who have acquired the infection through a presumed respiratory route. Such patients may occasionally report diarrhea and other gastrointestinal symptoms. It is unclear, however, whether this reflects the direct effect of the streptococcal infection, or is related to other possible pathogens, particularly as mishandling of food has been implicated as a factor in a number of outbreaks (see discussion of transmission pathways, below). The sequelae of post-streptococcal rheumatic fever and glomerulonephritis have not been reported with foodborne infections. If, as has been suggested, there are certain streptococcal subtypes (as defined by multilocus sequence typing [MLST]) that are more likely to be transmitted via a foodborne route [7], these same strain groups may also have a reduced propensity for causing autoimmune sequelae.

Outbreak reports have almost all focused on occurrence of pharyngitis caused by group A (or occasional group C or G) strains. However, there are reports of invasive infections, including sepsis and meningitis, caused by other streptococcal groups/species, where food and/or animal contact have been mentioned as possible

sources for the microorganism [13]. Such cases are, at best, rare, and may be more likely to occur among persons who are immunocompromised or have other chronic diseases.

Microbiology

Classification of streptococci remains complex, due in part to persistence of historical (but still useful) schemes for categorization of strains [14]. Early classification schemes dating to the 1930s focused on behavior of blood-containing agar (hemolytic activity, characterized as α-hemolysis [causes greenish discoloration of agar around colonies], β-hemolysis [complete hemolysis of red cells, with a zone of clearing around colonies], and γ-hemolysisn [no hemolytic activity]), and on serologic response to cell wall antigens (Lancefield groups [15]). Over the subsequent years (and, in particular, with the advent of molecular classification systems), it has become obvious that the various categories in each of these schemes contain multiple species; however, based on their clinical utility, these classifications continue to be used.

Clinically significant β-hemolytic strains in Lancefield group A are almost all within the species *S. pyogenes*. These are the strains linked with "strep throat" pharyngitis, and are the focus of the current chapter. Strains may be further characterized based on M-protein type, and by various molecular approaches, including MLST; there is at least a suggestion that foodborne *S. pyogenes* strains cluster together based on MLST type [7]. *S. agalactiae* are in Lancefield group B and may have β- or γ-hemolysis. These strains, which may colonize the vaginal tract and are an important cause of neonatal sepsis, have not been linked with foodborne transmission routes. *S. dysgalactiae* subsp. *equisimilis* and members of the *S. anginosus* species group may fall into Lancefield groups C or G, with the possibility of α-, β-, or γ-hemolysis; as noted above, some of these strains may be transmitted by food.

Exposure pathways

Introduction of group A streptococci/*S. pyogenes* into the food chain may be by respiratory droplet (transmitted to the food by coughing or sneezing), or from infected skin lesions. It appears likely that growth in a contaminated food product is necessary to reach an appropriate infectious dose. It is not uncommon for outbreaks to have had identifiable deficiencies in food handling practices, including preparation of food items well in advance of consumption, and subsequent inadequate refrigeration. Interestingly, there appears to be a slightly increased risk of egg and egg-containing products serving as vehicles for transmission of the microorganism in reported outbreaks, with a suggestion that eggs may serve as a particularly good growth medium for streptococci [3]. There is, however, a wide range of foods that

have been implicated in outbreaks (particularly "complex" foods that require more handling, providing more opportunities for contamination), and it is likely that virtually any food can serve as a vehicle if there is heavy enough contamination by an infected food handler.

While the primary source of infection in an outbreak may well be food, secondary transmission by infected persons via a "traditional" respiratory route is well recognized. In outbreaks, it is not at all uncommon to have a "ripple effect," with secondary cases reported within households or among persons with close personal contact with infected individuals [3,16].

Prevention and control

For the developing world, where pasteurization may not be universal (and for persons who insist on consuming raw milk), care must be taken to avoid collection of milk from animals with mastitis. However, for the developed world, this is a food handler disease: Prevention is dependent on assuring that persons preparing foods that will not be further cooked or processed are not carrying the microorganism. The greatest risk would appear to be individuals who have an acute pharyngeal infection, with cough, sneezing, and runny nose; or persons with an active skin infection. Ideally, such persons should be excused from work until the infection is resolved. At a practical level, however, this may be difficult, as symptoms of infection, particularly respiratory infections, may be non-specific. To minimize risk, any food handler with an active upper respiratory infection should be required to wear a surgical mask (which reduces the risk of droplet transmission). Active skin infections, which may reflect staphyloccal or streptococcal infections, should always be a contraindication to handling food; requirements that all employees wear gloves may help to mitigate this risk in cases where infections are not immediately obvious.

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CHAPTER

Aeromonas and *Plesiomonas*

14

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Introduction

Although the primary role of *Aeromonas* and *Plesiomonas* in gastroenteritis is still debated, and the incidence of gastroenteritis associated with these organisms is typically low, the ability to cause septicemia with a high mortality rate and a number of other extraintestinal infections is of concern. Blood-borne infections of *Aeromonas* spp. are particularly problematic due to a poor association of gastrointestinal symptoms with septicemic cases and resistance to first line antibiotics. Consumption of raw and undercooked seafood and foreign travel are the major risk factors for infections from these organisms; however, their ubiquitous distribution in water systems causes introduction into a large majority of food commodities.

Clinical features

Gastroenteritis

The primary disease associated with *Aeromonas* spp. and *P. shigelloides* is gastroenteritis. For *Aeromonas* spp., the severity of the disease is variable, ranging from mild, self-limiting, watery diarrhea to a rare, more dysenteric-like syndrome with bloody, mucus-containing, frequent stools, abdominal pain, and fever [1,2]. Most diarrheal patients infected with *P. shigelloides* exhibit invasive symptoms similar to colitis; i.e., bloody stools containing mucus and polymorphonuclear leukocytes, and present with cramping, vomiting and dehydration [3]. Rate of incidence of gastroenteritis due to *Aeromonas* spp. is routinely 2–4% of diarrheal patients, although slightly higher in children and higher in developing countries [4]. Less is known about the incidence of *P. shigelloides* gastroenteritis, but one study reported a rate similar to that seen for *Aeromonas* [5]. Cases with prolonged diarrheagenic episodes have been reported for both organisms, up to 7–10 days for *Aeromonas* spp. and as long as 3 weeks for *P. shigelloides* [6]. Rare cases of chronic diarrhea have

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been reported due to both organisms, as well as hemolytic-uremic syndrome, renal impairment, and relapsing diarrhea as sequelae for *Aeromonas* [7].

Fecal carriage rate of *Aeromonas* spp. can be as high as 4–8% in asymptomatic humans. The carriage rate of *P. shigelloides* in healthy individuals is usually lower (less than 1%), although higher in developing countries. Other pathogens are commonly isolated from stools of patients in which *Aeromonas* spp. are isolated, while this is usually not the case with *P. shigelloides*.

Extraintestinal infections

In addition to gastroenteritis, *Aeromonas* spp. and *P. shigelloides* can be involved in an impressive myriad of extraintestinal infections, including septicemia, cellulitis, urinary tract infections, peritonitis, meningitis, conjunctivitis, endocarditis, pneumonia, arthritis, endophthalmitis, and cholecystitis [2,6]. These invasive infections can quickly develop into bacteremia and sepsis, and be life-threatening without aggressive treatment. The predominant route of entry for septicemia infections is translocation from the gastrointestinal tract to the circulatory system, although wound infections and traumas have also been implicated, especially those incurred during water-based recreational activities or exposure [8,9].

Host susceptibility

A higher incidence of gastroenteritis infection with *Aeromonas* is seen in developing countries, suggesting sanitary conditions as a significant risk factor. Further, *Aeromonas* spp. have been reported as an important causative agent of travelers' diarrhea (TD), ranked only behind enterotoxigenic and enteroaggregative *E. coli* (ETEC, EAEC) and *Shigella* spp. [10,11]. Indeed, the most common risk factor of gastroenteritis from *Aeromonas* for healthy adults from developed countries is travel. *P. shigelloides* is also a causative agent of TD, particularly associated with travel to Southeast Asia [12]. Children are at particular risk for acute, watery diarrhea. *Aeromonas* spp. have been reported as the primary etiological agent for as high as 15–20% of children with diarrhea, but the prevalence is usually lower, i.e., 5–10% [13,14]. In developed countries, incidence of *Aeromonas* infection is highest among the elderly and immunocompromised [15]. Further, elderly patients have been shown to be at higher risk to develop chronic enterocolitis from *Aeromonas* gastroenteritis [16].

Septicemia from *Aeromonas* spp. is most commonly associated with underlying hepatic, biliary, and pancreatic disease, and malignancy [2]. The mortality rate is 30–60%, due to few infected individuals (approximately 10%) experiencing diarrheal symptoms at the time of or preceding the invasive disease, lack of clinical features which indicate *Aeromonas* spp. as the causative agent, and resistance to antibiotics routinely used as a first course to treat septicemia [2].

P. shigelloides infections are reported among all age groups; however, gastroenteritis and extraintestinal infection incidence rates are higher in immunocompromised patients [17]. Septicemia from *P. shigelloides* is almost always preceded by

symptomatic gastrointestinal illness, with deep wound infections as a rare port of entry for systemic disease. Neonatal infants are at risk of meningitis from *P. shigelloides*, being passed from the infected mother during childbirth.

Microbiology

Members of the genera *Aeromonas* and *Plesiomonas* are characterized as gram-negative, facultatively anaerobic, oxidase-positive bacilli, which ferment glucose. Historically, both groups were previously members of the family Vibrionaceae based on similarities of phenotypic traits and shared ecological niches between each other and the marine Vibrios. Genetic evidence, however, has established *Aeromonas* in a separate family, Aeromonadaceae, distinctly removed from the closely related Vibrios and Enterobacteriaceae [18,19]. Likewise, the genus *Plesiomonas* was been reclassified as a member of the Enterobacteriaceae, based on phlyogenetic studies [20].

Aeromonas

Aeromonads can be further described as catalase-positive, able to reduce nitrate to nitrite, producing gas from glucose fermentation, and possessing a single polar flagellum; several members also produce lateral flagella. Typically, the group is divided into two groups: non-motile psychrophiles such as *A. salmonicida*, which are pathogenic to fish and cold-blooded animals; and motile mesophiles, which include human pathogens, such as *A. hydrophila*. Historically, the taxonomy of *Aeromonas* has been convoluted. This is largely a consequence of phenotypic similarities among species, which placed current genospecies into a single phenospecies, and persistence of older phenospecies epitaphs in the literature. DNA-DNA hybridization studies, genome-scale fingerprinting, namely AFLP [21], and MLST [22] have led to the establishment and validation of several distinct (geno)species, which currently number 27 (for current status, please consult http://www.bacterio.cict.fr/).

Virulence factors of *Aeromonas* spp.

A number of virulence factors, including several extracellular enzymes and colonization factors, have been reported for *Aeromonas* spp. [23]. While the majority of these studies have focused on *A. hydrophila*, work has extended to other species of the genus. Extracellular enzymes include hemolytic *A. hydrophila* cytotoxic enterotoxin (Act); channel-forming toxin, aerolysin; beta-hemolytic hemolysin, HlyA; and cytotonic enterotoxins, Alt and Ast. There is a correlation between clinical strains of *Aeromonas* spp. and presence of both *alt* and *ast* genes. Other colonization factors include a type IV bundle forming pilus (Bfp), lateral flagella, type III secretion system (T3SS), and enolase. Additionally, *Aeromonas* spp. have been shown to invade tissue in culture.

Aeromonas spp. and clinical syndrome

Several studies have suggested a correlation between species type of *Aeromonas* and clinical syndrome. Eighty-five percent of all human clinical and 95% of septicemia isolates of *Aeromonas* are comprised of three species: *A. hydrophila*, *A. caviae*, and *A. veronii* biovar Sobria [1]. *A. jandaei*, *A. schubertii*, *A. trota*, and *A. veronii* biovar Veronii are considered minor human pathogens [2,24]. *A. hydrophila*, *A. caviae*, and *A. veronii* biovar Sobria, and to a lesser extent *A. veronii* biovar Veronii, *A. jandaei*, and *A. trota*, are associated with gastroenteritis. Dysenteric-like gastroenteritis is more frequently seen in infections with *A. veronii* biovar Sobria, while *A. caviae* is the most common species involved in pediatric diarrhea [25]. *A. schubertii* and *A. veronii* biovar Sobria are associated with wound infections [26].

Isolation of *Aeromonas*

Aeromonas spp. will grow on a wide array of laboratory media, including selective media for the isolation of enterics. For isolation of *Aeromonas* spp., cefsulodin-irgasan-novobiocin (CIN) agar is primarily used [27]. Routine subculturing onto blood agar (BA) plates is useful to distinguish *Aeromonas* spp. from isolates of *Yersinia* and *Citrobacter*, based primarily on a positive reaction for cytochrome oxidase, which can be performed from BA directly, but not CIN. Beta-hemolysis of *Aeromonas* spp. on BA is also a useful, but not exclusive, distinguishing characteristic. The use of ampicillin in culture media is now discouraged, since *A. trota* is susceptible to it, as well as some isolates of *A. caviae* [28].

In addition to certain members of the *Enterobacteriaceae* and *Pseudomonas* spp., it is possible that *Aeromonas* spp. are mistaken for *Vibrio* spp. *Aeromonas* spp. can be distinguished from *Vibrios* based on their ability to grow in 0% NaCl, their inability to grow above 6% NaCl, their resistance to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropyl pteridine, 150 μg), and a negative result from the "String" test. *Aeromonas* spp. are distinguished from *P. shigelloides* in that plesiomonads are susceptible to the O/129 vibriostatic agent, have positive reactions for lysine and ornithine decarboxylase and arginine dihydrolase, and are able to ferment inositol.

Most commercial clinical diagnostic systems incorrectly identify *Aeromonas* to the species level. The seven test dichotomous key, Aerokey II [29], is capable of efficiently differentiating all major human clinical isolates of *Aeromonas* spp. [30]. There are approximately 100 serogroups of *Aeromonas*, based on somatic O-antigens [31]. While serotyping is not commonly done, the O:11, O:16, and O:34 serogroups are most common among clinical, and environmental, isolates [32].

Plesiomonas shigelloides

In contrast to *Aeromonas*, the genus *Plesiomonas* consists of a single homogeneous species, *P. shigelloides*, formerly *Aeromonas shigelloides*. The name is indicative of the fact that strains of *P. shigelloides* can cross-react with antisera of *Shigella* spp., in

particular *S. sonnei*. Strains of *P. shigelloides* possess two to seven polar flagella, and lateral flagella have been observed.

Little is definitively known about the mechanisms of pathogenicity for *P. shigelloides*. Several virulence factors, such as enterotoxins and invasins, including an aerolysin-like hemolysin [33], have been studied and proposed. Lack of an appropriate animal model and contradictory results from human volunteer studies have hampered their acceptance as virulence factors and extend the proposition that, as for *Aeromonas* spp., not all strains are pathogenic [34].

Strains of *P. shigelloides* can be typed based on their somatic, or LPS, (O) and flagellar (H) antigens. Over 100 serovars of *P. shigelloides* have been described, and an international typing scheme has been proposed, which includes 76 O- and 41 H-antigens [35]. Two serotypes—O17:H2 and O24:H5—have been implicated in separate outbreaks [36].

Isolation of *P. shigelloides*

Like *Aeromonas* spp., *P. shigelloides* grows well on most media used for the isolation of enteric bacteria. However, the appearance of colonies on most of these is quite variable due to delayed lactose fermentation. The most commonly used media for the selective recovery of *P. shigelloides* is inositol-brilliant green-bile salts agar (IBB) and CIN [2,37]. Inhibition of growth in 6% NaCl, arginine dihydrolase, and L-histidine decarboxylase can be used to differentiate *P. shigelloides* and *Vibrio* spp. *P. shigelloides* is distinguished from other Enterobacteriaceae by a positive reaction for the cytochrome oxidase test.

Exposure pathways

Both *Aeromonas* spp. and *P. shigelloides* are autochthonous members of the aquatic bacterial community. While both members are occasionally isolated from marine waters, growth inhibition by high salt content restricts their primary ecological reservoir to fresh and brackish water ecosystems. *P. shigelloides* exhibits a limited temperature growth range, with an optimal growth temperature around 38 °C, and a minimum of 8 °C. *Aeromonas* spp. exhibit a wider temperature growth range, 5–45 °C, with an optimum of 28 °C, due to the heterogeneity of the group. As seen for most *Vibrio* spp., abundance of *Aeromonas* spp. and *P. shigelloides* and subsequent frequency of human clinical cases increase during the warmer months of the year, for example, May through October in the Northern hemisphere [2,38]. *P. shigelloides* is more commonly isolated in tropical and subtropical regions.

The primary entry of *Aeromonas* spp. and *P. shigelloides* into the food supply is through exposure to water in which the abundance of these organisms is high. *Aeromonas* spp. have been isolated from chlorinated and non-chlorinated water, biofilms, biosolids and sewage, fish and seafood, frogs, and leeches [39,40]. They also have been isolated from the feces of a wide variety of animals, including

pigs, cows, sheep and poultry, and even from some prepared foods, such as pasteurized milk. *P. shigelloides* is more commonly associated with contaminated water, fish, and seafood, particularly oysters. However, the organism has also been isolated from dogs, cats, cattle, pigs, snakes, shellfish, and fish.

The infectious dose of *Aeromonas* spp. to cause gastrointestinal disease in humans has not been definitively established. A limited number of case-control or human volunteer studies have been conducted. In these studies, the dose needed was very high and only a limited number of study volunteers developed disease. Likewise, a single human volunteer study failed to establish virulence of *P. shigelloides* as a primary cause of disease.

Food attribution

Currently, food attribution for infections by *Aeromonas* spp. and *P. shigelloides* is incomplete, primarily due to the sporadic nature of the epidemiology of these organisms. Infection due to *Aeromonas* is strongly associated with consumption of raw or improperly cooked or stored seafood, including fish, shrimp and prawns, and oysters [41]. *Aeromonas* has also been found in milk and milk products, including pasteurized milk; vegetables, such as lettuce, onions, celery, tomato, parsley, broccoli, spinach, alfalfa sprouts, and watercress; and meat and poultry [42]. The most common species isolated from food sources are *A. hydrophila*, *A. veronii* bv. Sobria, and *A. caviae*. Raw and undercooked seafood is the most common vehicle of *Plesiomonas* gastroenteritis outbreaks [3]. Both organisms are unlikely to grow in foods with high salt content and high acidity. It should be noted that the recent trend in water recycling and use of graywater may pose increased risks of exposure since retreatment is not usually practiced and bacterial abundance may increase in this setting. Of particular concern is the use of this type of water in crop irrigation.

Prevention and control

Since contamination of foodstuffs by *Aeromonas* and *P. shigelloides* is largely through exposure to contaminated water sources and given their ubiquitous distribution in water systems, prevention can be challenging. As one of the main risk factors for both organisms is consumption of raw or undercooked seafood, the FDA seafood HACCP (Hazard Analysis and Critical Control Points, instituted in 1995 and discussed in more detail in Chapter 35) is one of the primary preventive control measures against infections from *Aeromonas* and *Plesiomonas*. A HACCP plan is particularly important for the seafood industry, for which contamination is possible at several points along the farm to fork continuum. Contamination of retail meats, vegetables, and dairy products is most likely due to contact with contaminated water at post-slaughter/post-harvest handling and processing stages.

In conclusion, although these organisms are ubiquitous in natural water systems, abundance of these organisms in municipal and treated water is usually low. Therefore, strict adherence to good hygienic practices, such as good personal hygiene and proper storage, preparation and cooking of foodstuffs, will greatly reduce bacterial proliferation and cross-contamination, and the number of *Aeromonas* and *Plesiomonas* infections.

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CHAPTER

Brucellosis 15

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Introduction

Brucellosis is one of the world's most widespread zoonotic diseases. This facultative, gram-negative, intracellular pathogen has been known to afflict mankind since its first isolation from humans in 1887, but likely it has caused illness throughout our history [1,2]. Brucellosis in humans, also known as "undulant fever," is a febrile disease whose clinical presentation can mimic many other infectious and non-infectious diseases [3,4]. The early identification of cases and prompt initiation of appropriate therapy is complicated by its non-specific clinical features, difficulties in laboratory diagnosis, and relatively high prevalence in countries with common occurrence of other febrile illnesses and poor public health and animal health infrastructures. This compounds the impact of a disease that tends toward relapse and chronicity even with prompt and careful case management. Brucellosis in livestock causes abortion, weight loss and decreased milk yield in female animals, sterility in male animals, and enormous economic losses.

Transmission of brucellosis to humans mainly occurs by consuming raw (unpasteurized or unboiled) milk and dairy products made from raw milk from infected animals, and through occupationally related exposures. After entering the human body, *Brucella* species can spread throughout the body via the lymphatic and circulatory systems. Potentially every organ and system can be affected by brucellosis. Treatment of brucellosis in humans requires long periods of therapy with two or more antimicrobial drugs. Although brucellosis rarely is fatal, it can be severely debilitating and disabling. All attempts to produce a safe and effective human vaccine against infection with *Brucella* have been unsuccessful, and ultimate control of human brucellosis requires control of brucellosis in livestock.

Clinical features

Brucellosis in humans is a systemic bacterial disease that has non-specific signs and symptoms, requires prolonged combined antimicrobial treatment, and often

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is misdiagnosed, resulting in inadequate therapy and illness of long duration [1,3–5]. Symptoms of disease may last for several days to more than a year. While the onset of clinical disease can be abrupt or develop slowly, the infection itself is a chronic granulomatous process. It has a wide spectrum of clinical manifestations, depending on the state of the disease and the organ systems involved. The clinical onset of brucellosis in humans generally is insidious (rather than abrupt) and follows a variable incubation period of weeks to months, commonly 5 to 60 days. The early stage of disease is associated with bacteremia and systemic spread of infection. Fever is almost universally present and may be spiking and accompanied by shaking chills during bacteremia, or may be relapsing (undulating) or mild and constant. Many cases initially present as fever of unknown origin clinically compatible with influenza-like disease or septicemia, and they report variable non-specific symptoms such as fever with or without chills, profuse sweating, fatigue, malaise, anorexia, weight loss, headache, joint pain, and generalized aching.

Clinical signs observed in cases of brucellosis include arthralgias, lymphadenopathy, hepatomegaly, splenomegaly, anemia, leukopenia, and thrombocytopenia [3–5]. Localized suppurative and chronic granulomatous lesions may occur in the liver, spleen, and other organs. Elevations of C-reactive protein (an acute-phase protein found in blood in response to inflammation), elevated erythrocyte sedimentation rate, and positive rheumatoid factor also are commonly observed. Antibodies of the IgM class against the *Brucella* smooth lipopolysaccharide (LPS) appear during the first week of infection, followed by IgG antibodies in the second or third week; both peak during the fourth week of infection and are suppressed by early administration of effective antimicrobial agents. IgM antibodies typically remain elevated for months following infection, but persistent IgG and IgA antibodies indicate chronic infection.

Later clinical manifestations may include osteoarticular, dermal, gastrointestinal, respiratory, cardiovascular, and neurologic disorders mimicking many other infectious and non-infectious diseases, and chronic course, focal complications, and relapses frequently occur [4,5]. Osteoarticular manifestations account for over half of the focal complications of brucellosis, and include sacroiliitis, spondylitis, and non-erosive arthritis of the knees, hips, ankles, and wrists; prosthetic joints also can be affected. The most common osteoarticular finding in children is arthritis in only one joint (usually knees or hips), whereas in adults, sacroiliitis is most frequent. Osteoarticular complications may be more common in persons with human leukocyte antigen (HLA) B39.

Genitourinary complications (epididymoorchitis, glomerulonephritis, and renal abscesses) can be found in about 10% of cases [4,6]. Tropism to reproductive tissues is the hallmark of brucellosis in domestic animals but localization in reproductive tissues appears less common in humans. Because *Brucella* grows well in tissues enriched with erythritol, which includes placenta and male and female reproductive tissues in ungulates (e.g., cattle, goats, sheep, and pigs), abortion and reproductive failure are common in livestock infected with *Brucella* species.

However, levels of erythritol are not elevated in reproductive tissues and placenta in humans, and reproductive complications are less frequent in human brucellosis. Nonetheless, abortions can occur, especially in infections during the first and second trimester, and medical intervention is important. Limited data suggest that vertical transmission of human brucellosis and transmission via breast milk can occur, but they appear uncommon.

Complications in two systems, the cardiovascular system and the central nervous system, are associated with most human mortality linked to brucellosis, but the overall case-fatality rate is low, generally in the range of 1–3% [4]. Endocarditis, an uncommon complication in most clinical series, generally involves the aortic valve and frequently is associated with fatal outcome. Neurologic findings occur in 10% of cases or less in most case series and can include peripheral neuropathies, chorea, meningoencephalitis, meningovascular disease, cranial nerve compromise, brain abscesses, demyelinating syndromes, and psychiatric manifestations.

While severity and duration of illness are highly variable, susceptibility to infection is general. Interferon-gamma is important for controlling infection and persons who are homozygous for the IFN-gamma(+874)A allele may be more susceptible to infection. In contrast, persons with HIV/AIDS do not appear to be at increased risk of opportunistic infections with *Brucella* species [4]. Brucellosis can lead to chronic infections with debilitating recurrent clinical episodes in the absence of effective treatment [2,4,5]. WHO recommends an oral treatment regimen consisting of 200 mg doxycycline along with 600–900 mg rifampicin daily for a minimum of 6 weeks, and an oral/parenteral scheme of 6 weeks of doxycycline plus 15 mg/kg body weight streptomycin daily for the first 2–3 weeks of the 6-week course of doxycycline [3,7,8]. Treatment failure and relapse rates of more than 20% have been reported in some case series, but there is little evidence that antimicrobial resistance is a problem, and relapses can be treated with the same antimicrobial cocktails used to treat the primary infection. However, the requirement for protracted and repeated therapy increases the burden of disease. Alternative therapy regimens have been suggested but data are mixed and nothing appears to be more consistently successful than the current WHO recommendations.

Microbiology

The bacterium *Brucella* (in the family Brucellaceae within the order Rhizobiales of the class alpha-Proteobacteria) is a gram-negative, facultative intracellular coccobacillus [2,5,8–10]. Members of the genus *Brucella* are genetically homogeneous and species are distinguished by host preference and phenotypic characteristics. The preferred hosts of the currently recognized *Brucella* species are cattle for *B. abortus*; goats and sheep for *B. melitensis*; pigs, hares, reindeer, and rodents for *B. suis*; dogs for *B. canis*; sheep for *B. ovis*; desert rodents for *B. neotomae*; seals for *B. pinnipedialis*; dolphins and porpoises for *B. ceti*; voles for *B. microti*; and unknown for *B. inopinata*. However,

host preference is not absolute (and for *B. suis* varies by biovar), and most of the species have been isolated in multiple different hosts.

In addition to its preferred hosts, *Brucella* has been isolated from humans, bison, elk, feral swine, wild boar, fox, hare, camels, African buffalo, reindeer, caribou, chamois, and ibex. *Brucella melitensis*, *abortus*, and *suis* account for most human infections acquired through any route. Most foodborne infections are associated with consumption of milk and dairy products contaminated by *melitensis* and *abortus*. Molecular characterization and genomic techniques are clarifying genetic relationships among species and biovars, and providing information useful in diagnostic strategies, epidemiologic investigations, and studies of pathogenic mechanisms.

In most infections, elements of host innate (non-specific) immunity recognize pathogen-associated molecular patterns (PAMP) present on bacterial pathogens and products of host cell damage, and respond by arming bactericidal mechanisms and by bolstering the adaptive immune response [11,12]. Macrophages, which constitute a first line of defense of the innate immune response, eliminate foreign particles by phagocytosis followed by fusion of the phagosome with lysosomes to create a highly degradative phagolysosome. Normally after interacting with pathogens, host dendritic cells undergo a maturation process leading to cytokine secretion, presentation of bacterial antigens to T-lymphocytes to stimulate adaptive immunity, and activation of natural killer T-lymphocytes. *Brucella*, however, thwarts host defenses by a variety of mechanisms, including interfering with the functions of these two cells.

Brucella virulence mostly resides in its ability to enter, survive and replicate within phagocytic and non-phagocytic cells. Analysis of the complete genomes of *B. melitensis*, *suis*, and *abortus* have not identified classic virulence factors such as endotoxins, exotoxins, fimbriae, and capsules [1,3–5,11–14]. *Brucella* enters the host via ingestion, inhalation, or through penetration of the conjunctiva or abraded skin, comes into contact with humoral mediators, and is phagocytized by neutrophils, macrophages, and dendritic cells. While many of the invading *Brucella* cells are killed at that point, some survive because their non-classical LPS and outer membrane protein modifications by the BvrR/BvrS gene sensing system induce negligible levels of inflammation, insignificant levels of complement fixation, low levels of pro-inflammatory cytokines and chemokines, low recruitment of neutrophils and weak bactericidal activity of neutrophils and macrophages. The outer membrane proteins and production of AMP and GMP help inhibit phagolysosome fusion, activation of bactericidal substances, and TNF production. *Brucella* thus sequestered within host cells spreads throughout the body and remains in its vacuole until its release from the host cell by hemolysins and induced cell necrosis.

The type IV secretion system, VirB, produces effector molecules that direct *Brucella* to the host cell endoplasmic reticulum with which it establishes a sustained interaction to create a protected vacuole permissive for bacterial replication (a strategy also employed by *Legionella*). In addition, *Brucella* prevents programmed host cell death to prolong its replicative state, which accounts for

chronic infections of the spleen, liver, bone marrow, and other sites. Vaccination of livestock with attenuated live *Brucella* before exposure to wild type *Brucella* subverts all of these nifty adaptations because a Th-1 type cell-mediated response and activated macrophages effectively kill intracellular *Brucella.* Activation of macrophages and adaptive immunity later in the course of infection may be unable to clear already established brucellosis, although granuloma formation in conjunction with B cell and T cell activities may eventually overcome the infection [11,14].

Because the clinical presentation of brucellosis can be misleading, the cornerstone of clinical diagnosis lies in taking a detailed history and paying particular attention to possible exposure (consumption of contaminated milk and dairy products and/or contact with infected animals) and proximity to other cases already diagnosed [3,4,13]. Ill persons who live in endemic areas or who have traveled to endemic areas, or who have consumed potentially infectious foods from endemic areas, should be considered possible cases of brucellosis. Diagnosis of brucellosis in one person should lead to a search for other cases among those similarly exposed. When human brucellosis is a diagnostic possibility but the exposure potential of the source animals or milk is unknown, communication between public health and animal health officials is important both for proper diagnosis and epidemiologic follow-up.

Classically, detection and identification of *Brucella* have been based on growth on culture followed by biochemical tests that demonstrate small, gram-negative, oxidase-positive, and urease-positive coccobacilli [3–5,13]. While they may be catalase-positive, this test should not be performed as it may nebulize particles and infect laboratory personnel. *Brucella* species are cultured in biphasic media and should be subcultured for at least 4 weeks. Sensitivity of blood culture has been reported to be as high as 90% and as low as 30%, depending on methods, phase of the disease, and previous use of antibiotics. Bone marrow cultures may be more sensitive, yield faster culture times, and have other advantages, but bone marrow aspiration is a painful, disagreeable process. *Brucella* also can be isolated from pus, tissue samples, CSF, pleural fluid, joint fluid, and ascitic fluid. Because isolation methodology can be difficult and success uncertain, and because of laboratory-acquired infections when it is grown in the laboratory, non-cultural diagnostic methods have been developed and are commonly used to diagnose brucellosis. Each method has strengths and weaknesses, including false negative results, and false positive results due to cross-reactions with other bacteria, prior infections, and incorrect cutoff values. Ease of use of various diagnostic methods may be an issue in poorly resourced laboratories common in regions with the greatest problems with brucellosis.

Antibody response to *Brucella* LPS is a useful diagnostic tool, but appears to play a limited role in overall host response to infection [2–4]. Agglutination tests, including the Rose Bengal test, the serum agglutination test and Coombs' test, are based on the reactivity of antibodies against smooth LPS. Sensitivity and specificity are reasonably high, but antibodies to smooth LPS tend to persist for a long time, making these agglutination tests difficult to interpret in endemic areas. Using

higher cutoff values increases the specificity and predictive value of a positive test in endemic areas, but patients with acute brucellosis or persisting and relapsing disease may present with low antibody levels and be falsely determined to not have brucellosis. Looking for seroconversion or a 4-fold titer change between paired serum samples can address this problem, but requires two interactions between the patient and laboratory separated by 2 or more weeks. Additional diagnostic considerations for the serum agglutination test include its failure to pick up *B. canis* infections, as well as cross-reactions with *Francisella tularensis, Escherichia coli* O:116 and O:157, *Salmonella urbana*, *Yersinia enterocolitica* O:9, *Vibrio cholerae*, *Xanthomonas maltophilia*, and *Afipia clevelandensis*, and the failure of some patients to demonstrate antibodies by this method.

Cross-reacting IgM antibodies can be addressed by using the 2-mercaptoethanol test to measure only the more specific IgG antibodies, but many patients develop only low IgG titers. Indirect enzyme-linked immunosorbent assays (ELISA) measure IgM, G, and A immunoglobulins, which allows for superior interpretation of the clinical picture and prognosis, and may be more sensitive and specific than the serum agglutination test, especially in cases with neurobrucellosis. ELISA is an increasingly popular assay but, like the agglutination tests, targets antibodies against smooth LPS, and cutoff values may need to be adjusted in endemic areas. Commercial ELISA assays for brucellosis have been poorly evaluated, making their use problematic. Very good polymerase chain reaction (PCR) methods have been developed that address many of the shortcomings of serum tube agglutination, although closely related *Ochrobactrum* species cross-react in some of them. Multilaboratory validation studies are needed for many of the newer diagnostic tests.

Developments in culture and serologic diagnostic methods and the availability of advanced molecular detection and typing methods are improving laboratory diagnosis of brucellosis and epidemiologic traceback to sources of infection [2,3]. Some of the newer diagnostic methods are simple, robust, and affordable, and may improve case finding, source identification, and cost-of-disease estimates. Automated culture systems, such as the lysis centrifugation method, have increased sensitivity and reduced culture times compared with traditional biphasic culture methods; however, some may generate infectious aerosols and laboratory-acquired infections. New point-of-care tests like fluorescent polarization immunoassay and lateral flow assay are promising and are widely used in some animal disease control programs. Currently available tests are poor for neurobrucellosis and prediction of treatment outcome and relapse, although real-time PCR assays show promise; PCR methods also appear useful for subtyping and will have epidemiologic value.

Exposure pathways

Brucellosis occurs worldwide, especially in Middle Eastern countries, southern Europe and North Africa, countries in South and Central Asia, sub-Saharan Africa, Mexico, the Caribbean, and countries in South and Central America [3–5,8,15,16]. Its

prevalence remains very high among ruminant livestock in many developing countries, although its incidence and prevalence are largely underestimated. In these areas, prevalence rates in humans that exceed 10 cases per 100,000 population are documented, despite brucellosis being commonly undiagnosed and under-reported. The sources of human infection and the predominant species and biovar of *Brucella* causing infection vary according to prevailing animal infections in the geographic region. Both sporadic cases and outbreaks occur in humans. *B. abortus* infections in cattle have been eradicated, or at least controlled, in most economically developed countries. *B. melitensis* infection in goats and sheep remains a problem in some of these economically developed countries, as well as in many economically developing countries.

Virtually all human *Brucella* infections result from direct or indirect exposure to infected animals or their secretions via ingestion, via inhalation or conjunctival contamination by aerosols, or via bacterial invasion of cuts and abrasions (a method of exposure controllable by wearing gloves and other personal protective equipment, and attention to hygiene) [2,4,5,8,17]. The leading source of human infections in most endemic countries (as well as in countries that have achieved good levels of control) is consumption of infectious raw milk and dairy products made from raw milk, especially fluid raw milk, soft cheese, butter, and ice cream. *Brucella* persists for several days in milk, even when it turns sour, and in soft cheese, and can persist for weeks in ice cream and butter. Although muscle tissue rarely is positive for *Brucella*, the infectious dose for brucellosis is low and meat of animals with brucellosis may be a source of infection if contaminated meat is uncooked or insufficiently cooked.

Crushing the umbilical cord of newborn lambs and kids with one's teeth also has been associated with infection; whether these cases are considered foodborne or occupational depends on perspective. Hunters who accidentally or intentionally consume blood or raw tissues of wildlife they have killed also occasionally are infected with *Brucella* species. Brucellosis is an occupational disease in shepherds, abattoir workers, veterinarians, dairy-industry personnel, and personnel in microbiology laboratories, but many of these people in endemic areas also consume raw milk and dairy products, which complicates attribution.

Although *Brucella* species are identified, in part, by their host preferences, those preferences are not absolute. Where management systems allow contact among farmed species, cross-over infections of cattle with *B. melitensis* and *B. suis* biovar 1 occur, as do swine infections with *B. abortus,* complicating control efforts and challenging epidemiologic assumptions about source of human infections that are based on usual species preferences. *B. melitensis* can colonize the bovine udder and human infections associated with drinking raw cow milk have become a problem in southern Europe and the Middle East. *B. suis* biovar 1 also can colonize the bovine udder, and causes similar public health problems in Mexico and South America. In some regions camel brucellosis due to *B. melitensis* or *abortus* is another important source of human foodborne and occupational exposure. Factors influencing prevalence in livestock include production systems, ecologic zones, husbandry and management practices, and contact with infected wildlife, resulting in spillover

infections. Human-to-human transmission by tissue transplantation (including blood transfusions) and sexual contact have occasionally been reported but are not important means of spread. Humans rarely contract brucellosis from another human.

In domestic and wild animals, *Brucella* causes a generalized infection with a bacteremic phase followed by localization in the reproductive organs and macrophages [2,5,15]. Information about geographic distribution of brucellosis in domestic animals is available on the World Organization for Animal Health (OIE) website (http://www.oie.int), although lack of consistent diagnostic and surveillance capacity in many countries makes the data difficult to interpret and compare. Infections in natural hosts often are subclinical and manifest themselves primarily through abortions, infertility, weight loss, and reduced milk yield. Aborted fetuses, birth fluids and tissues, vaginal secretions, colostrum, and milk all may be heavily contaminated and represent routes of animal-to-animal and animal-to-human transmission. Venereal spread is possible, but is restricted mainly to *B. suis* infections. Spread between herds usually occurs by the introduction of asymptomatic chronically infected animals, or animals recently infected and not yet detectable by diagnostic testing.

All major species of livestock are susceptible and eradication of the disease in some areas is complicated by infections in wildlife. Wildlife infections may represent unsustainable spillover from infected domestic animals [18] or sustainable infections, such as those in bison and elk in the Greater Yellowstone area of the United States that occasionally infect otherwise *Brucella*-free cattle sharing the same rangeland (USDA, 2011). In economically developed countries, including Australia, the United States, Canada, and much of Northern Europe, brucellosis has been controlled or eradicated through a long-term, census-based slaughter surveillance strategy, combined with area and community herd testing, elimination of reactor animals, and use of vaccination to increase herd immunity. Control of imported animals and animal products, and universal pasteurization of milk for human consumption also have played a major role in mitigating transmission of and eliminating brucellosis. In economically less-developed countries, however, test-and-slaughter programs may not be sustainable because of many factors, including the inability of the government to compensate animal owners and emotional attachment by owners to the livestock to be killed.

Prevention and control

Control of brucellosis in humans requires the consistent, concurrent, and long-term application of a number of interlocking human health and animal health programs [4,8,15,16]. Because human brucellosis is not sustainable, measures to prevent brucellosis in humans ultimately require eliminating the disease from domestic livestock and wildlife sources. Preventive measures that mitigate foodborne brucellosis always include universal pasteurization or boiling of milk before it is consumed or

enters into commerce, and educating the public—especially residents of and tourists to endemic areas—not to drink raw milk and not to eat dairy products made from raw milk.

The specific approach to control, prevention, or eradication of brucellosis in a country or region will depend on many factors, such as the level of infection in herds and flocks, type of husbandry, economic resources, public health impact, and potential implications for trade in animals and animal products [15]. Formal disease control, surveillance, and eradication programs should be science-based and utilize effective epidemiologic and diagnostic testing strategies. Risk assessments undertaken to determine the best strategies for control of infections in humans must include site-specific factors important to human exposure, infections in local domestic livestock, and risk associated with commercially and non-commercially imported animals and animal products [19,20]. Global trade and travel make brucellosis in economically developing nations an important international public health issue. While the epidemiologic features necessary for evaluating control strategies for brucellosis in cattle, goats, and sheep are relatively well established in some parts of the world, brucellosis in other species that are important as sources of milk in some endemic areas (e.g., camels and yak) is largely unstudied. Likewise, enhanced investigation, surveillance, and diagnosis of fevers of unknown etiology in humans is badly needed in areas where brucellosis in humans coexists with malaria, typhoid, and other common infectious causes of febrile illness [21,22].

Eradication of brucellosis in animals can be achieved in principle by a combination of vaccination of all breeding animals followed by test-and-slaughter programs [15]. The principal goals of brucellosis control and eradication efforts are to protect public health, ensure food safety, and maintain the economic viability of livestock industries in national and international trade. Documenting the burden of human disease and the economic costs of brucellosis in livestock is important to maintain government support for necessary program activities and for public education and awareness campaigns. On the human health front, this requires access to medical care and adequately resourced diagnostic facilities; public health infrastructure sufficient to provide reference and subtyping laboratory support, rapid and competent epidemiologic follow-up of cases, regular reporting of accurate incidence, prevalence, and health impact data; and routine prevention and treatment effectiveness assessment [3].

On the animal health front it requires sufficient veterinary infrastructure to support effective disease control, surveillance and eradication strategies in susceptible domestic and wild animals. Effective strategies often include appropriate use of brucellosis vaccines, and implementation of animal or herd quarantines and movement controls to prevent spread of disease outside of brucellosis-affected regions. Control strategies should be based on species-specific incidence and disease prevalence rates, and take into consideration local agricultural economics and practices, available resources and adequate communication and coordination with the surrounding area and external trading partners [15]. Reasonably good live attenuated vaccines exist for cattle, goats, and sheep (but not for humans, reindeer, water buffalo, yaks, camels, and swine) and they are effective parts of control and eradication programs [23].

However, they can be pathogenic for the humans administering them and, depending on its physiologic state, the ruminant receiving the vaccine, and can interfere with serologic tests used in eradication efforts [5,8].

Brucellosis in humans, livestock, and wild animals poses a number of challenges in designing an effective surveillance program [15]. The infection can be chronic and cases may exhibit no, or variable, signs and symptoms. Laboratory confirmation of disease is an essential component of surveillance; however this may be difficult to obtain for domestic and wild animals and even for human cases in many regions of the world where brucellosis exists. The link between confirmed and suspected human cases and a potential animal source may be difficult to establish because of the variable incubation periods and limited epidemiologic and laboratory capacity in these regions as well. In many areas where control of brucellosis is most critical from a human health perspective, animal populations are poorly identified and enumerated, veterinary infrastructure is deficient, and animals are inaccessible for long periods because of weather, bad roads, and transhumance.

The characteristics of coordinated human and animal brucellosis surveillance systems will vary based on a number of factors, but clearly each system must have well-described standardized definitions and methods, clearly stated agreed-upon objectives, adequate long-term stakeholder support and resources to achieve stated objectives, routine methods for providing validated data to interested parties and the public, and the ability to evaluate the effectiveness of surveillance and adapt to changing conditions. Existing surveillance efforts for human disease have sometimes revealed alarming rates of seropositivity and active disease in human populations, and special studies conducted in resource-poor regions refute the small numbers in official reports for both humans and domestic animals [16,24].

In the United States, a longstanding bovine brucellosis eradication program achieved designated Class-Free status for all 50 States, Puerto Rico, and the Virgin Islands in July 2009. However, the presence of sustainable brucellosis in the wild elk and bison populations in the Greater Yellowstone Area states (Idaho, Montana, and Wyoming) has resulted in the occasional finding of brucellosis-affected cattle herds in that area. Effective disease control and eradication strategies (including herd quarantines, repetitive herd testing, and removal of any test positive animals), movement controls and use of appropriate vaccination protocols are implemented in these herds to assure brucellosis is eliminated from the herds and does not spread outside the area. In addition, the United States maintains vigilant import requirements to mitigate the introduction of brucellosis from countries where disease is present [25]. The United States is considered free of *Brucella melitensis* infections in small ruminants. Interestingly, most of the approximately 100–120 cases of human brucellosis reported annually in the United States are due to *B. melitensis* infections, most commonly acquired during foreign travel or from consumption of illegally imported dairy products made with unpasteurized milk. Occupation-related infections with *B. abortus* and *B. suis* also occur in small numbers [16,17,26].

Compared with the United States, the European Union (EU) has had fewer decades in which to achieve uniformity among its member States. EU States in the

North and Northwest have achieved better control of brucellosis in livestock than those EU States in the East and South, and report commensurately fewer human infections [16,27]. Nonetheless, the number of infected cattle, goats, and sheep infected with *B. abortus* and *B. melitensis* is declining EU-wide and there has been a significant declining 5-year trend, from 2005 to 2009, in the number of confirmed human cases of brucellosis in the EU. In 2009 a total of 401 confirmed cases of human brucellosis were reported in the EU [27].

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CHAPTER

Cronobacter Species (formerly *Enterobacter sakazakii*)

16

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Introduction

Cronobacter is a newly described bacterial genus that includes opportunistic pathogens formerly known as *Enterobacter sakazakii*, and includes seven described species: *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis* (subsp. *dublinensis*, *lausannensis*, and *lactaridi*), *C. condimenti*, and *C. universalis* [1,2]. While these pathogens have been detected in a wide variety of foods, reconstituted, temperature-abused powdered infant formula (PIF) remains the most commonly linked source for cases and outbreaks of neonatal and infantile meningitis, necrotizing enterocolitis (NEC), and septicemia [3–5].

Clinical features of illness associated with infection

Cronobacter spp. are capable of causing invasive diseases, and are primarily associated with meningitis, necrotizing enterocolitis (NEC), and septicemia. Premature infants, low birth-weight neonates, and those with underlying medical conditions are at highest risk for developing *Cronobacter* infections [3,6]. Mortality rates among this group range from 40–80%, and infants who survive frequently suffer developmental delays, hydrocephaly, mental retardation, and other neurological sequelae [3,5].

Although *Cronobacter* spp. have been primarily associated with infections in infants, recent reports have emphasized the risk posed to immunocompromised adults, particularly the elderly [7]. *Cronobacter* infections in elderly patients include wound infections, urinary tract infections, septicemia, vaginitis, and aspiration pneumonia. Additionally, the prevalence of *Cronobacter* spp. infections in adults is increased in the elderly who have experienced strokes that have affected

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their abilities to swallow (dysphagia), and who may use rehydrated protein supplements as part of their diet [6]. This is a problem that is likely to become more common because of the aging of the world's population, and as trends for consumption of synthetic, dehydrated nutritional supplements for such patients increase.

Microbiology

General features

There is considerable diversity with respect to both genotypic and phenotypic characteristics among different isolates of *Cronobacter* spp. Members of the genus *Cronobacter* comprise a diverse group of Gram-negative bacilli that are approximately 3 µm long and 1 µm in diameter, oxidase-negative, catalase-positive, facultatively anaerobic, and motile by peritrichous flagella [1]. Growth occurs between 6° and 45 °C in brain heart infusion broth. However, current isolation schemes recommend using a growth temperature between 37° and 44 °C. Most strains of *Cronobacter* spp. produce yellow pigmented colonies on non-selective agar media, due to the presence of the *Pantoea*-related carotenoid biosynthetic operon, *crtE-idi*-XYIBZ [8,9]. Additionally, two different colony types can be exhibited: smooth and rugose [8,10]. The rugose phenotype in other enteric organisms has been shown to provide resistance to desiccation and killing by antimicrobial agents such as hypochlorite and increased ability to form and persist within biofilms [11–14].

Thermoresistance properties

Thermoresistance of *Cronobacter* spp. has been well documented and studied [15,16]. Recently, an 18-kbp region has been reported that contains 22 open reading frames that are upregulated under heat adaptation conditions [17]. The major feature of the region is a cluster of conserved genes—most of them having significant homologies with known bacterial proteins involved in some type of stress response, including heat, oxidation, and acid stress.

Isolation and identification

Since *Cronobacter* spp. exist in low numbers and, almost certainly, as injured cells in PIF, other related food products/ingredients, and the environment, it is important to grow these cells initially in either a non-selective or a selective enrichment step that can resuscitate injured cells. An initial non-selective broth, such as modified buffered peptone water incubated at 35–37 °C, is acceptable but a selective enrichment broth that can inhibit Gram-positive and other competing Gram-negative bacteria is preferred.

Various chromogenic plating media have been developed which use α-glucosidase activity of *Cronobacter* spp., unique among the enterics, as a differential characteristic. For example, Druggan, Forsythe, and Iversen agar (DFI) [18] uses the chromogen

5-bromo-4-chloro-3-indoxyl-α-D-glucopyranoside (X-α-Glc). However, some strains of *Cronobacter* spp. may not produce, or are weak producers of, α-glucosidase activity when grown on DFI and its derivatives, and this may lead to potential false-negative results [19]. As an alternative, *Enterobacter sakazakii* chromogenic plating medium (ESPM, R&F Labs, Chicago, IL) includes a second chromogen, 5-bromo-4-chloro-3-indoxyl-β-D-cellobioside (X-β-Cell), used to detect β-cellobiosidase activity of *Cronobacter* spp. [20]. Presumptive isolates of *Cronobacter* spp. can be confirmed using many commercial diagnostic automated microbial identification kits such as Vitek 2.0 Compact (bioMerieux, Inc., Marcy l'Etoile, France). Additionally, presence of a genus-specific zinc metalloprotease, zpx, can be used as a molecular diagnostic tool [21] to screen cultures.

It is important to note that some members of the closely related *Enterobacter* spp., such as *E. pulveris*, *E. helveticus*, and *E. turicensis*, may produce presumptive positive *Cronobacter*-like colonies on the chromogenic agars [22,23]. API20E, Rapid ID32E, and Vitek 2.0 Compact (bioMerieux, Inc.) can reliably distinguish these species from *Cronobacter* spp.; however, API20E may falsely confirm some isolates of *E. cloacae*, *E. hormaechei*, and *E. asburiae* that give positive presumptive colonies using ESPM or DFI as *C. sakazakii* [22–24].

Species-specific identification

Isolates of *Cronobacter* spp. can be further characterized using a species-specific PCR assay based on polymorphisms within the *rpoB* gene [25]. A seven loci multilocus sequence typing (MLST) scheme has also been developed for species identification, which has revealed that over half (29/53) of *C. sakazakii* strains examined belonged to one sequence type, ST4, which appears to be a highly stable clone with a strong association with neonatal meningitis [26].

Serotyping

Molecular O-antigen serotyping assays have been reported for *Cronobacter* species [27,28], and up to 10 serogroups have been described. There are no commercial antisera for *Cronobacter* spp.; however, a serological scheme has been proposed by Sun et al. [29] that correlated well with the molecular schemes proposed by Mullane et al. [27] and Jarvis et al. [28]. In general, *Cronobacter* species possess unique O-antigen serogroups, but some species share highly similar O-antigen determinants [28]. Some serogroups of *C. sakazakii* are more commonly found among clinical isolates, namely O1 and O2 [27,28].

Virulence mechanisms

The mechanism of *Cronobacter* neonatal meningitis and the roles of potential virulence factors remain largely unknown. Currently, our knowledge is largely derived from experimental animal models of meningitis and results of tissue culture assays.

Studies using a newborn rat infection model suggest that enterocyte apoptosis, controlled by the induction of high levels of nitric oxide synthase, may be responsible for triggering apoptosis events leading to NEC [30,31]. Furthermore, results from a rat expression microarray confirmed these *in vitro* findings. Additionally, ingested cells of *Cronobacter* may transcytose across both the human intestinal and blood–brain barriers [32,33]. The outer membrane proteins, OmpA and OmpX, are involved in the invasion process [33]. Similar to other enteric invasive organisms, host cell cytoskeleton components such as actin and microtubulin polymerization/depolymerization are involved in adherence and invasion [34].

In silico analysis revealed the presence of a large RepFIB-like plasmid in the genome sequence of two clinical isolates of *Cronobacter* spp. [35]. Several putative virulence factors are contained on this plasmid, which was found to be harbored by most (97%) strains of *Cronobacter* spp. Common features include the presence of two iron acquisition systems (*eitCBAD* and *iucABCD/iutA*); *cpa*, a plasminogen activator-like (omptin) gene that is associated with *C. sakazakii* and *C. universalis*; a type VI secretion system locus that is only associated with strains of *C. sakazakii* and is variable in length; and a large region encoding a filamentous hemagglutinin gene (*fhaB*), its specific transporter gene (*fhaC*), and associated putative adhesins, associated with some strains of all species, but more specifically associated with *C. malonaticus* and *C. turicensis* isolates [35].

Exposure pathways

Cronobacter spp. are regarded as ubiquitous organisms and have been found in a variety of environments and foods. The natural habitat of *Cronobacter* spp. is presently not known; however, an environmental niche related to eukaryotic plants and/or plant material has been proposed, based on the presence of physiological features such as ability to produce a yellow pigment that is thought to protect the cell against UV radiation, ability to express capsules and fimbriae that aid in adherence to surfaces, and ability to survive under high osmotic stress and desiccation [36,37].

As mentioned above, a number of *Cronobacter* spp. outbreaks in neonatal intensive care units have been traced to reconstituted, temperature-abused, PIF contaminated with *Cronobacter* spp. [4]. Following the Tennessee outbreak of 2001 that epidemiologically linked *C. sakazakii* to the consumption of reconstituted PIF, several surveys of PIF were conducted. For example, a 3-year study (2002 to 2005) found that 9.3% of PIF samples analyzed were contaminated with *Cronobacter* [36]. Extrinsic contamination of opened PIF cans and bottled water supplies has also been reported [38]. In addition to PIF and its manufacturing plant environments, other environments and foods from which *Cronobacter* spp. have been isolated are follow-on formulae, weaning foods, formula preparation equipment (blender, spoons), milk and sodium caseinate powders, rice seed, dried herbs and spices, spiced meats, dried flour or meal (corn, soy, potato, wheat, and rice), dried infant and adult cereals, dried vegetables, grains, tofu, powdered ice tea, mixed

salad vegetables, tomato harvesting bins, chocolate and candied cough drops, pastas, hospital wards, and household vacuum cleaner dust [39–42].

The infectious dose is currently unknown due to lack of volunteer challenge data. However, a case study reported by Parra Flores et al. [43] involving two 4-month-old infants with hemorrhagic diarrhea showed that the microorganism was present at levels of 0.33 most probable number (MPN)/g and 24 MPN/ml in PIF and rehydrated PIF, respectively. These quantities suggest that the total ingested exposure dose for a day before the onset of the diarrhea was between 2160 and 3600 CFU. Several reports confirm that an oral infectious dose of approximately 100 CFUs in rehydrated PIF can cause septicemia and colonize the brains of several strains of mice [44].

Prevention and control

PIF is not a sterile product and can pose a significant risk if it is prepared and handled inappropriately. Reconstituted PIF provides an ideal environment for the growth of harmful bacteria, which greatly increases the risk of infection. Because of its implication as the main route of exposure, most preventive efforts regarding *Cronobacter* spp. infections have focused on the use and manufacture of PIF. The United Nations (UN) Food and Agriculture Organization (FAO) and World Health Organization (WHO), in response to a request for scientific advice from the Codex Alimentarius Committee on Food Hygiene, have established guidance to PIF manufacturing companies and consumers regarding the prevention of *Cronobacter* infections [6].

In recent years, Hazard Analysis and Critical Control Point (HACCP) programs (see Chapter 35) designed for the management of contamination have been widely accepted and applied in all food manufacturing facilities and are mandatory in most of the food industry. However, at this time infant formula producers have not been required to have HACCP quality system standards in place. Nevertheless, all infant formula industry-related manufacturers are required to adhere to good manufacturing practice (GMP) and practice-related quality control protocols, which help with the monitoring of the quality of PIF during the production processes.

Poor hygiene during preparation and feeding has been reported as the most likely cause of some *Cronobacter* outbreaks [45]. Following simple good hygiene practices, such as hand washing and washing of feeding and preparation equipment (e.g., cups, bottles, teats, and spoons), preparing formula fresh for each feeding with boiled water that has been allowed to cool to no less than 70 °C, and discarding unused formula, should limit both intrinsic and extrinsic contamination of PIF.

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SECTION

Foodborne Infections: Viral

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CHAPTER

Noroviruses 17

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Introduction

Human noroviruses (NoVs), plus-strand RNA viruses 7.4–7.7 kb in length, are emerging pathogens of public health concern. They are estimated to be responsible for over 95% of non-bacterial epidemic gastroenteritis outbreaks, and 50% of all gastroenteritis outbreaks, worldwide. In fact, the Centers for Disease Control and Prevention (CDC) recently ranked NoVs as the most common cause of foodborne disease outbreaks [1]. It is becoming increasingly clear that NoVs are also a major cause of severe childhood diarrhea in developing nations. Noroviruses are extremely transmissible and can spread through multiple routes, including via exposure to contaminated food, water, fomites, or aerosolized vomitus particles and through person-to-person contact (Figure 17.1). They are also exceptionally stable in the environment, resistant to many common disinfectants, and cause incapacitating albeit typically acute disease. Based on these collective features of NoV infections, they are classified by the National Institute of Allergy and Infectious Diseases (NIAID) as Category B Priority/Biodefense Pathogens.

The extreme environmental stability of NoVs facilitates their prolonged survival in water and soils, and ultimately their contamination of food sources such as shellfish and produce. Ready-to-eat (RTE) foods are also common sources of NoVs, through their contamination by infected food handlers. Overall, NoVs have emerged as major global contributors to not only gastroenteritis outbreaks but also severe childhood diarrheal disease. As such, research into effective food processing techniques to eliminate NoVs, enhanced NoV attribution methods, and treatment options are all highly justified.

Clinical features

Disease manifestations

The course of NoV infection is typically rapid—following a short incubation period of 24–48 hours, people experience vomiting and diarrhea for 12–72 hours [2].

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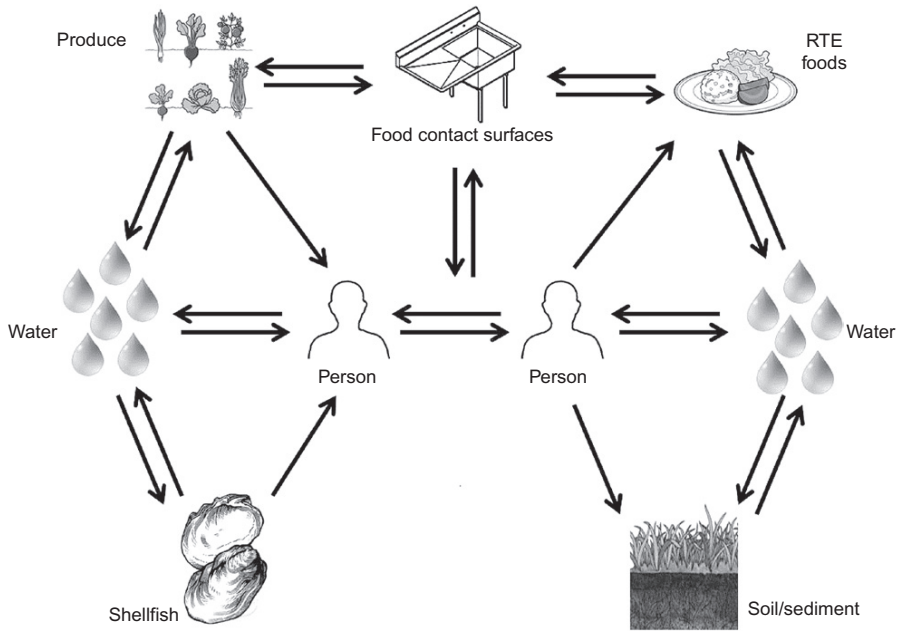

FIGURE 17.1

Routes of NoV transmission. Human NoVs spread efficiently person-to-person and through exposure to contaminated foods (e.g., produce, ready-to-eat [RTE] foods, and shellfish), water, and fomites. They are extremely stable in water and soils, and thus these environmental sources as well as infected people can all act as NoV reservoirs.

A proportion of individuals also present with nausea, abdominal cramps, low-grade fever, and malaise. Certain risk groups including infants and young children, transplant patients and other immunosuppressed individuals, and the elderly can experience more severe and prolonged disease upon NoV infection (reviewed in [3]). Noroviruses are also now considered likely contributors to travelers' diarrhea. Improved NoV diagnostics in recent years has correlated with an increased number of anecdotal reports of NoV associations with clinical outcomes other than gastroenteritis, including infantile necrotizing enterocolitis, encephalopathy, benign pediatric seizures, post-infectious irritable bowel syndrome, and exacerbation of inflammatory bowel disease (reviewed in [3]). Particularly concerning, based on the ability of related animal viruses to cause fatal hemorrhagic-like and systemic diseases, a number of afflicted individuals during a 2002 human NoV outbreak presented with headaches, neck stiffness, light sensitivity, diminished alertness, and disseminated intravascular coagulation in addition to gastroenteritis [4]. These reports clearly do not prove causation but they do collectively emphasize the need to consider NoVs as potential etiologies of diseases other than gastroenteritis.

Genetic diversity

Noroviruses constitute a genus within the Caliciviridae virus family; another calicivirus genus that causes significant numbers of gastroenteritis in humans is the sapovirus genus described in Chapter 21. The NoV genus is subdivided into five genogroups (GI-GV), with members sharing at least 40% identity in their capsid sequences [5]. The GI, GII, and GIV NoVs are primarily human pathogens [5,6]. Exceptions include several porcine GII NoVs [7,8], a canine GIV NoV [114], and a lion GIV NoV [9]. The other genogroups contain animal NoVs—GIII NoVs are bovine-specific [10,11], and GV NoVs are murine-specific [12].

The NoV genogroups are further subdivided into 32 genotypes in which members share at least 56% capsid identity: 8-GI, 19-GII, 2-GIII, 2-GIV, and 1-GV genotypes [5,13]. Noroviruses displaying 0–14% capsid identity are considered a single strain. This degree of genetic variability among members of a single genus is unusually high, even when compared with genera of other plus-strand RNA virus families [5]. Current knowledge indicates a lack of inter-genogroup cross-protection and even intra-genogroup cross-protection is limited [14], undoubtedly posing a major hurdle to future NoV vaccination attempts.

Epidemiology

Noroviruses are a leading cause of gastroenteritis outbreaks worldwide. Outbreaks are most common in semi-closed communities such as long-term care facilities, restaurants, schools, hospitals, and cruise ships (Figure 17.2). They are the most prevalent cause of hospital ward closures associated with nosocomial outbreaks [15]. Other settings that are highly susceptible to NoV outbreaks include military settings and disaster relief evacuation centers. Norovirus outbreaks can affect large numbers of people because they are highly contagious, infectious at low doses, shed for extended periods in symptomatic and asymptomatically infected people, and extremely stable in the environment. They also fail to elicit lasting homologous protective immunity in at least a subset of people; thus re-infections are common.

Because of these collective features of NoV infections, they spread efficiently at the regional and global levels. In fact, there have been at least five documented NoV pandemics since 1995 [3]. Interestingly, all of the pandemic NoV strains are GII.4 (genogroup II, genotype 4) variants, with GII.4 NoVs accounting for 70–80% of all NoV outbreaks since at least 2002 [16]. It is unclear why this particular strain has become so globally dominant but possibilities include more rapid evolution [17], increased virulence and/or infectivity [18,19], and higher titers of shed virus contributing to increased transmission [20]. The GII.4 NoVs have been documented to spread predominantly through person-to-person transmission, in contrast to other NoV genotypes that are also commonly associated with foodborne and waterborne outbreaks [21,22].

While NoVs have not historically received significant attention as a cause of sporadic gastroenteritis, recent advances in NoV diagnostics (see a detailed discussion below) have led to the generally well-accepted view that NoVs are second

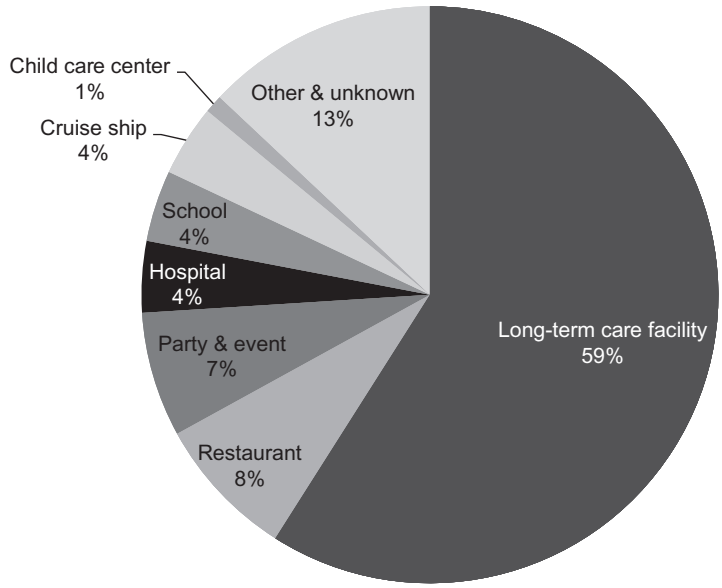

FIGURE 17.2

Settings of NoV outbreaks. The Centers for Disease Control collected data from 1518 confirmed NoV outbreaks in 2010 and 2011. The results of this surveillance are summarized here for the type of setting affected by the outbreak.

Data are reproduced from http://www.cdc.gov/.

only to rotaviruses in causing severe childhood diarrheal disease [23,24]. A recent epidemiological survey of severe diarrhea in clinical settings estimated that NoVs cause over one million hospitalizations and 200,000 deaths in young children in developing nations annually [25]. In just the past year, numerous other studies have reported that NoVs can be detected in 3–20% of children <5 years of age hospitalized with diarrhea in underprivileged settings. Interestingly, there are distinctions in the epidemiology of NoVs even among strains within the same genogroup. For example, while GII.4 strains are the most prevalent cause of NoV-induced gastroenteritis outbreaks, GII.3 strains are often the dominant genotype associated with sporadic NoV infections, especially in pediatric cases [26].

Because of the increased numbers of NoV cases during GII.4 pandemics and the realization that NoVs are a major cause of severe childhood diarrhea, there has been heightened interest in NoV epidemiology recently. This has led to the establishment of national (CaliciNet) and global (NoroNet) surveillance networks that will undoubtedly play major roles in elucidating NoV epidemiological patterns in the future. For example, a novel GII.4 variant, GII.4 New Orleans, was first identified by CaliciNet in October 2009; it was the dominant NoV strain circulating in the United States through at least May 2010 simultaneous to the decreasing

prevalence of the GII.4 Minerva variant that was the predominant cause of outbreaks from 2006–9 [27].

Host susceptibility

During multiple human volunteer studies since the 1970s, it has been observed that a subset of the population is resistant to NoV infection, potentially reflecting a host genetic component to susceptibility. In 2002, this postulate was confirmed when Hutson et al. identified an association between a person's histo-blood group type and his or her risk of NoV infection [28]. It has subsequently been established that many human NoVs associate with histo-blood group antigens (HBGAs), a polymorphic family of neutral complex carbohydrates that are expressed on the surface of intestinal epithelial cells and secreted into the intestinal lumen [29]. For certain NoVs, susceptibility to infection has been demonstrated to correlate with host expression of specific HBGAs [30], although HBGA binding is not sufficient for virus entry [31,32] so other as-yet-unidentified receptors must exist. Norovirus recognition of HBGAs is virus strain-specific, and there is a complex array of binding profiles between human NoVs and HBGAs. In fact, it has been shown that NoV strains can bind a shared HBGA using distinct binding interfaces, and they can bind distinct HBGAs using a shared binding interface (recently reviewed in [33]). There is evidence for a shared intra-genogroup binding interface, at least for the GII NoVs.

In evolutionary terms, the current paradigm in the field is that herd immunity to a circulating NoV strain drives antigenic escape by the virus, leading to altered HBGA usage [16,34]. The resultant NoV variants have the advantage of not only escaping herd immunity but also potentially infecting a naïve subset of the population that expresses a distinct type of HBGA. This pattern of evolution—typified by periods of epidemic spread of a dominant virus strain followed by periods of stasis until an antigenically distinct viral variant emerges—is referred to as "epochal evolution." Consistent with epochal NoV evolution, only a minimal number of amino acid changes in the NoV capsid protein can alter HBGA usage, providing NoVs' great flexibility in evolving to infect new populations [35]. Moreover, this alternating pattern of high NoV activity followed by quiescent periods has clearly been observed for GII.4 pandemics between 1995 and the present day. It is interesting to note that GII.4 NoVs generally display a broader pattern of HBGA binding compared with other NoV genotypes, potentially a major contributing factor to their global dominance since this translates into a larger effective target population [14].

Microbiology

Norovirus diagnostics

Because NoVs remain uncultivable, the most commonly used NoV detection assays are enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR)-based approaches. A further complication in NoV

diagnostics is the extreme genetic diversity within the genus, requiring effective assays to display a broad range of specificity. The most common approach currently used in NoV detection is RT-PCR of RNA extracted from stool samples targeting the junction between ORF1 and ORF2 in the viral genome; this region has been shown to be highly conserved across GI and GII NoVs [36]. The capsid open reading frame is also a common RT-PCR target for NoV genotyping.

Factors that can impede NoV detection using RT-PCR include the presence of PCR inhibitors in stool and degradation of NoV genomes due to suboptimal sample collection or storage conditions. Another concern is the lab-to-lab variability in methodology specifications. While ELISA-based approaches are not as sensitive as RT-PCR-based assays, they are still commonly employed for NoV diagnostics based on their commercial availability, high-throughput capability, and relative technical simplicity [37]. Another strategy for NoV detection and genotyping that is gaining popularity is the use of NoV-specific microarrays, or NoroChips [38]. While this approach is not currently in a form useful for wide-scale NoV diagnostics, it promises to be the preferred methodology of the future.

Exposure pathways

Reservoirs

People can become infected with NoVs through multiple routes of transmission (see Figure 17.1): (1) direct contact with an infected individual; (2) exposure to aerosolized vomitus particles from an infected person; (3) ingestion of fecally contaminated foods or water; and (4) exposure to fecally contaminated fomites. Water and soils can become contaminated through exposure to raw or minimally treated human sewage upon discharge of wastewater, illegal dumping of human excrement, or unintentional discharge due to urban, rural, and agricultural runoff [72]. Failure of water and sewage treatment plants has been clearly linked to NoV outbreaks [39–41]. Moreover, numerous studies have shown that common wastewater treatment methods can be ineffective at eliminating enteric viruses including NoVs [42–45]. This problem exists primarily because water treatment was designed with a focus on bacterial pathogens and continues to be validated using bacterial fecal indicators even though these indicators do not correlate with reductions in viral load [46,47].

Norovirus stability in water

Once NoVs are seeded into the natural environment, their hardy nature allows them to persist for long periods of time. Noroviruses have been detected in both marine and freshwater samples [48,49] and are stable in groundwater, river water, mineral water, and tap water for months [50–52]. A study examining the stability of the commonly used murine NoV (MNV) surrogate observed that MNV maintained infectivity up to one month in potable water [53]. Both environmental surveys and laboratory experiments have linked temperature to the survival of NoVs in

water: The virus is more prevalent in the northern hemisphere waters during colder months [54,55], and degradation in mineral and tap waters occurs more slowly at colder temperatures (4 °C and –20 °C) compared with ambient temperature [51].

Norovirus stability in soil

In addition to water, sediments and soils may serve as environmental reservoirs of human NoVs similar to other enteric viruses [56]. While NoVs have only occasionally been detected in environmental sediment samples [57], numerous studies have experimentally evaluated their survival in different soil types. Murine NoV stability studies in soil have demonstrated no significant reduction in viral concentration after 5 days, and retention of infectivity for at least 12 days [53,58]. Environmental conditions contributing to the longevity of NoVs in soils include lower temperatures and dry conditions [53,59]. Conditions such as soil composition, sunlight exposure, and rainfall influence survival of other enteric viruses [60–62], and are also likely to impact environmental survival of NoVs. A recent study by Bruggink and Marshall reported a significant correlation between higher rainfall amounts and increases in local NoV disease [63], suggesting that events causing sediment re-suspension (e.g., storms, tides, and strong winds) may also cause re-suspension of NoVs in water.

Direct person-to-person and aerosolized NoV spread

Evidence of direct person-to-person spread of NoVs is well supported by investigations of outbreaks where a significant, and sometimes sole, risk factor for becoming infected was contact with an infected individual. Person-to-person contact is also evidenced by the high secondary attack rate of NoVs among close contacts, ranging from 14–33% in representative outbreaks [64–67]. Another contributing factor to the high incidence of secondary infections is undoubtedly the extremely high levels at which NoVs are shed in feces; e.g., two representative studies detected as high as 10^{10} NoV genome copies per gram of feces using RT-PCR-based approaches [68,69]. There has also long been evidence for NoV spread through aerosolized vomitus particles.

Points of entry and movement from farm to fork

The majority of foodborne NoV disease results from consumption of fresh produce, molluscan shellfish, and RTE or prepared foods [70,71], all of which will be discussed in detail in the following sections. Entry of NoVs into the food chain occurs at most every point in the process including cultivation, harvesting, processing, preparation, and service of foods (see Figure 17.1). Although poor personal hygiene remains the leading contributor to the spread of NoV disease, many foods can become contaminated at the point of cultivation. Two food categories where NoV outbreaks are associated with contamination from the natural environmental are shellfish and produce.

Shellfish

Noroviruses are the pathogen most commonly implicated in shellfish-borne gastroenteritis outbreaks. Shellfish are exposed to NoVs when they are grown in fecally contaminated harvest waters. This contamination can occur through illegal dumping of sewage, failing septic systems in coastal communities, or the discharge of treated or untreated wastewater and sludge [72]. While most studies of shellfish-associated NoV outbreaks have inferred that shellfish are the source of infection based on epidemiological data collected from patients, at least one study has provided phylogenetic support for a direct chain of infection [48].

In particular, contaminated oysters are frequently implicated in NoV outbreaks across the globe. This is at least in part because of the minimal processing and cooking of oysters prior to consumption [64,73–77]. However, it should be noted that more heavily processed oyster meat has also been associated with multiple NoV outbreaks [78]. Another reason oysters are common sources of gastroenteritis-causing pathogens is their filter feeding nature that allows them to passively concentrate bacteria and viruses. While bacterial loads in contaminated oysters can be reduced 95% by simple depuration, one study observed only a 7% reduction of a NoV using the same purification process [79], suggesting that more stringent interactions are involved in the bioaccumulation of NoV particles in oysters. Indeed, it has been elucidated that oysters express a carbohydrate in their digestive tracts that is indistinguishable from a human HBGA, and that this facilitates a specific interaction between NoV particles and oyster tissue [80]. There is seasonal variation in the expression of this HBGA-like carbohydrate in oysters [81].

Produce and RTE foods

Produce has also been widely implicated in NoV outbreaks, particularly leafy greens and soft red fruits. As with shellfish, produce can become contaminated early in the food supply chain. For example, NoVs have been detected in groundwater used for irrigation [82], suggesting the possibility that irrigation waters may serve as a vehicle for transmission.

Food handling by a NoV-infected individual represents the primary point of virus entry onto produce as well as RTE foods, reflected by numerous direct links between symptomatic food handlers and foodborne NoV outbreaks. For example, NoV outbreaks associated with contaminated produce, e.g., salad, fresh cut fruits, and coleslaw [83–85], and RTE foods, e.g., boxed lunches, sandwiches, cake, pasta, potato salads, and deli meats [86–92] have all been linked to symptomatic food handlers. The spread of NoVs by food handlers is certainly facilitated by the high concentrations of NoVs shed by both symptomatically and asymptomatically infected people for weeks following infection [68,69,93–95], and a low infectious dose [96].

Infected food handlers can also contaminate food preparation surfaces and NoVs can survive on these surfaces for long periods of time. Determination of virus stability is challenging to assess for human NoVs due to the lack of a cell culture system and thus infectivity assays. It should be noted that many studies have attempted to correlate human NoV stability with RT-PCR detection of virus

genomes, but recent work with the cultivable MNV surrogate clearly argues against such an association [53]. Thus, we limit our discussion here to stability analyses of cultivable surrogate viruses. Both MNV and feline calicivirus (FCV) have been shown to remain infectious for weeks on food preparation surfaces especially at colder temperatures [53,97,98]. Furthermore, MNV is stable across an extremely broad pH range (pH 2 to 10) [97] and resistant to commonly used industrial sanitizers [99]. Underscoring the need for highly stringent disinfectant protocols in food handling environments, there have even been cases of outbreak initiation following transfer of a human NoV from purportedly cleaned food surfaces onto food [92]. These observations collectively underscore the stringency that food handlers must adhere to in order to prevent NoV transmission.

Approaches to food attribution

Multiple features of NoV infections complicate the identification of outbreak sources, including their numerous routes of transmission, high secondary attack rates, and environmental stability [100]. Even when a food product is implicated as the cause of an outbreak, the presence of a NoV can be difficult to confirm due to diagnostic challenges. In general, it can also be difficult to detect pathogens in foods due to the complexity of the food matrix and the low dose of the pathogen which can be distributed unequally throughout the product.

In spite of these challenges, improvements in molecular diagnostic techniques (reviewed above), increased communication among governments, and increased international food safety standards are resulting in better traceability of NoVs in foods. In particular, the high degree of genetic variability among NoV strains is being exploited to determine origins of foodborne outbreaks and to reveal common sources of geographically distinct NoV outbreaks, highlighting the effect of globalization of the food market on pathogen dissemination [100]. In one such example, a gastroenteritis outbreak in France was linked to oysters contaminated with a novel GII.b NoV; following their international distribution, oyster-associated GII.b outbreaks or cases were reported in Denmark, Finland, the Netherlands, Sweden, Italy, Belgium, Germany, the United Kingdom, Spain, and Slovenia (reviewed in [99]). There is a strong rationale for enhanced NoV attribution methods since identification of foods contaminated early in the supply chain (e.g., during cultivation or production) could reduce the occurrence of geographically disseminated outbreaks. Thus, this will surely be a continued area of active research in the near future.

Prevention and control

Food processing to eliminate noroviruses

Processing and cooking are the primary means to reduce microbial contamination of the food supply. These methods have only recently begun to be evaluated for their ability to reduce or eliminate NoV contamination now that surrogate

cultivable NoVs are available. The most common food processing technique used in industrial, commercial, and residential settings is basic *washing of food* prior to consumption. Washing of produce artificially contaminated with a NoV has been shown to reduce virus load, but the reduction was often minimal and varied depending on the disinfectant used in the wash water [101–104].

Refrigeration and freezing are other treatment methods commonly applied to produce, RTE foods, and seafood. However, NoVs can survive in food products for long periods of time at refrigeration temperatures with little or no reduction in virus levels [105], and the concentration of MNV on vegetables remains unaffected even after 6 months of deep freezing [102].

Heat treatment is one of the oldest and most widely used methods of food preservation and has shown some efficacy against NoVs: Both mild and hot pasteurization temperatures reduce concentrations of MNV and a human NoV, but mild temperatures were only sufficient if contamination levels were low [97,102,106]. Neither boiling nor steaming caused significant reductions of a human NoV in artificially contaminated mussels, but the read-out in this study was RT-PCR of viral genomes in contrast to a more relevant infectivity assay using a surrogate NoV [107].

Of the processing methods examined to date, the most effective in reducing NoV concentrations are *hydrostatic pressure* and *radiation treatment* although their effectiveness varies depending on the specific virus and food product tested. For example, FCV concentrations can be significantly reduced at low hydrostatic pressures in fruit juices whereas MNV concentrations are only reduced at high pressures [108,109]. High hydrostatic pressures are also required to reduce MNV concentrations and infectivity in oyster meat [110,111]. Electron beam radiation also reduces MNV infectivity when high radiation levels are applied; this processing technique was found to be significantly more effective when applied to virus suspended in liquid media compared with virus inoculated onto foods, suggesting that food matrices enhance NoV survival [112]. Collectively, NoVs are able to withstand many of the processing methods used to control pathogens in food. Even treatments that are able to reduce viral numbers may not be sufficient to prevent NoV disease given the low infectious NoV dose [96], highlighting once again the importance of preventing initial contamination of food products.

In the United States, foodborne pathogens are controlled through the use of a preventative approach called hazard analysis and critical control points (HACCP). HACCP is used as a means of pathogen detection at critical steps during the production of a given food product. For example, water quality is a critical control point in both harvest and post-harvest processing of fruits and vegetables because it is so widely used and can easily become contaminated. Although HACCP has been quite successful in reducing the distribution, and therefore the consumption, of certain pathogen-contaminated foods, it is important to recognize that most of the HACCP systems currently in place are designed to identify strictly bacterial pathogens. Consequently, the ability of HACCP to reduce NoV disease is questionable for two primary reasons: (1) NoV contamination of foods often occurs immediately preceding its consumption as a result of handling by an infected

individual; and (2) bacterial indicators are used to assess fecal contamination but these have a poor correlation to the presence of enteric viruses. There remains a critical need to establish testing for viral pathogens, particularly in fresh produce, seafoods, and RTE foods.

Advances in norovirus vaccination and development of therapeutics

There are currently no specific NoV therapies or licensed vaccines. However, there has been extensive investigation into the potential to use non-replicating virus-like particles (VLPs) composed of the NoV capsid protein as an immunogen to elicit protective immunity. In fact, [113] recently published promising results of a randomized, double-blind, placebo-controlled phase II clinical trial demonstrating modest efficacy for at least 3 weeks post-vaccination. There are currently no available specific NoV therapeutics and the most effective control measures during NoV outbreaks currently remain to be hand washing and isolation of infected individuals.

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CHAPTER

Hepatitis A

18

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Introduction

Viral hepatitis is an important global health problem associated with significant morbidity and mortality. Although there are at least five major and distinct human hepatitis viruses characterized to date, which are referred to as hepatitis A, B, C, delta, and E, only hepatitis A (HAV) and E viruses are primarily transmitted by the fecal-oral route, by either person-to-person contact or consumption of contaminated food or water. This chapter will provide information on the epidemiology, clinical symptoms, diagnosis, treatment, and prevention of HAV infection along with points important or unique to foodborne outbreaks.

Clinical features

HAV can cause an acute and usually self-limited infection. The average time from exposure to onset of symptoms for HAV infection is approximately 28 days (range: 15–50 days) [1]. The minimum infectious dose required for HAV infection in persons is unknown [2]. The likelihood of developing a symptomatic HAV infection is related to age: Only 30% of children less than 6 years of age have symptomatic infections; 70% of older children, adolescents, and adults manifest symptoms [3–5]. Symptomatic HAV infection may last on average 2 months, though prolonged or relapsing disease is possible for up to 6 months [6,7]. Symptomatic illness from HAV infection usually starts abruptly and is manifested by fever, malaise, nausea, anorexia, abdominal discomfort, dark urine, and jaundice [8]. HAV does not result in chronic infection or chronic liver disease. Persons with underlying chronic disease are at increased risk for developing an acute liver failure from HAV infection [9–11]. The case-fatality from HAV infection is 0.3–0.6% (with 1.8% among persons aged ≥50 years) [3]. Immunity after infection for HAV persists for life.

Because HAV infection is typically self-limited and does not result in chronic infection or chronic liver disease, treatment is supportive. Hospitalization may be necessary for patients who develop dehydration or fulminant hepatitis.

Foodborne Infections and Intoxications.

Microbiology

Hepatitis A virus (HAV) is a positive-strand, 27–32 nm in diameter, RNA virus of the Picornaviridae family [12]. Genotypes I and III are the most prevalent genotypes isolated from humans. Only a single serotype of HAV exists, despite genetic heterogeneity at the nucleotide level [13]. HAV displays a high degree of antigenic and genetic conservation and does not appear to accumulate the high frequency of genetic changes seen in many RNA viruses. HAV isolated from the first cases in an extended outbreak is genetically the same as HAV isolated from the last cases [14–16]. However, there is enough heterogeneity in several HAV genome regions to differentiate the relatedness of isolates circulating within and between communities over time, including within subgenotypes [16,17].

HAV is viable on fomites for about a week. HAV can also survive in water and sewage for months. HAV can survive on the surface of produce (lettuce, carrots, and fennel) for 4–9 days; washing significantly reduces the level but does not fully eliminate detectable HAV [18]. The virus can be killed by disinfection of surfaces with solutions containing bleach, quaternary ammonium, hydrochloric acid, formaldehyde, or glutaraldehyde or by heating the virus to 185 °F (85 °C) for 1 minute [19–21].

Viral hepatitis A, B, delta, C, and E are clinically indistinguishable; therefore, only laboratory tests would allow determining the etiology. Acute HAV infection can be confirmed by a serologic test detecting immunoglobulin (IgM) antibody to capsid proteins of HAV (also known as IgM anti-HAV). IgM anti-HAV typically is detectable 5–10 days before onset of symptoms. As false-positive IgM anti-HAV results have been reported in the past, current recommendations encourage limiting laboratory testing for acute HAV infection to persons with clinical symptoms of hepatitis A or known exposure to the virus [22,23].

IgG anti-HAV appears early in the course of infection and remains detectable for the person's lifetime, providing lifelong protection against the disease [24–26].

Nucleic acid amplification methods also are used to identify HAV RNA in the blood and stool during the acute phase of illness; however, these tests are performed only in a few research laboratories and are not used for diagnostic purposes [3]. HAV RNA sequencing and phylogenetic sequence analysis help to determine the relatedness of HAV isolates during epidemiologic investigations and are typically utilized during outbreak investigations [15,16,27].

Due to slow growth of HAV in cell culture, and generally low level of contamination in environmental samples, virus detection is feasible only with use of sensitive nucleic acid detection methods [13]. HAV has been successfully isolated from bivalve mollusks, oysters, mussels, and clams [13]. However, HAV detection in food is not included as a routine analysis during outbreak investigations due to long incubation periods—the implicated foods usually have been consumed or discarded by the time the outbreak is recognized.

Exposure pathways

The epidemiology of HAV infection has been well described, and the prevalence of HAV infection is generally associated with socioeconomic development and standards of hygiene among different populations [13]. HAV infection is endemic in many developing countries where most persons are infected during early childhood, developing asymptomatic infections, and therefore HAV transmission is often not recognized. In contrast, the prevalence of HAV is low in developed countries; hence, HAV infection typically occurs at older ages when clinical symptoms become more apparent [28]. Asymptomatic and non-jaundiced HAV-infected persons, especially children, are an important source of HAV transmission, often associated with outbreaks of HAV infection [29]. Presently in the United States, the most frequently reported risk factor for HAV infection is international travel; however, nearly half of all reported hepatitis A cases have no specific risk factor identified.

Food products can become contaminated with HAV at any point during cultivation, harvesting, processing, distribution, or preparation [2]. Unlike bacteria, which continue to replicate after the contamination of food, HAV that has contaminated food undergoes no further replication. It can be challenging to recognize a foodborne outbreak from surveillance data because of the long incubation period; imperfect or inconsistent recollection of food items consumed several weeks before the illness; the presence of asymptomatic illness; immunity because of past infection or vaccination; or because exposed persons may be geographically dispersed in more than one state [2]. In the United States, relatively few reported cases (only 2–3% cases per year) are identified as part of common source outbreaks of diseases transmitted by food or water [30]. When such outbreaks do occur, an infected food handler or an infected food source is typically identified [2,31]. Between 1992 and 2000 among 38,881 adults with hepatitis A, 8% of all cases were identified as food handlers, including 13% of the 3292 adolescents aged 16–19 years [2]. A person with HAV infection is considered to be contagious during the 1-week period before the onset of jaundice or elevation of liver enzymes and 2 weeks after, when the concentration of virus in the stool is highest [32,33].

In the past, hepatitis A infections and outbreaks have been associated with consumption of strawberries, green onions, and oysters and other shellfish [15,34–40]. Since 1988, there has been one large oyster-related outbreak of hepatitis A reported in the United States, which occurred in 2005. This was the first outbreak of hepatitis A in which an identical HAV sequence was obtained from both the implicated food product and the case patients in the United States [41]. Such a small number of oyster consumption-related outbreaks of hepatitis A since the late 1980s suggests that National Shellfish Sanitation Program guidelines have been effective in reducing HAV transmission via shellfish in the United States. Outbreaks of hepatitis A linked to consumption of oysters in the United States have been attributed to illegal

harvesting from sewage-contaminated areas [35,41] or unrecognized contamination of regulated harvest areas by sewage runoff associated with heavy rains and flooding [38,41]. Other HAV outbreaks linked to oysters were reported in Australia in 1997 and in France in 2007, with 444 and 111 persons acquiring HAV infections, respectively, after consuming contaminated oysters. The source of contamination of oysters in those outbreaks was sewage water [37].

Recently, two large foodborne outbreaks of hepatitis A have been reported in the United States. In 1997 frozen strawberries were implicated as the source of a multistate outbreak involving 262 persons [15]. Another large outbreak of hepatitis A occurred in 2003 in four states with 601 persons ill, 3 deaths, and at least 124 hospitalizations due to consumption of HAV-contaminated green onions imported to the United States [40].

Food handlers with IgM anti-HAV positive results are regularly identified through routine viral hepatitis surveillance. Food handlers with hepatitis A can infect several hundred patrons. Therefore such surveillance reports often lead to epidemiologic investigations to evaluate the magnitude of the risk of HAV transmission from the foodhandler to patrons. Of 816 outbreaks reported during 1927 to 2006 where food workers were implicated, hepatitis A was the second most common virus (after norovirus), causing 84 outbreaks involving 5046 cases [31].

Waterborne outbreaks of HAV are less common than foodborne outbreaks in the United States [2]. Nevertheless, unregulated and untreated water supplies if used in agricultural settings may pose a risk for contamination by foodborne pathogens, particularly when used for produce that does not undergo disinfection after harvesting [42].

Prevention and control

General measures for the prevention of hepatitis A include the maintenance of good personal hygiene standards, improved sanitation, provision of clean drinking water, washing of potentially contaminated foods, and use of immunoglobulin (IG) or hepatitis A vaccine [2,43].

IG is a sterile preparation of concentrated antibodies prepared from pooled human plasma processed by cold ethanol fractionation [44]. Prophylactic administration of IG provides passive protection against HAV infection for up to 6 months. When given within 2 weeks of exposure to the virus it is 80–90% effective in preventing the disease [3].

An effective and safe vaccine against hepatitis A became available in the United States during 1995–96 [43]. Vaccination programs have resulted in dramatic declines in hepatitis A cases in the United States from 31,582 (national incidence 12 per 100,000 population) in 1995 to 2979 (national incidence 1 per 100,000 population) in 2007 [30]. Almost all vaccine recipients develop protective antibody levels after one dose of vaccine with the second dose resulting in a high level of antibody production in all recipients [45–49]. Studies show that even 10 years after receiving the vaccine, 99% of persons have protective antibody levels and disease has not been recorded

among vaccinated people over 20 years after immunization [50]. Currently, universal vaccination against HAV is recommended for all children at age 1 year as well as for high-risk groups including men who have sex with men, users of illegal injection and non-injection drugs, persons who have underlying liver disease, or persons who frequently travel to countries where the virus is endemic [3,51].

Post-exposure prophylaxis (PEP) refers to an attempt to prevent or treat a disease after someone has been exposed to the pathogen. This can be achieved by administering vaccine (active immunization) or antibodies (passive immunization). When administered within 2 weeks of exposure, the vaccine provides protection equivalent to IG [52]. An additional benefit of hepatitis A vaccine is the long-term protection it provides. ACIP (Advisory Committee on Immunization Practices) recommendations for prevention of HAV infection state that for previously unvaccinated persons aged 1–40 years, a single dose of hepatitis A vaccine, within 2 weeks of exposure, is preferred to IG [51]. Efficacy of the vaccine administered after 2 weeks post-exposure has not been established. A second dose of vaccine should be administered according to the vaccine manufacturer's licensed schedule. Children under 1 year, due to lack of efficacy of the vaccine, and adults aged >40 years, due to lack of data, should continue to receive a dose of IG [51]. IG also should be used for immunocompromised persons, persons with chronic liver disease, and persons for whom vaccine is contraindicated [51].

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CHAPTER

Hepatitis E

19

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Introduction

Hepatitis E virus (HEV) causes waterborne epidemics and sporadic illness in developing countries with significant mortality among pregnant women. In developing countries, every year an estimated 20 million infections occur with 3.3 million symptomatic hepatitis E cases and 70,000 deaths [1]. However, in developed countries the prevalence of antibody against hepatitis E virus (anti-HEV) is high, but clinical disease is uncommon [2,3]. Over the last two decades locally acquired hepatitis E has occurred in many developed countries [4]. There is growing direct and circumstantial evidence that HEV infection may be transmitted by food [5–7]. HEV isolated from cases of locally acquired hepatitis E in the USA demonstrated close similarity to HEV isolates from domestic swine [8]. Furthermore, molecular epidemiologic investigations have linked hepatitis E to consumption of raw or undercooked meat and liver from pork, deer, and wild boar [5,7]. This chapter will provide data on the epidemiology, clinical laboratory diagnosis, and prevention of foodborne HEV infection.

Clinical features

HEV infection can result in a spectrum of illness ranging from asymptomatic infection to fulminant hepatitis. The minimum infectious dose of HEV has not yet been determined. However, studies of non-human primates have demonstrated that the clinical presentation, immunological response, severity of symptoms, and biochemical markers of liver damage increase with increasing inoculum size [9]. The incubation period of HEV infection ranges from 15 to 60 days (mean 40 days). The clinical presentation of hepatitis E is indistinguishable from acute illness caused by other hepatitis viruses. Hepatitis E is generally an acute disease with abrupt onset of non-specific symptoms followed by right upper quadrant pain, jaundice, anorexia, malaise, nausea, and vomiting. HEV infection in pregnant women is typically severe during the third trimester of pregnancy [10] with mortality rates ranging

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from 10–25% [11]. Persons with advanced liver disease, including cirrhosis, can develop acute hepatic failure when superinfected with HEV [12]. Chronic hepatitis E after infection with HEV genotype 3 has been reported among persons receiving immunosuppressive treatments following organ transplants [13]. The mortality rate from hepatitis E during an epidemic ranges from 0.5–4% [14].

Laboratory abnormalities in liver enzymes and liver function tests in persons infected with HEV are similar to those that occur with other forms of acute viral hepatitis and include elevated serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin, and γ-glutamyltransferase [15]. Abnormalities in liver function typically coincide with onset of clinical symptoms and resolution generally occurs within 1 to 6 weeks after onset of illness.

HEV infection elicits both immunoglobulin M (IgM) and G (IgG) antibodies against HEV. The IgM anti-HEV response is rapid, occurring about 1 month after infection and peaking at the time of onset of biochemical abnormalities and/or symptoms [16]. HEV RNA can be detected in both blood and stool.

There is no specific therapy for hepatitis E; treatment is supportive. The disease typically resolves within 4–6 weeks of the onset of symptoms, usually without any long-term consequences. To date immune globulin has not been demonstrated to be effective in preventing HEV infection [17].

Microbiology

HEV is a spherical, non-enveloped, single-stranded, positive-sense RNA virus that is approximately 32–34 nm in diameter [18]. The organization of the HEV genome is substantially different from other viruses and it has its own family, the Hepeviridae, genus *Hepevirus*, species HEV [19]. The HEV genome is arranged in three overlapping open reading frames (ORF). The three coding frames are used to express different proteins. HEV is relatively stable in the environment, and can be recovered from sewage samples [20,21]. Compared with the hepatitis A virus, HEV is less resistant to environmental conditions such as temperature. HEV is classified into at least four major genotypes 1–4 and 24 subtypes [18]. However, HEV has only one serotype. To date, genotypes 1 and 2 have been found only in human populations while genotypes 3 and 4 have been found in both human and animal populations.

Enzyme immunoassays (EIA) have been developed to detect IgM and IgG anti-HEV. During acute HEV infection, IgM anti-HEV becomes detectable days before the onset of symptoms and disappears over a 4–6 month period [22]. IgG anti-HEV appears soon after the IgM response. In addition to serologic assays, nucleic acid amplification methods can be used to identify HEV RNA in the blood and stool of infected individuals. HEV RNA is detectable in blood at the peak of abnormal liver function tests beginning about 2 weeks before onset of jaundice to a week after onset of jaundice. HEV RNA tends to appear in stool later than in blood and disappear from stool about 2 weeks after it became undetectable in blood [23]. These

tests are usually performed in specialized or research laboratories. Nucleotide sequence from the ORF 1 or ORF 2 can be sequenced to determine phylogenetic relatedness.

Exposure pathways

Foodborne or zoonotic transmission of HEV genotype 1 has not been established. Cross-species transmission studies found that human HEV genotypes 1 and 2 can be transmitted to non-human primates but not to other animals. In contrast, HEV genotype 3 has been experimentally transmitted from pigs to non-human primates and vice versa [24]. Zoonotic reservoirs for HEV genotype 3 and 4 have been demonstrated.

Use of molecular epidemiologic methods has suggested an association between consumption of undercooked pork, undercooked venison, and undercooked pig liver and hepatitis E in Japan and Germany. These studies identified phylogenetically similar HEV RNA from source food and clinical specimens from cases [18,25–31]. HEV RNA has been isolated from pig livers in grocery stores, suggesting that consumption of undercooked pig liver could result in exposure to HEV infection [32,33]. Figatellu, traditional pig liver sausage available in supermarkets in France, was found to have an HEV sequence similar to that recovered from patients who reported eating raw figatellu [25].

A case-control study identified consumption of shellfish as a risk factor for an outbreak of hepatitis E on a cruise ship [6]. The link with shellfish, however, was not confirmed by molecular epidemiological techniques. Another study demonstrated a significant relationship between anti-HEV seroprevalence and consumption of unwashed raw vegetables [34].

Although the epidemiology of waterborne HEV infection has been well described, little is known about foodborne hepatitis E. The epidemiology of hepatitis E varies distinctly between developing and developed countries [35]. The majority of acute hepatitis E cases in developed countries is imported by persons who travel to developing countries. In the late 1990s, locally acquired hepatitis E cases were reported from developed countries where the prevalence of an antibody against HEV was high but clinical disease was uncommon [36]. Locally acquired hepatitis cases in developed countries tend to be common among middle-aged or older men [37] and persons who consume excessive amounts of alcohol [36].

Prevention and control

In hepatitis E outbreaks, as in other fecal-orally transmitted infection outbreaks, the provision of clean drinking water and improving the sanitary disposal of human waste are the two most important prevention approaches. Based on limited observations that consumption of undercooked pig and boar liver, undercooked meat, and

undercooked venison is associated with hepatitis E, thorough cooking of such items is recommended. The incidence of foodborne hepatitis E is not well established and no specific interventions have been tested. As evidence for person-to-person transmission of HEV is increasing, it is prudent that strategies focused on reducing transmission from this route—such as soap and hand washing encouragement—be implemented in an effort to reduce transmission during outbreaks [35]. However, because the success of these interventions as assessed in outbreak situations is currently quite limited, the need to develop, test, and provide a reliable hepatitis E vaccine is imperative [38]. There are at least two hepatitis E vaccine candidates that have shown promise in clinical trials [39,40].

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CHAPTER

Astroviruses as Foodborne Infections

20

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Introduction

Astroviruses (AstV) are one of the leading causes of viral gastroenteritis in the young, elderly, and immunocompromised [1] and are estimated to be associated with ~15,000 cases of foodborne diarrhea each year in the United States [2]. This chapter will provide information on astroviruses including burden of disease, prevalence, and the potential role for food- and waterborne transmission.

Clinical features of illness

Human astroviruses (HAstVs) predominantly affect children under the age of 2, the elderly, and immunocompromised individuals and are thought to be involved in up to 20% of the cases of sporadic non-bacterial diarrhea and 0.5–15% of diarrheal outbreaks [3]. AstV gastroenteritis is usually a mild, self-limiting disease that does not require hospitalization. Clinical symptoms normally last between 2 and 4 days and consist of watery diarrhea and, less commonly, vomiting, headache, fever, abdominal pain and anorexia [4,5]. However, severe disease can occur, including intussusception (a form of intestinal obstruction in which a segment of the bowel prolapses into a more distal segment of the intestine) [6]; chronic diarrhea primarily in immunocompromised patients [7]; and necrotizing enterocolitis [8]. Several recent studies also suggest that HAstVs may be able to go systemic [9,10]. Of great concern, especially when considering food safety, is the fact that HAstV infections can be asymptomatic [11] suggesting that infected food handlers could unknowingly shed virus.

Microbiology

In 1975, two groups described 28–30 nm small round viruses (SRV) in the feces of infants with mild diarrhea and vomiting [12,13]. These viruses had a distinctive five- or six-pointed star-like appearance for which they coined the term "astrovirus"

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(*astron* meaning "star" in Greek). Similar viruses were also reported in diverse species of young animals with diarrhea [14]. These star-like projections are present in less than 10% of the viral population, suggesting that the use of electron microscopy for AstV diagnosis could result in under-reporting and misidentification [15]. In 1993, Monroe and colleagues sequenced the AstV RNA and proposed the classification of AstVs as a new family of RNA viruses designated as Astroviridae [16].

To date, AstVs are thought to infect in a species-specific manner and are classified based on the species from which the strain was isolated. Until 2008, human infections were thought to be limited to eight closely related HAstV genotypes now referred to as the "classic" HAstV (Figure 20.1). HAstV-1 appears to be the predominant circulating serotype worldwide followed by types 2–5 and occasionally 8 depending on the region. HAstV-6 and -7 are rarely detected [11,17–19]. To date there is no information on the association between disease symptoms and HAstV genotype.

In 2008 and 2009, a variety of new HAstV genotypes were identified through pathogen discovery, including MLB1 (AstV-MLB1; [20]), AstV-VA1 [21], and

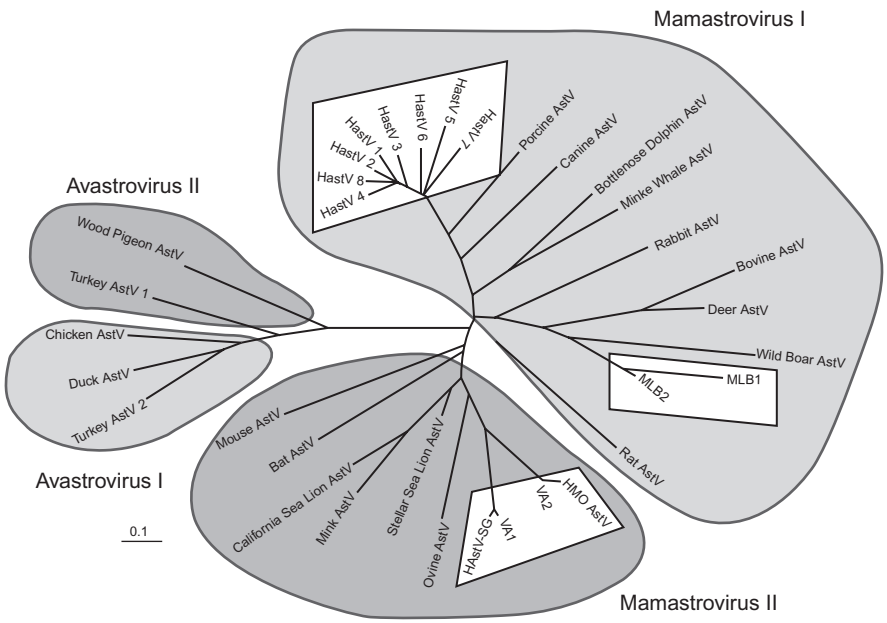

FIGURE 20.1 Phylogenetic tree of Astroviridae family.

The complete nucleotide sequence of the astrovirus capsid region (ORF2) was aligned using MEGA4 software utilizing the neighbor-joining method and a Kimura 2-parameter model with pair-wise deletion. This alignment clearly demonstrates the classification of the Astroviridae family into two genera: *Mamastrovirus* and *Avastrovirus.* Black boxes show the position of the current human astrovirus genotypes.

HMOAstV types A, B, and C [22]. Genetic characterization demonstrated that AstV-MLB1 was highly divergent from known HAstVs, comprising a new genogroup within the Mamastrovirus I family [20]. The AstV-VA and HMOAstV viruses are quite distinct from the classic HAstV and AstV-MLB viruses and cluster within the Mamastrovirus II family. Of concern is that it is evident that there are at least three clades of AstVs co-circulating in humans: classic, MLB1-like and VA1-like. It is possible that co-infection by two different genotypes in one person could lead to the generation of a new type of AstV as has occurred within the classic HAstV strains [23].

Diagnosis/Detection

Current detection strategies include RT-PCR and real-time RT-PCR applications using primers to the conserved region of the viral capsid protein or ELISA. However, most of the published and commercial assays are specific to the "classic" HAstV strains and fail to detect the new genotypes. Further complicating the issue, we currently have no *in vitro* culture system for the non-classic viruses. Thus, much of our prevalence data is based solely on the classic HAstV strains. With this information in mind, AstV prevalence ranges from ~10% of non-bacterial diarrheas with rates as high as 30% in some developing countries. Several epidemiological studies suggested that the highest rates of infection are in the winter months [17,19], although HAstV can also be detected in the spring and summer seasons [24].

Exposure pathways

Human volunteer studies demonstrated that HAstVs are transmitted through the fecal-oral route [25] and infectious particles can be detected in stool for months. AstVs can persist on dry, inanimate surfaces for up to 90 days [26] and, due to the lack of a lipid membrane, are exceptionally hardy to many commonly used forms of disinfection [27], suggesting they are incredibly stable in the environment. AstV particles are stable at a pH of 3 and resistant to chloroform and a variety of detergents and lipid solvents; hence they are resistant to food processing/preservation techniques that are designed to inhibit or inactivate other pathogens [1,28,29]. While chlorine-based disinfectants are considered to be the most effective against enteric viruses, AstVs are resistant to common levels of chlorine [30] and HAstV remains infectious for up to 10 minutes of heat treatment at 60 °C and viral particles appear to be stable at ultralow temperatures (−70° to −85 °C) for 6–10 years. Thus, these viruses are resistant to refrigeration and freezing and may be highly prevalent in undercooked or raw foods; however, virus particles may be disrupted by repeated freeze and thaw cycles [31].

Human enteric viruses such as AstVs are recognized as important pathogens in causes of waterborne illness [28]. HAstVs have been found in both untreated and

treated samples from wastewater facilities, indicating that current treatment methods may be inadequate to remove the virus from the water supply [32–34]. Once in the water supply, they are extremely stable and infectivity decreases only 10-fold after 30 days in groundwater samples [30].

HAstVs have been identified in urban wastewater and treated water samples in a number of countries [33–38] as well as in environmental [33,39] and surface water; in water used for irrigation [40]; and in water used for recreational purposes. Indeed, AstVs have been epidemiologically associated with drinking water and recreational waterborne disease outbreaks [41–43]. Enhanced surveillance using the most updated molecular diagnostic tools that recognize all the HAstV genogroups is required to truly understand the prevalence of AstVs in water and outbreaks associated with contaminated water supplies.

Obviously, poor water quality continues to be a threat to human health and may be a source of foodborne contamination as was found in a foodborne outbreak in Japan [44]. Foods may be contaminated with viruses in two ways. First, contamination may occur at the source of growing and harvesting. While no evidence is currently available on the prevalence of AstVs in consumer meat products coming from infected animals, there is evidence that the virus can be isolated from the muscle tissue of turkeys infected with a strain of turkey AstV [45]. However, it is unknown whether an AstV found in the muscle tissue or milk of an infected animal could cause disease in humans. Fruits and vegetables can be contaminated with polluted water or sewage runoff during irrigation or fertilization practices; however, no outbreaks from viral gastroenteritis being caused by AstVs have been reported from these sources [28].

One particular foodstuff, bivalve mollusks (oysters, clams, mussles, and cockles), is of particular concern as AstV-associated foodborne outbreaks of gastrointestinal disease. Due to the nature by which they are grown and harvested, molluscan shellfish are particularly susceptible to becoming fomites [28]. AstVs has been found in shellfish from Asia, Europe, and Africa, including oysters, clams, and mussels [46–49]. The majority of AstV contamination occurred in the winter months; however, AstV could be detected in the shellfish year round [46,48,49]. The prevalence of AstVs in shellfish is detailed in Table 20.1.

While the sampling sites for these studies varied, numerous locations were chosen because they were frequently impacted by contaminants; and these shellfish were often used for environmental monitoring rather than market production. One study investigated samples from direct-to-market growing and found that 16.4% of the mussels, clams, and oysters intended for human consumption were positive for AstVs. Indeed, AstVs was the only pathogen detected in oysters collected from growing areas classified for direct-to-market sale [47]. In terms of shellfish-associated gastroenteritis outbreaks, AstVs have been detected in both stool samples and outbreak-associated shellfish; however, up to seven different virus species could be detected in individual stool specimens and up to six viruses were detected in outbreak-related selfish so the exact cause of the outbreak remains unknown [51,53]. Finally, food contamination may also occur during preparation and handling, often stemming from infected food handlers [28].

Table 20.1 Reported Astrovirus Contamination of Shellfish

Country	Types of Shellfish	Production or Sampling Site	Environmental Conditions	Prevalence	Detected	Reference
Japan	Oysters	Naturally grown		15/112 (13.3%)	Winter	[50]
	Japanese clams	Supermarket		Not detected	N/A	[48]
Tunisia	Clams	Naturally grown		61%	Year round	[46]
	Mussels			23–25% of total tested		
Greece	Mussels		Market	24%		
Italy	Oysters			13.6%		
	Oysters			19.3% in environmental samples		
	Oysters			16.4% in direct market samples		
France	Oysters	Naturally grown		17%	Year round	[49]
	Mussels			50%		
	Oysters	Restaurant, private home production	Outbreak		Outbreak-associated	[51]
	Clams	Naturally grown	Environmental monitoring	14.7%	N/A	[47]
Spain	Mussels	Wild and cultured	B and C EC conditions	5.8% in wild mollusks		[52]
	Clam			Not detected in cultured mollusks		
	Cockles					

N/A = not available

To date, no studies have shown AstV contamination due to mishandling, but this may be due to under-reporting or limited detection techniques during an outbreak.

Prevention and control

The only way to prevent AstV infection is to remove the common source and to interrupt transmission. This is especially important in locations such as hospitals, daycare centers, and food preparation areas where person-to-person transmission

is likely to occur. Universal hygienic procedures can help to prevent the spread of these viruses; however, hand washing with soap and water is highly preferable to the use of alcohol-based antiseptics as AstVs are quite resistant to alcohol disinfection [54,55]. No antiviral therapy for AstV infection is currently available; however, potential immunization strategies are being explored.

While members of Astroviridae may not be associated with as much foodborne disease as caliciviruses, because of their prevalence, stability, and potential for asymptomatic transmission in adults, it is clear that they pose a public health threat and that a better understanding of foodborne outbreaks of AstVs is warranted. There are many obstacles to overcome if we want to truly understand the prevalence of AstV-associated food and waterborne disease, including (1) developing low-cost and rapid diagnostic tests with sufficient sensitivity to detect the presence of diverse AstVs in food and water sources; (2) formulation of guidelines to ensure the virological quality of selected commodities in specific scenarios; (3) understanding the role of animal AstVs in human disease; and (4) developing effective methods to inactivate AstVs in food and water sources. The cooperation of virologists with the food and water commodity groups will be needed to ensure that our food and water are safe from AstVs, and other enteric viruses, for future generations.

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CHAPTER

Rotavirus 21

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Introduction

Rotavirus is the most common cause of severe gastroenteritis in young children worldwide, leading to an estimated 2 million hospitalizations and 453,000 deaths every year among children less than 5 years of age [1,2]. Most of these deaths occur in Africa and Asia, where rehydration therapy is not readily available. Mortality due to rotavirus in developed settings is much lower, but health care utilization and costs are still substantial. For example, prior to the introduction of rotavirus vaccination in the United States in 2006, an estimated 55,000–70,000 hospitalizations, 205,000–272,000 emergency department visits, and almost half a million outpatient visits associated with rotavirus gastroenteritis occurred annually, resulting in a total cost to society of approximately $893 million, of which $319 million were direct costs to the US health care system [3].

Despite differences in sanitation and safe food and water, the incidence of rotavirus infections is similar in both developed and developing countries, and early exposure to rotavirus is universal. This suggests that person-to-person spread is the most important mode of transmission, and contaminated food and water play a secondary role. In the USA, rotavirus accounts for less than 1% of all foodborne illness, causing an estimated 15,433 gastroenteritis episodes and 348 hospitalizations annually [4]. In Australia, about 2% of rotavirus disease is thought to be foodborne, causing an estimated 4700 gastroenteritis episodes and 70 hospitalizations annually [5]. In 1995, the incidence of foodborne rotavirus disease in England was estimated to be 2.5 illnesses per 1000 person-years, with an estimated 8979 cases, 1497 general practitioner visits, 46 hospitalizations, and 4 deaths due to rotavirus foodborne disease occurring in that country in 2000 [6]. The economic burden of foodborne illness associated with rotavirus has been estimated recently for the United States. In 2010, rotavirus foodborne disease cost an average of $1154 per episode in direct health care expenses and lost productivity, equating to a total cost of $18 million [7].

Foodborne Infections and Intoxications.

Clinical features

Age distribution

The vast majority of symptomatic rotavirus disease occurs in young children, with disease incidence peaking in children between 6 months and 2 years of age. However, rotavirus can cause gastroenteritis among adults and the elderly, often among those who care for young children [8]. About 3–5% of hospital admissions due to gastroenteritis among adults are estimated to be due to rotavirus [9]. In the USA, approximately 24,000 rotavirus hospitalizations are estimated to occur annually among individuals aged 5 years or more [10].

Clinical presentation

In children, after a short incubation period of 1–3 days, disease begins with acute onset of fever and vomiting, followed shortly by profuse, non-bloody, watery diarrhea. Rotavirus gastroenteritis is a self-limiting illness, lasting 3–8 days. In moderate to severe disease, especially when vomiting precludes oral intake, diarrhea can lead to dehydration and electrolyte abnormalities, and may be life-threatening.

In adults, rotavirus infections are commonly asymptomatic or mild, likely due to immunity from previous infection. This could be particularly relevant in foodborne transmission, as asymptomatic food handlers may promote the spread of infections by unknowingly shedding rotavirus in their stool. However, it has been demonstrated that smaller quantities of rotavirus are shed during asymptomatic rotavirus infections [11]. When adults are symptomatic, clinical presentation is similar to that of children, although manifestations may vary [9]. Patients with human immunodeficiency virus infection or other immunocompromising conditions may develop persistent diarrhea or prolonged asymptomatic rotavirus shedding [12,13].

Microbiology

Rotavirus structure

Rotavirus, named for its wheel-like appearance (from the Latin word *rota* meaning "wheel"), is a non-enveloped virus belonging to the *Rotavirus* genus of the Reoviridae family. The rotavirus genome is composed of 11 segments of double-stranded ribonucleic acid (RNA), with each segment coding for a non-structural or structural viral protein (VP). The genome is protected by three concentric protein layers: the inner core, an internal capsid, and an outer coat (Figure 21.1). The inner core is composed of VP1, VP2, and VP3, and encloses the genome. The inner core is surrounded by an internal capsid, comprised mainly of VP6. The outermost layer, or outer coat, is made of VP7 and VP4, which form the protein shell and protruding spikes around the virion, respectively. VP7 and VP4 induce type-specific neutralizing antibodies involved in protective immunity, and are used to define serotype nomenclature.

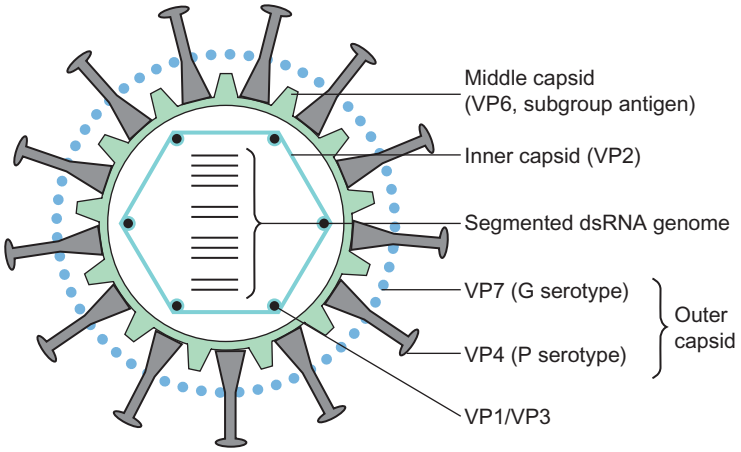

FIGURE 21.1

Schematic representation of a rotavirus particle structure [14].

Rotavirus classification

Rotaviruses are classified into serotypes based on the VP7 and VP4 outer capsid antigens. VP7, a *g*lycoprotein, is also referred to as the "G-type antigen," while VP4, a protease-sensitive protein, is referred to as the "P-type antigen." Ten G serotypes and 11 P genotypes are important for humans, with G1 and P[8] being the most common worldwide and in the USA. The segmented nature of the rotavirus genome allows for gene re-assortment, allowing for the possibility of multiple G and P protein combinations. For example, a novel rotavirus strain, thought to be derived from zoonotic (porcine) transmission with subsequent re-assortment, has been shown to cause illness [15]. However, interspecies transmission of rotaviruses between humans and animals is uncommon and rarely produces severe clinical disease.

Strain prevalence

Four strains cause the majority of infections around the world. Among samples collected between 1973 and 2003, the most common circulating strains associated with rotavirus gastroenteritis were G1P[8], G2P[4], G3P[8], and G4P[8], accounting for 88% of rotavirus gastroenteritis in children [16]. In recent years, G9 emerged as an important strain worldwide, and has been considered the fifth most important serotype globally [14]. Similar serotype prevalence has been seen in the USA, with G1P[8] also predominating [17].

Diagnosis

Given that the presentation of rotavirus and that of other enteropathogenic viruses and bacteria are similar, it is not possible to diagnose rotavirus disease exclusively

on clinical grounds. Laboratory diagnosis of rotavirus infections is fairly easy, however, as a large number of virus particles (10^{12} per gram of stool) are shed early during illness. The most widely used diagnostic tool is antigen detection in diarrheal stools by enzyme-linked immunoassay (EIA), which targets an epitope on VP6. EIAs are highly sensitive and specific, easy to use, provide results quickly, and are inexpensive. Molecular techniques, such as real-time reverse transcriptase polymerase chain reaction (RT-PCR), have been used mostly for strain characterization, and employ rotavirus-specific primers complementary to regions of VP4 and VP7.

Rotavirus detection in food and environmental samples is challenging. Although cell culture-based methods are available, viral detection by nucleic acid amplification has been increasingly adopted to test for rotavirus in food [18]. Molecular techniques have improved sensitivity and are faster compared with cell culture but have some limitations as well. They can only be done routinely in specialized laboratories, methods are not standardized for different food products, enzyme inhibition is a concern, and detection of nucleic acid cannot distinguish between infectious and inactivated viruses [19,20]. Advantages of PCR methods include detection of group B and C rotaviruses [21], and the option of strain typing, which can be used in outbreaks to establish links between cases and a suspected source [22].

Immunity

Repeated rotavirus infections occur throughout childhood and confer natural immunity. Each new infection provides greater protection against subsequent and severe disease [23]. Initial antibody responses to rotavirus are serotype-specific, with limited production of cross-protective antibodies. Subsequent infections increase heterotypic antibody responses [24].

Nearly all adults have antibodies against rotavirus, and thus symptomatic disease is relatively uncommon in this population. Illnesses do occur, however, and may be due to uncommon strains, poor heterotypic or waning immunity, or increased susceptibility to a virulent strain. As an example, a few rotavirus outbreaks among adults due to relatively less prevalent G2 strains have been reported in both the USA and Japan [22,25,26].

Exposure pathways

Rotavirus is largely transmitted person-to-person, via the fecal-oral route. However, several characteristics of rotavirus suggest the potential for transmission through food. The infectious dose is low (as few as 10 virus particles can cause infection), a high number of viral particles are shed in the stool, it is fairly stable in the environment, and to some extent it is resistant to disinfectants. For example, under controlled laboratory conditions at room temperature, simian rotavirus (SA-11)

has been shown to survive on lettuce up to 25 days [27], and for several hours in non-acidic fresh fruit juices [28]. SA-11 has also been shown to survive up to 10 days on aluminum surfaces, and up to 60 days at colder temperatures (4 °C) [29]. Finally, the level of rotaviral disinfection from environmental fomites that is achieved by multiple commercially available disinfectants may be suboptimal [30]. Despite these characteristics, however, the substantially fewer number of foodborne illnesses and outbreaks related to rotavirus compared with norovirus gastroenteritis likely reflect the fact that most adults are immune to rotavirus and do not play an equally important role in disease transmission.

Contamination of food with fecal material, leading to rotavirus foodborne illnesses, can occur anywhere along the farm-to-fork continuum, including viral contamination at the source (e.g., using polluted water for irrigation, crops being exposed to inadequately treated sewage sludge, or improper fertilization practices) or due to poor hygiene of food handlers [31,32]. For instance, rotavirus has been detected in lettuce and other vegetables [33,34], and 43% of infectious rotavirus particles placed on human fingers survive for 60 minutes, and can subsequently contaminate environmental surfaces [35].

Several examples of foodborne rotavirus outbreaks have been published. During April 2000, a rotavirus gastroenteritis outbreak occurred among adults in Japan after eating at a restaurant [26]. Another outbreak involving 55 individuals, suspected to be due to salad vegetables contaminated with rotavirus, has also been reported [36]. A recent report implicated a contaminated potato stew in a gastroenteritis outbreak in a mother and child sanatorium; comparison of rotavirus amplification sequences obtained from the suspected food vehicle and stool from an infected child were identical [37]. Finally, group C rotavirus was associated with a large outbreak in Japan affecting schoolchildren. This outbreak was suspected to be foodborne, as cases presented simultaneously, and because all affected children ate at a common food court [38].

Most documented viral foodborne outbreaks have been traced to an infected food handler. For rotavirus, this scenario has been illustrated in a few published reports. An outbreak of rotavirus gastroenteritis among college students in Washington, DC, was associated with eating deli sandwiches that were likely contaminated by a food handler [22]. In addition, two confirmed rotavirus foodborne outbreaks, reported to the Centers for Disease Control and Prevention's (CDC) Foodborne Disease Outbreak Surveillance System, have implicated a food handler. One occurred in Colorado in 2009 and involved 28 schoolchildren and a catering company; the second outbreak occurred in New York in 2005 and involved 30 children and adults at a banquet facility [39].

Specific food attribution for foodborne rotavirus outbreaks is limited due to scant reporting. Enteric virus outbreaks have been commonly associated with foods which are served raw or only lightly cooked, including fruits, vegetables, and salads. Food vehicles implicated in a few rotavirus outbreaks have included tuna and chicken sandwiches [10], salads [36], and a potato stew likely infected after preparation [37].

Prevention and control

Endemic childhood rotavirus disease

Given the tremendous burden of rotavirus disease worldwide, vaccine development became a public health priority in the late 1990s. Two rotavirus vaccines are currently recommended for use in children in all countries by WHO [40]. In the USA, where childhood rotavirus vaccination was introduced in 2006, a 64% reduction in the number of positive rotavirus tests at the CDC's surveillance laboratories [41] and a 60% decline in the rate of rotavirus-coded hospitalizations were documented by 2009 [42]. Furthermore, an unanticipated effect of rotavirus immunization has been the herd immunity conferred to unvaccinated older individuals. Small but significant reductions in rotavirus hospital discharges among adults and the elderly have been seen during the peak rotavirus month of March [10]. Thus, it is plausible that the observed overall decrease in circulation and transmission of rotaviruses may also result in a reduction of foodborne rotavirus disease, especially illness linked to food handler-associated transmission.

Foodborne rotavirus disease

A large proportion of foodborne rotaviral infections likely arise from a combination of an infected food handler and minimal subsequent processing of the implicated food. Thus, it makes sense that strict hygienic control would be most important to prevent foodborne viral outbreaks. This is particularly true for food handlers in contact with sick children, who must understand the possibility of asymptomatic transmission, the possibility of prolonged shedding, and the importance of methodical hand washing [19]. The 2009 Food Code currently recommends exclusion of symptomatic food handlers with vomiting and diarrhea until 24 hours after illness resolution [43]. Food handlers should be educated about microbial safety and hygiene guidelines at all levels of the food chain, including the agricultural, food manufacturing, catering, and food service industries.

At the agricultural level, primary and raw produce must be protected from contamination by human and animal waste, and water for irrigation of crops should be of adequate quality. In order to decrease contamination during and after processing, food manufacturing industries should follow viral food safety management systems and encourage handlers to follow food safety guidelines [19]. Catering and food service industries should emphasize good hygiene practices of food handlers. Environmental spread in kitchens can be avoided using effective disinfectants like hypocholorite or 70% ethanol solutions. Individuals and consumers should follow the WHO recommended five keys to safer food [44] that include washing hands and sanitizing surfaces and utensils, cooking food thoroughly, and using safe water to wash raw materials.

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CHAPTER

Sapovirus

22

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Introduction

Although far less is known about sapoviruses than other enteric viruses, such as noroviruses and rotaviruses, there is increasing evidence that they contribute to both sporadic cases of gastroenteritis and outbreaks. Since their discovery 35 years ago, sapoviruses have been generally thought to cause relatively minor disease almost exclusively among young children [1]. However, increasing use of molecular-based diagnostics have improved understanding of the impact of sapoviruses on human populations, including outbreaks in a variety of settings, sporadic disease across all age groups, and a worldwide distribution [2–4]. Sharing many similarities with the closely related noroviruses, which are recognized as a leading cause of foodborne disease, sapoviruses may also pose a risk of disease through contaminated foods. With over one-third of all foodborne disease outbreaks reported in the United States lacking either a confirmed or suspected etiology [5], some of these outbreaks may in fact be due to sapoviruses. Thus, sapoviruses merit further attention and investigation to better appreciate their relative role in foodborne disease and identify potential opportunities for prevention.

Clinical features

Sapovirus infection manifests with typical viral gastroenteritis symptoms, characterized primarily by diarrhea and vomiting often accompanied with fever, nausea, and abdominal cramps. Diarrheic stools tend to be watery and non-bloody, which can help distinguish viral infections from some common enteric bacterial infections. A prospective study reported diarrhea in 95% of sapovirus gastroenteritis patients, vomiting in 60%, and fever in 43% [6]. Sapovirus infections may also be asymptomatic, while shedding copious amounts of virus (10^6–10^9 viral copies per gram of stool), a feature that is particularly problematic when occurring among food handlers [7].

Foodborne Infections and Intoxications.

Sapovirus gastroenteritis is clinically indistinguishable from other forms of viral gastroenteritis, such as those associated with norovirus and rotavirus infections; however, symptoms of sapovirus disease tend to be milder with a lower average severity score [1,8,9]. The incubation period for sapovirus is similar to that of norovirus, with illness onset usually occurring 12–48 hours after exposure. Illness duration can vary, perhaps based on host factors, with reported median durations ranging from 2–6 days [1,6]. Virus is typically shed in the stool for up to 2 weeks after illness onset [6,10], although some infected individuals may shed high volumes of virus ($\geq 10^5$ viral copies per gram of stool) for as long as 4 weeks [11]. It is unclear, however, what role prolonged post-symptomatic shedding plays in sapovirus transmission. No long-term sequelae of sapovirus infection have been reported.

Sapoviruses were previously considered to be exclusively pediatric pathogens; however, there is increasing evidence that sapoviruses cause disease across all age groups. Sapovirus disease burden estimates have been limited by the availability of clinical diagnostics, so there are few published reports of sapovirus incidence. A US study estimated nine sapovirus cases in the community and one sapovirus-associated outpatient visit each year per 1000 population, representing 2% of all acute gastroenteritis among all ages [12]. Simlarly, a prospective study in the UK among all age groups estimated sapovirus incidence at 26 community cases and 2 cases presenting to general practice per 1000 person-years [13]. Sapoviruses appear to be a common infection in infants with seroprevalence similar to that of genogroup II noroviruses [14], and infections are often mild or asymptomatic [9]. A prospective study of Finnish children <2 years of age reported sapoviruses in 9% of sporadic gastrointestinal illness, compared with rotavirus in 29% and norovirus in 20% [1].

Outbreaks of sapovirus illness have been reported in a variety of settings including schools [15], child care facilities [16], adult long-term care facilities [4], and hospitals [10], as well as occasional foodborne outbreaks [17]. The broad age range of individuals affected in these outbreaks suggests that sapovirus infection is not restricted to young children.

Microbiology

Viruses with typical cup-like depressions (*calyx* means "cup" in Latin) similar to previously characterized animal caliciviruses were first described by different groups in the UK and in Sapporo, Japan, using electron microscopy of stool samples from children with gastroenteritis (Figure 22.1) [18,19]. The Sapporo virus became the prototype strain for the "Sapporo-like viruses" or "classical caliciviruses," known now as sapoviruses. The family Caliciviridae includes small, non-enveloped single-stranded positive-sense RNA viruses divided into five genera: *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus*, and *Nebovirus* [20]; only *Norovirus* and *Sapovirus* include human pathogens. The sapovirus genome contains two major open reading frames (ORFs) and encodes the major capsid protein (VP1) contiguous with the large non-structural polyprotein in ORF1 and a relatively small ORF near the 3′-end of the genome that encodes the minor structural protein (VP2) [15].

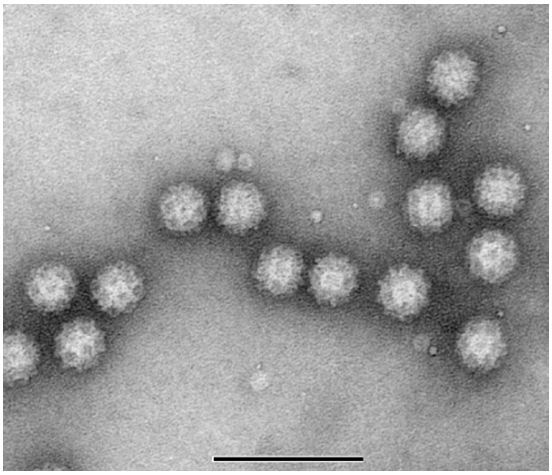

FIGURE 22.1

Negative stain electron micrograph of sapovirus particles with cup-like depressions or typical calicivirus morphology; bar marker represents 100 nm.

Courtesy of Charles Humphrey and Jackie Noel, Centers for Disease Control and Prevention.

Sapoviruses are divided into seven genogroups, four of which (genogroups I, II, IV, and V) contain viruses that infect humans, while GIII, GVI, and GVII infect porcines (Figure 22.2) [21]. Like noroviruses and other RNA viruses, new sapovirus variants may emerge and cause an increase in outbreaks. In recent years, genogroup I genotype 2 viruses have been associated with an increase in outbreaks among the elderly across Europe [22]. Both inter- and intra-genogroup recombination events may underpin the molecular evolution of sapoviruses [23].

TaqMan-based reverse transcription-polymerase chain reaction (RT-PCR) assays that allow simultaneous detection and quantification have largely replaced electron microscopy and conventional RT-PCR for the detection of human sapoviruses [24]. Several enzyme immunoassays for detection of sapovirus antigens have also been developed [25–27]; however, when compared with RT-PCR, these assays exhibit poor sensitivity and are generally not recommended for clinical diagnosis of sporadic gastroenteritis cases [26]. No sapovirus RT-PCR assays are cleared by the US Food and Drug Administration (FDA) for clinical laboratories so their use is generally restricted to public health laboratories and research settings.

Exposure pathways

Sapoviruses are transmitted primarily through direct person-to-person contact via the fecal-oral route, although indirect transmission through contaminated food, water, or environmental surfaces is also possible. Transmission may also occur from ingestion of vomitus. Foodborne transmission is thought to cause a very

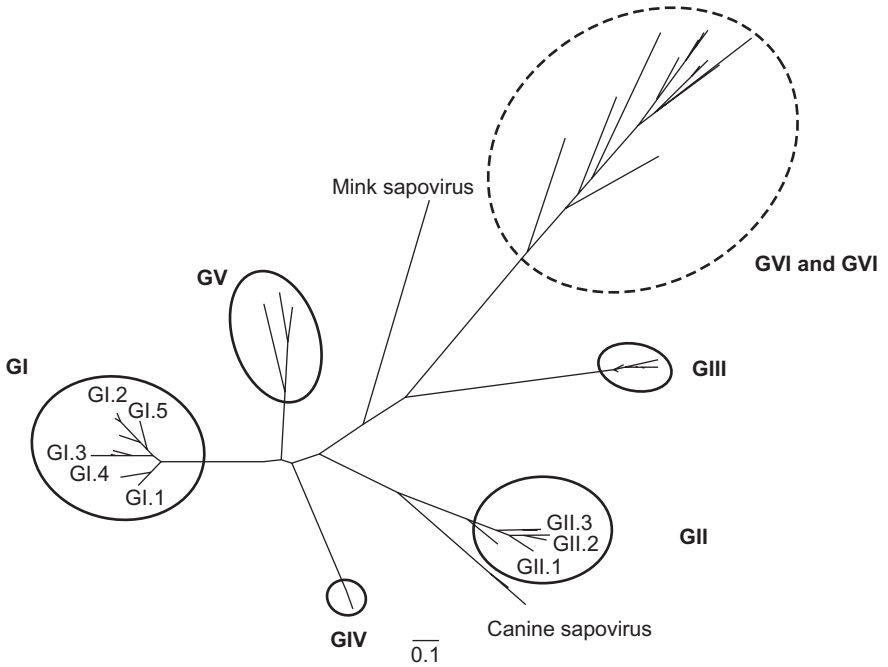

FIGURE 22.2

Classification of sapoviruses into at least seven genogroups (GI–GVII) based on complete VP1 sequencing; human strains cluster within GI, GII, GIV, and GV. Porcine strains cluster within GIII and GVI/GVII (dashed circle).

Courtesy of Everardo Vega and Jan Vinjé, Centers for Disease Control and Prevention.

small proportion (<1%) of all sapovirus disease [28]. A case-control study in the Netherlands identified contact with persons with gastroenteritis as the only significant risk factor for sapovirus disease, further underscoring the importance of person-to-person transmission [29]. There is no evidence of zoonotic or foodborne transmission of porcine sapoviruses to humans.

Sapoviruses may enter the food supply at any point along the farm-to-fark continuum, although, like other enteric viruses, most foods are thought to be contaminated by an infected food handler, who may or may not display symptoms [30]. Sapovirus may also be introduced onto foods during production if sewage-contaminated waters are used for growing or irrigating products consumed raw, such as shellfish and fresh produce. A Japanese study identified sapoviruses in raw market oysters that were genetically similar to those found in fecal specimens from gastroenteritis patients and wastewater samples from the same region [31]. Oysters contaminated with gross sewage often contain an assortment of viruses from the community, and thus may result in outbreaks associated with multiple viruses or genogroups [32]. Like all

foodborne viruses, sapoviruses do not propogate outside of their human hosts, thus viral amplification does not occur on contaminated foods.

Attribution of sapovirus disease to specific foods is limited by the dearth of foodborne sapovirus outbreaks reported. Furthermore, as with norovirus outbreaks, it is often difficult to implicate a specific food in an outbreak when an infected food handler is involved who may have contaminated multiple food items. The most commonly implicated foods in norovirus outbreaks are leafy vegetables, fruits, and shellfish, although essentially any ready-to-eat food item may cause disease if contaminated after the final cooking steps [36]. With more widespread use of sapovirus diagnostics during foodborne disease outbreak investigations, the etiologic role of sapoviruses and attribution to specific food vehicles may become better elucidated.

Prevention and control

Efforts to prevent sapovirus disease are directed at interrupting the person-to-person transmission cycle, even in the case of foodborne disease. Sapoviruses and other causes of viral gastroenteritis are essentially transmitted via the ingestion of infectious fecal (or, less commonly, vomitus) material, so standard santitation and hygienic precautions are key steps. These include frequent hand hygiene, environmental disinfection, proper disposal of fecal- or vomit-soiled materials, and limited contact with ill persons. Washing with soap and water is the preferred method of hand hygiene to prevent norovirus and sapovirus transmission, with alcohol-based hand sanitizers useful only as an adjunct when hands are not visibly soiled. Whenever possible, contact with ill persons should be limited during the period of peak infectiousness (acute illness through 48 hours post-recovery). Parents or other individuals changing soiled diapers should practice heightened hygiene precautions.

Food handlers, as well as health care workers, should be excluded from work until at least 48 hours after symptom resolution. Shellfish should be adequately cooked and fresh produce washed thoroughly before consumption, given the potential for these foods to be contaminated during production. Potentially contaminated environmental surfaces should be disinfected using a chlorine bleach solution with a concentration of 1000–5000 ppm (1:50–1:10 dilution of household bleach [5.25%]) or other approved disinfectants.

No specific treatment exists for sapovirus gastroenteritis. Similar to that for other causes of viral gastroenteritis, recommended supportive treatment includes therapy for dehydration and electrolyte imbalances. First line therapy for uncomplicated viral gastroenteritis should be oral rehydration solutions, while severe dehydration or shock may warrant intravenous fluid therapy [33]. Antiemetics, antimotility agents, and antibiotics are generally not recommended [34,35]. Interestingly, sapovirus episodes observed during a rotavirus vaccine trial were less severe and of shorter duration in vaccinated children, although the vaccine had no impact on overall sapovirus incidence [8]. There are no sapovirus vaccines currently on the market or in advanced-stage clinincal trials.

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SECTION

4 Foodborne Infections: Parasites and Others

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CHAPTER

Toxoplasma gondii 23

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Introduction

Toxoplasma gondii is an intracellular coccidian parasite and one of the most successful parasites worldwide. Sexual reproduction occurs only in felids, but virtually all warm-blooded animals can act as intermediate hosts. In humans, both congenital and acquired toxoplasmosis can cause major health problems. Based on the disease burden *T. gondii* ranked second out of 14 foodborne pathogens in the USA [1], and first in the Netherlands [2]. The parasite is a major cause of abortion in sheep, and thereby the cause of substantial economic losses.

Toxoplasmosis

Toxoplasma gondii was discovered in 1908 by Nicolle and Manceaux in Tunis and Splendor in Brazil. The first report of what was probably a case of congenital toxoplasmosis was described by Janků in 1923, but it took until 1939 for *T. gondii* to be conclusively identified as a cause of human disease (as reviewed by [3]).

Congenital toxoplasmosis

If a woman is infected for the first time during pregnancy, the parasite may be transmitted to the fetus. The incidence of primary infection during pregnancy varies roughly between 1 and 10 per 1000 pregnancies, and subsequent transmission occurs in about 29% of the cases [4]. The probability of transmission increases with gestational age, while the severity of the effects decreases. Although most children are asymptomatic at birth, transmission can result in death of the fetus, central nervous system abnormalities, or chorioretinitis. In Europe the risk of fetal death is estimated at 2%, the risk of ocular lesions in the first year of life at 14%, and that of intracranial lesions at 9% [4]. About 75% of congenitally infected children are born asymptomatic, but these children are at risk of developing chorioretinitis later in life. The incidence of ocular toxoplasmosis in the first 4 years after congenital infection is much higher in Brazil than in Europe (0.334 vs. 0.076/child-year at risk) [5].

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Acquired toxoplasmosis

T. gondii infections occur worldwide although the prevalence varies regionally. Foci of high seroprevalence (>40%) exist in Latin America, parts of Eastern/Central Europe, the Middle East, parts of Southeast Asia, and Africa; relatively low seroprevalences (<20%) are reported in North America, China, and Scandinavia [6]. In immunocompetent individuals the acute phase of *T. gondii* infection usually passes asymptomatically or with transient lymphadenopathy and mild flu-like symptoms. Severe outcomes such as encephalitis, pneumonia, myocarditis, or disseminated infections are uncommon. In immunocompromised individuals—like patients treated with corticosteroids or cytotoxic drugs, i.e., patients with hematological malignancies, transplants, or AIDS—these severe outcomes are more common and potentially fatal.

In the past all cases of ocular toxoplasmosis were considered sequelae of congenital transmission. Nowadays, it is suggested that at least two-thirds of ocular toxoplasmosis cases were acquired post-natally [7]. The incidence of eye disease in immunocompetent people infected with *T. gondii* is uncertain. Data are limited to those from a large waterborne outbreak: In the course of a year, 20 patients presented with ocular toxoplasmosis, which is estimated to reflect 0.3–0.7% of the infected individuals [8]. The incidence and severity of ocular toxoplasmosis is higher in Brazil, and may depend on *T. gondii* genotype [9].

T. gondii has a predilection for nervous and brain tissue, and in mice *T. gondii* infection appears to specifically reduce their fear of cats (e.g., [10,11]. In humans *T. gondii* infection has been associated with schizophrenia [12], Alzheimer disease [13], traffic accidents, and behavioral changes [14], although the cause-effect chain has not yet been conclusively shown.

A series of fatal cases of *T. gondii* infection in immunocompetent individuals was reported from French Guyana [15]. This may be explained by poor adaptation of humans to strains of *T. gondii* circulating in a forest-based cycle, and shows that newly emerging strains are a potential threat to human health.

Toxoplasmosis in animals

Similarly to *T. gondii* infection in humans, the infection usually remains asymptomatic in most animal species, including cats. Congenital transmission resulting in abortion or offspring born with abnormalities is the most commonly observed problem, and *T. gondii* is an important cause of abortion, especially in sheep [16]. For Australian marsupials [17] and New World monkeys [18] primary infection with *T. gondii* is often fatal. Female Pallas cats can transmit *T. gondii* to their offspring when chronically infected, which often leads to fatal toxoplasmosis in kittens [19]. In addition, some pigeon breeds or species are highly susceptible to clinical toxoplasmosis, and canaries show an unusually severe eye infection with symptoms varying from blindness to complete ocular atrophy [20].

Diagnosis and detection

None of the clinical signs can be considered pathognomonic, and various laboratory techniques are used to aid diagnosis. Serologic (e.g., Sabin–Feldman dye-test,

ELISA, IFA, and immunoblotting), microscopic, and PCR-based techniques are available. Bioassay in mice or cats and tissue culture are uncommon methods nowadays. Detection of parasitic DNA in blood is only possible in a short period of time and cannot be used for routine diagnostics. IgG antibodies may persist lifelong, thus detection demonstrates prior exposure, without information on timing. Although presence of IgM and low IgG avidity is suggestive of recent infection, persistence of IgM is common and maturation of avidity can be delayed [21]. Demonstration of a significant increase of IgG titers in two consecutive serum samples is more conclusive to determine active infection. To diagnose fetal infection, amniotic fluid or placenta can be tested by PCR or bioassay. Post-natally, IgM detection in neonatal blood and comparison of mother and child immunoblotting patterns can be applied.

In immunocompromised individuals and cases of ocular toxoplasmosis, clinical signs usually result from reactivation of a latent infection, thus serology is of limited use in these cases. For ocular toxoplasmosis, an algorithm involving clinical presentation, detection, and comparison of antibody levels in serum and intraocular fluids, immunoblotting, and PCR has been proposed [22]. To diagnose toxoplasmosis in immunocompromised patients, blood, CSF, or biopsies are tested by PCR, microscopy, or bioassay.

To detect *T. gondii* infection in livestock, serologic assays can be applied. With the exception of cattle [23], the presence of antibodies and tissue cysts is assumed to correlate well. Meat or tissue samples can be tested by bioassay or PCR. However, because the concentration of tissue cysts in meat is low, large samples need to be tested and standard DNA isolation methods do not suffice. Detection of oocysts in cat feces, water, or soil samples is based on concentration (e.g., sedimentation-flotation or antibody-based magnetic capture) followed by microscopy, PCR-based detection, or bioassay [24]. *T. gondii* oocysts cannot be differentiated from *Hammondia* or *Neospora* oocysts morphologically, thus molecular techniques need to be applied.

Microbiology

Nicolle and Manceaux named *Toxoplasma gondii* after its bowed shape (toxon = bow, plasma = life) and the original host (the gundi). *T. gondii* is the only known member of the genus *Toxoplasma*, but as a protozoan parasite it belongs to the phylum of Apicomplexa together with other coccidian species, piroplasms, and plasmodia.

Life cycle

The life cycle of *T. gondii* includes both sexual and asexual multiplication (Figure 23.1). Sexual multiplication takes place in the gut of felines, making them the definitive hosts [26]. If a cat ingests a *T. gondii* infected prey animal or meat,

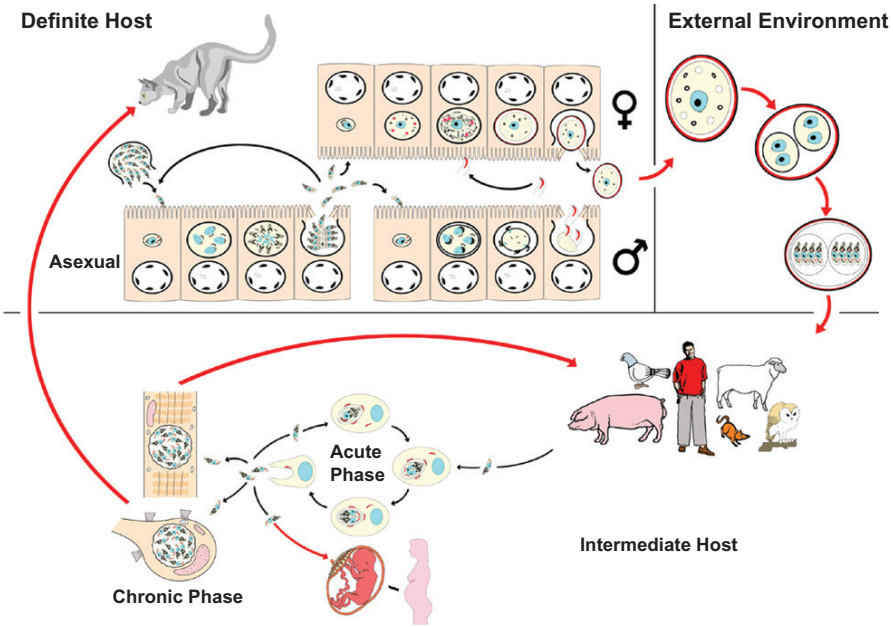

FIGURE 23.1 The life cycle of *Toxoplasma gondii*.

Key: bold arrows, parasite movements between hosts; thin arrows, parasite developmental stages within host.

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bradyzoites are released from the tissue cysts contained in their meal. In the previously uninfected cat, these bradyzoites invade epithelial cells of the cat's small intestine, where they start multiplying. After five stages of multiplication, macrogamonts and microgamonts are formed. Upon fertilization, a zygote and an oocyst wall are formed. The zygote nucleus divides and two sporoblasts, with two nuclei each, are formed. As the epithelial cells rupture, millions of oocysts are discharged into the intestinal lumen and eventually shed. Depending on temperature and humidity, sporoblasts sporulate within 1 to 5 days to become infectious sporozoites.

Sporulated oocysts, containing eight sporozoites, are infectious to cats [27], and to a range of intermediate hosts; probably all warm-blooded animals can be infected. If an intermediate host ingests sporulated oocysts or tissue cysts, sporozoites or bradyzoites will be released into the gut lumen and pass through the gut epithelium to enter cells in the lamina propria. Sporozoites and bradyzoites then transform into tachyzoites that rapidly divide inside host cells until released, and continue to infect neighboring cells. Tachyzoites disseminate through the body, and finally transform into slowly dividing bradyzoites

surrounded by a cyst wall, especially in nervous and muscle tissue. Tissue cysts seem to remain present lifelong in most hosts, although individual cysts are thought to rupture occasionally. Both felines and intermediate hosts are susceptible to infection via tissue cysts, which means that intermediate hosts are infectious to each other.

If a host is primary infected during pregnancy, tachyzoites may be transmitted transplacentally to the fetus. This is mainly of clinical importance. However, it has been shown that congenital transmission in mice (e.g., [28,29]) and possibly sheep (e.g., [30]) may happen from chronic infection. This way, a population of mice can remain *T. gondii* positive for years without an external source of contamination, thus sustaining parasite survival.

Genetic variation

Early genetic studies demonstrated that mouse virulent strains all belonged to a single clonal genotype. Typing more strains showed a highly structured population with nearly all strains belonging to one of three clonal lineages (types I, II, and III) [31]. More recently, *T. gondii* (especially from South America, but to a moderate extent also from Africa and the Middle East) has been shown to be genetically more divergent; a new type that was isolated from sea otters in California (and afterwards also shown to be present in terrestrial carnivores) is now considered dominant in wildlife in North America [32]. Nowadays high clonality and low diversity is associated with a high degree of anthropization and a dominance of domesticated cats and intermediate hosts [33]. Currently 14 separate haplogroups are recognized, but variation is partitioned geographically and large regions of the world remain underrepresented in current surveys of genetic diversity [34].

Subtyping

The genome of *T. gondii* is about 65 Mbp long, haploid, and spread over 14 chromosomes [35]. For most loci only two alleles exist, one of which is shared by two clonal types. Because of shared alleles and sexual recombination, multilocus typing is essential for strain differentiation. Typing techniques vary but are usually based on restriction fragment length polymorphism (RFLP) analysis, length polymorphisms for microsatellites, or single nucleotide polymorphisms detected by sequencing. Sequencing provides the greatest resolution for estimating genetic diversity [34].

Exposure pathways

Sources of infection

Like all intermediate hosts, humans can be infected by congenital transmission of tachyzoites and by ingestion of oocysts or tissue cysts.

Oocysts

Infectivity of cat feces was demonstrated in 1965 [36] and led to the identification of sexual development of *T. gondii* in the cat intestines (reviewed by [37]). Oocysts are spread in the environment by vectors, wind, or rain and transported to water by runoff. They can survive up to 18 months in soil and up to 54 months in water at 4 °C, and up to 28 days of freezing at −21 °C [24]. People may ingest oocysts while cleaning the cat litter box more than one day after use, from consuming unwashed fruits and vegetables, from contact with soil, and from drinking untreated water. Dogs [38,39] and raw shellfish and other filter feeders [40] may play a role in transmission of cat-shed oocysts to humans. Aerosolized transmission of oocysts has been suggested in an outbreak of toxoplasmosis [41].

Tissue cysts

Carnivorism was suggested as a possible mode of *T. gondii* transmission as early as 1937 [42] and confirmed in 1965 by a high incidence of *T. gondii* infection in a hospital where a raw meat diet was given therapeutically [43]. All warm-blooded animals can harbor viable tissue cysts, but they have been detected predominantly in sheep, pigs, and poultry with outdoor access and various wild animals, and are considered very rare in cattle. If infected meat is consumed without prior freezing or proper heating (core temperature over 67 °C) *T. gondii* can be transmitted [44]. Salting, fermenting, drying, and smoking also reduce tissue cyst viability, but the exact conditions needed to inactivate *T. gondii* are less well established.

Milk

Raw milk can contain *T. gondii* parasites. It is generally thought that milk will contain mostly tachyzoites, which are less resistant to pepsin digestion [45] and therefore considered less infectious. Nonetheless, cases in humans due to consumption of raw milk, specifically goat's milk, have been reported [46–49].

Other sources

In addition to these natural routes, infection is possible in laboratory accidents and through organ transplantation or blood transfusion. Transmission by organ transplantation is most likely with heart transplants [50]. Although there is a parasitemic phase in infection, reports of transmission via blood or leucocyte transfusion are scarce [51].

Source attribution

To set up effective strategies to reduce human risk of infection, the relative importance of the potential sources needs to be clarified. There are different approaches to source attribution (as detailed in Chapter 2 and by Pires et al. [52]). Those applied in the field of *T. gondii* are detailed below.

Risk-factor analysis

A review of risk-factor analyses showed that very few report population attributable fractions [53]. In a case-control study that included five European centers it was

estimated that, depending on the center, 30–63% of the infections were acquired through the consumption of meat and 6–17% was soil-borne [54]. In North America the strongest risk factors are also related to consumption of rare meat [55]. Outside Europe and the USA waterborne infections are important.

Outbreak investigation

Combining data from Smith [56] and AFSSA (2005), 27 outbreaks of *T. gondii* infection have been reported. These outbreaks were attributed to raw goat's milk (5), lamb or mutton (5), beef (3), pork (1), wild boar (1), venison (1), unspecified meat (5), geophagy (2), water (2), and contact with infected cats (2).

Risk assessment

In a microbial risk assessment, information on the prevalence and concentration of a pathogen in the sources is combined with the number of effective contacts with those sources. To calculate the number of predicted infections from the exposure, information on the dose–response relation is required. Depending on *T. gondii* strain and the animal model, the dose resulting in a 50% probability of infection varied from 1.8 to 2529 bradyzoites and from 0.47 to 3.7 oocysts (AFSSA, 2005).

Because the concentration of tissue cysts in meat is low, bioassay was considered the only method sensitive enough to detect tissue cysts. Because bioassay results are not quantitative, *T. gondii* risk assessments were limited to a qualitative study that has shown the effectiveness of freezing, heating, and salting in inactivating *T. gondii* [57]. Recently a sensitive PCR-based technique for quantitative detection of *T. gondii* in meat has been developed, and the results were used for a quantitative risk assessment for meatborne *T. gondii* infection in the Netherlands [58]: 40% of all predicted infections were attributed to unheated meat products. Preferably, environmental transmission should be included in the assessment. However, detection of *T. gondii* oocysts in soil and water samples has limited sensitivity that depends on the type of sample [24]. Estimation of oocyst contamination levels based on cat population size, incidence of *T. gondii* infection, the amount of oocysts produced upon infection, and the fraction of feces deposited outside might be more feasible. The annual quantity of oocysts deposited on US soil was estimated at 779–1328 oocysts/m^2 [59].

Expert elicitation

Two relevant expert elicitation studies have been performed. Experts attributed 56% (26–88) of *T. gondii* infections to food, 36% (6–66) to environmental transmission, 1% (0–1) to human-to-human transmission, 3% (0–3) to direct animal contact, and 5% (0–9) to travel-associated transmission [60]. The other study focused only on foodborne transmission [1]. In both studies pork and beef or lamb were identified as the major sources of foodborne transmission.

Source-specific diagnostics

A serological assay that can specifically detect oocyst-acquired infections in recently infected individuals (up to 6–8 months post infection) has been developed

[61]. Use of this test has shown a high rate of oocyst-derived infections in the USA (78%) [62] and Chile (43%) [63].

Prevention and control

Measures to reduce the disease burden due to *Toxoplasma gondii* fall into two main categories. Firstly, prevention of infection can be attempted by health education or by reducing the infection pressure in the sources (i.e., meat or the environment). Secondly, it is possible to try to lessen the effects of infection by early detection and treatment. This approach is limited to congenital toxoplasmosis by screening of pregnant women or newborns.

Primary prevention

Health education

T. gondii-related health education is typically aimed at pregnant women or high-risk patients and focuses on the various options to prevent exposure to *T. gondii*: proper heating or freezing of all meat, hygiene around gardening or other soil contact, delegation of or daily cleaning of the cat litter box wearing gloves followed by proper hand washing, and proper washing of vegetables and fruit consumed raw. The few studies that look at the effect of *T. gondii*-related health education show no clear effect on *T. gondii*-related knowledge, behavior, and risk of seroconversion in pregnancy [64,65].

Now that acquired infections are recognized as a cause of chorioretinitis, it can be argued that prevention measures should also target the general population. Health education can be extended to the total population, but with questionable effectiveness, so other measures should be considered.

Reduction of infection levels in meat

Freezing of meat for at least 3 days at less than −12 °C prior to sale could be very effective [44], either for all meat, for meat that is likely to be consumed undercooked, or, after implementation of a monitoring program, for meat from animals that tested positive. Other options for effective decontamination of meat are irradiation and high-pressure pasteurization.

To prevent infection in livestock, exposure to *T. gondii* should be limited, for example by limiting outdoor access, by use of sterilized feed and bedding and clean drinking water, by removing cats from the farm, and by strict rodent control [44].

In the future, vaccination of livestock or cats at farms may help reduce the infection levels in meat. Toxovax®, the vaccine to prevent ovine abortions [66], may help prevent tissue cyst formation from natural exposure, but this has not been tested. Development of new vaccines for livestock is ongoing [67].

Reduction of environmental contamination by cats

To reduce environmental contamination with oocysts it is important to limit the number of cats, to reduce the incidence of *T. gondii* infection in cats, and to prevent fecal deposition in the environment. Therefore, cat owners should be stimulated to have their cats castrated and the stray cat population should be controlled. Because hunting is the most important risk factor for infection in cats, this behavior should be limited by, for example, keeping the cat indoors or equipping the cat with a bell or a bib [68]. To avoid oocysts ending up in the environment, cats should be stimulated to use a litter box, and the filling is best disposed of with household waste.

In a farm-based experiment, an experimental vaccine for cats has been shown to decrease the exposure of pigs to *T. gondii* [69]. The vaccine strain is produced in mice, has limitations to the scale of production and the shelf life, requires frozen storage until administration, and could be hazardous to the people administering it [67]. Development of a better utilizable vaccine that prevents oocyst shedding by cats is highly desirable, as limiting environmental contamination will reduce the risk of infection for many intermediate hosts.

Secondary prevention

Prenatal screening

In Europe, a prenatal screening program is available in France, Austria, Lithuania, Slovenia, and parts of Italy [70]. In France, the program consists of a serological test before or early in pregnancy, and monthly follow-ups for those who tested negative. Women that seroconvert during pregnancy are treated with spiramycin and tested for transmission to the fetus. If amniocentesis is positive, spiramycin is replaced by pyrimethamine-sulfadiazine treatment until delivery.

If most women are seronegative at reproductive age, many will require follow-up during pregnancy, causing the program to be costly, and the psychological burden from false positive test results tends to be high [71]. Moreover, the effect of treatment on transmission and symptoms is uncertain. A large meta-analysis of cohort studies showed a weak association between early treatment and a reduced risk of transmission, but no effect of prenatal treatment on clinical manifestations [4]. Later, an effect of prenatal treatment on the occurrence of serious neurological sequelae and death was shown [72], but randomized controlled trials are still lacking.

Neonatal screening

Screening of neonates is offered in Massachusetts and New Hampshire [73] in the United States and in the Poznan region of Poland [70] and used to be offered in Denmark [74]. Neonatal blood is tested for the presence of anti-*T. gondii* IgM antibodies. Neonatal screening has the advantage that it requires less testing, but the effectiveness of treatment of infected newborns is even more uncertain [75]. This lack of a demonstrable benefit of treatment was the reason for the termination of the program in Denmark [74].

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CHAPTER

Mycobacterial Species 24

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Introduction

Mycobacteria are well-known human and animal pathogens, with spinal and rib lesions consistent with tuberculosis (TB) found in ancient mummies and DNA in a 17,000-year-old bison [1]. Prior to bovine TB eradication programs, approximately 40% of cattle in the United Kingdom were infected with *Mycobacterium bovis*, leading to approximately 2500 deaths annually [2]. In the United States, eradication efforts started in 1917, when approximately 10% of all cattle were infected, leading to approximately 15% of all human TB cases [3].

While eradication programs have been amazingly successful, reports to the World Organization for Animal Health indicate that 56% of countries in the world, including the United States, the United Kingdom, and France, still have areas of bovine tuberculosis infection [4]. In addition, a number of additional mycobacteria, called non-tuberculous mycobacteria (NTM), cause disease in humans; while many of these are environmental pathogens, others are food- and waterborne [5].

Clinical features

Mycobacterium bovis is the primary foodborne mycobacterium. Lesions are typically divided into primary and post-primary, as well as pulmonary and extrapulmonary. Granulomatous cervical lymphadenitis (scrofula), chronic dermatitis (lupus vulgaris or tuberculosis luposa), gastroenteritis, and other extrapulmonary forms are more common from ingestion of infected milk [6]. Pulmonary lesions due to *M. bovis* are indistinguishable from *M. tuberculosis*; these typically consist of areas of caseation and cavitation that may calcify with time [5]. After initial infection, reactivation can occur, leading to post-primary disease.

Typically, individuals with primary *M. bovis* infection are younger and have a history of ingestion of unpasteurized milk. In a recent study in the United States, groups at a higher risk were either born outside the USA, Hispanic, under 15 years old, infected with HIV, or had extrapulmonary disease [7].

Foodborne Infections and Intoxications.

Bacteria in the NTM group tend to cause cervical lymphadenitis, generally in young children, especially during teeth eruption [8]. However, these can also cause pulmonary lesions similar to tuberculosis, solitary extrapulmonary lesions, and, in immunosuppressed patients, widespread systemic disease [5]. In one study, approximately 1500 AIDS patients per day are exposed to *M. avium* via tap water [9].

There have been a number of studies examining the role of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a cattle pathogen, in a number of diseases, including Crohn's disease [10] and type I diabetes [11]. However, other studies have found no link, including finding no difference in gastrointestinal signs, between patients treated empirically with antibiotics to treat NTM and control cases [12], and further research into the relationship between MAP and human disease is necessary.

Microbiology

Mycobacteria are gram-positive, aerobic, non-spore forming, non-motile, 0.2–0.6 μm × 2–10 μm slightly curved to straight rods [13]. The genus *Mycobacterium* contains more than 120 described species. The *Mycobacterium tuberculosis* complex (MTB) is a subset of eight species, including *M. tuberculosis* and *M. bovis*, which are more than 99.5% similar at the nucleotide level [14]. These appear to share a recent common ancestor that diverged from the other mycobacteria, with *M. tuberculosis* originating farthest in the past and *M. bovis* originating more recently [1,14].

Historically, organisms were divided into Runyon groups (I–IV) based on their ability to synthesize carotenoids in light or dark, and their growth rate. However, as molecular typing has become more common, species have been found to bridge Runyon groups, making this classification less useful.

Identification of *Mycobacterium* is typically via acid-fast staining and culture of sputum, body fluids, abscess contents, or biopsy materials. However, caution is warranted, as contaminants such as tap water may contain acid-fast bacteria that may contaminate test results [13]. Many mycobacteria are slow growing, which can complicate culture and make decontamination necessary. A number of commercial culture systems are available, using biphasic systems, radiometric systems, fluorescence, or automation to increase speed and sensitivity. Cultures are typically grown for six to eight weeks before being diagnosed as negative. However, *M. bovis* does not grow well on some culture media [6]. While *M. bovis* is generally pyrazinamide-resistant, using this to differentiate from *M. tuberculosis* is only 82.5% sensitive [7].

In addition to culture, a number of molecular tests are available, including γ interferon testing, analysis of mycolic acid production via high-performance liquid chromatography (HPLC), amplification of *hsp65* and restriction analysis, and examination of species-specific genome deletions [15]. These allow for identification of organisms to the species level.

Exposure pathways

The most common foodborne exposure to *M. bovis* is via ingestion of infected unpasteurized milk [6]. While pasteurization is common in most developed countries, some mycobacteria, although not *M. bovis*, can still be cultured from both pasteurized and ultra-high temperature (UHT) processed milk [16]. Also, there have been movements in many developed countries touting health benefits of raw milk, and raw milk may be used in making cheese and other dairy products [17].

In countries with bovine TB surveillance programs, infection of cattle is typically due to a wildlife reservoir. In the UK, these are typically badgers [2]; in the USA, deer are the source of the most recent cattle outbreak [18]. These wildlife reservoirs highlight the need for continued monitoring despite a low prevalence of disease, as cattle may become infected despite apparent disease control. *M. bovis* can survive in the environment for days to weeks, although direct sunlight can kill bacilli in hours [19]. Aerosol transmission occurs from humans-to-humans, animals-to-animals, animals-to-humans, and humans-to-animals; animal-to-animal transmission is the most common way infection spreads between cattle.

As the NTM are typically environmental contaminants, they can enter the food chain anywhere from the source to the end consumer. *Mycobacterium mucogenicum* has been found in hot sauce [20], and a variety of mycobacteria have been isolated from frozen fish [21]. *M. kansasii*, *M. simiae*, and *M. xenopi* are generally transmitted by drinking water systems; however, *M. xenopi* grows in hot water, with optimum growth at 45 °C [22]. One of the main concerns with a link between MAP and human disease is its persistence in food products; a number of studies have found that up to 8% of retail dairy products are culture-positive for MAP [23].

Given the lack of reporting and the difficulty in differentiating between *M. bovis* and *M. tuberculosis*, the proportion of cases of mycobacteriosis that are due to foodborne illness rather than airborne spread is difficult to determine. However, studies in the USA [7] and New Zealand [24] have found two distinct infected populations. The first is older individuals who may have been infected as children prior to implementation of control measures; these are generally pulmonary infection. The second are young individuals all born after control measures were implemented. In the USA, these are generally extrapulmonary infections, suggesting ingestion of unpasteurized milk. The biggest risk factors for developing foodborne *M. bovis* infections are intensive dairy farming (enabling cow-to-cow transmission), lack of adequate veterinary infrastructure, and lack of pasteurization of milk [6]. While numbers vary, approximately 1–2% of all TB cases and approximately 8–10% of all MTB-associated cervical lymphadentitis cases are associated with *M. bovis*.

Prevention and control

The main methods of prevention of *M. bovis* infections are pasteurization of milk, testing cattle, and culling infected animals, as up to 30% of infected cattle can

shed intermittently [25] and animals with tuberculotic mastitis can shed enough bacteria to make pooled milk from 100 cattle infective [2]. Heating milk to 63.5 °C for 30 minutes, or 72 °C for 15 seconds, completely inactivates *M. bovis*. These measures were effective in reducing the number of cattle infected from approximately 4% in 1922 to 0.5% in the late 1930 s, preventing 25,000 deaths per year [3]. However, this is difficult to accomplish in regions with inadequate veterinary medical care, lack of infrastructure, and a predominance of small family farms, which, when coupled with wildlife reservoirs, may allow the disease to persist long-term.

Recent advances in molecular biology have allowed testing bulk tanks for *M. bovis* as an aid to tuberculin-based control programs [25]; however, given its sensitivity, these techniques may be useful in the future in preventing entry of *M. bovis* into the human food chain.

Treatment of mycobacterial infections is complex and extended, due to the resistant nature of the organism, the difficulty of reaching adequate concentrations of chemotherapeutics at the site of infection, and possible resistance to one or more drugs. Current first-line antibiotics for both pulmonary and extrapulmonary infections are isoniazid, rifampin, ethambutol, and pyrazinamide; however, as *M. bovis* is resistant to pyrazinamide, this may be dropped if *M. bovis* infection is confirmed. However, mycobacteria frequently acquire resistance, and single drug therapy should be avoided to avoid development of multi-drug resistance. Monitoring of treatment to prevent development of antibiotic resistance (direct observed treatment, or DOTS) is recommended [26]. Therapy may need to be adjusted in HIV+ patients to prevent drug interactions.

Treatment of NTM varies depending on the organism, and testing to determine the species of *Mycobacterium* involved in a particular infection is strongly recommended. If the infection is localized (such as in cervical lymphadenitis), surgical excision is recommended and typically has a high cure rate without chemotherapeutics. Clinical response to therapy correlates well with *in vitro* susceptibility in some species (such as *M. kansasii*) but poorly with many others [22].

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CHAPTER

Trichinella 25

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Introduction

Trichinella belongs to a group of nematode parasites in the superfamily Trichinelloidea, family Trichinellidae, and includes eight recognized species and 12 genotypes (Table 25.1) [1,2,4–6]. Infection with adult or larval stages of *Trichinella* is known as trichinellosis, but may also be referred to as "trichinosis" or "trichiniasis." Human infection is common and has been documented in eight genotypes, including seven recognized species worldwide (Table 25.1) [2,4,7]. Human infections result from ingesting *Trichinella* larvae in raw or undercooked meat. The most common source of human infection is pork from domestic pigs; however, due to maintenance by sylvatic cycles and cultural differences throughout the world, trichinellosis also may occur from ingesting raw or undercooked meat from other mammals and reptiles [4]. The risk of acquiring a *Trichinella* infection from commercially processed pork is very low in the United States and most other developed countries [8]. The Centers for Disease Control and Prevention (CDC) reported a 97% decrease in trichinellosis cases from the 1940s to 2000s in the United States, which was attributed to good farm management practices and greater awareness of the disease [9].

Clinical features

Trichinellosis is clinical disease caused by infection of *Trichinella* larvae in humans [4,10]. Due to the parasite life cycle, there are two main phases of *Trichinella* infection and trichinellosis in humans: the intestinal phase and the muscular or systemic phase. The intestinal phase begins once infective *Trichinella* larvae have been ingested via raw or undercooked meat. The larvae are released from the meat and move into the small intestine where they penetrate the intestinal mucosa and develop into sexually mature adults 4–5 days post-infection; however some reports indicate maturation as early as 1–2 days post-infection. Within 7 days post-infection, copulation occurs and females deposit larvae into the intestinal mucosa,

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after which the larvae migrate to the lymph nodes and into the venous blood supply via the thoracic duct. The larvae then enter the peripheral blood circulation and penetrate the skeletal muscle fibers. This is known as the systemic or muscular phase. There are nine species or genotypes of *Trichinella* that encapsulate, producing a collagen capsule around the muscle-embedded larvae, and three species or genotypes that do not (Table 25.1). Once encapsulated or embedded in muscle, the life cycle is complete and the larvae remain within the muscle tissue until ingested by another host [10,11].

Table 25.1 Genotypes and Species of *Trichinella,* Including Geographical Distribution, Primary Hosts, and Reported Cases of Human Infection[a]

Species	Genotype	Larvae Encapsulated in Muscle	Distribution	Primary Hosts	Documented Human Infection
T. spiralis	T1	Yes	North and South America, Europe, Asia, Egypt, New Zealand	Mammals	Yes
T. native	T2	Yes	North America, Europe, Asia	Mammals	Yes
T. britovi	T3	Yes	Europe, Asia, Northern and Western Africa	Mammals	Yes
T. pseudospiralis	T4	No	North America, Europe, Australia	Mammals, birds	Yes
T. murrelli	T5	Yes	North America	Mammals	Yes
	T6	Yes	North America	Mammals	Yes
T. nelson	T7	Yes	Africa	Mammals	Yes
	T8	Yes	Africa	Mammals	No
	T9	Yes	Japan	Mammals	No
T. papuae	T10	No	Papua New Guinea	Mammals, reptiles	Yes
T. zimbabwensis	T11	No	Africa	Mammals, reptiles	No
	T12	Yes	Argentina	Mammals	No

[a] *[1–5].*

Trichinellosis consists of acute and chronic stages of disease. These stages coincide with the intestinal and muscular phases of infection, and the convalescent stage is reached once the life cycle is complete and the larvae are embedded in muscle.

Acute disease occurs during the period of larval ingestion and subsequent migration of newborn larvae. Clinical signs vary between patients and seem to differ according to the number of larvae ingested, age and health of the patient, and tissues invaded by migrating larvae [10]. Onset of initial symptoms usually is sudden and may resemble non-specific gastroenteritis, making a correct diagnosis difficult without an accurate history of exposure. Later stage symptoms may last for weeks, and include severe headaches and persistent fever, which may reach 104 °F (40 °C) and last from 10 days up to 3 weeks depending on the severity of infection. Additional symptoms include chills, fatigue, periorbital facial edema, conjunctivitis, myalgia followed by maculo-papular rash and formication, and, in some instances, diarrhea. Splinter hemorrhages beneath the nails are also a common sign of trichinellosis. Varying degrees of eosinophilia have been reported in most cases, as well as an increase in immunoglobulin (Ig) E levels [10,11].

Chronic disease occurs once the larvae are embedded in the striated muscle. At this stage, complications such as myocarditis, encephalitis, muscular atrophy, and neurological disorders, all of which can also arise during acute stages of the disease, have been reported. Pneumonia or other respiratory complications may occur, along with continued formication and conjunctivitis [2,10,11]. Immunoglobulin G can be detected in serum and there is an extensive inflammatory response in affected muscle fibers. Myalgia can continue in the convalescent stage of the disease for up to 6 months, after which the affected host becomes an asymptomatic carrier of the parasite [11].

Biology of *Trichinella*

Trichinella spp. are unique nematodes in that they use the same host to not only complete their life cycle but also to serve as a reservoir for future hosts that can accommodate the next generation. Adults are found within the intestinal epithelium and have an esophagus comprised of large, granular cells or stichocytes (Figure 25.1A). Males average 1.4–1.6 mm in length and have distinct copulatory appendages at the posterior end (Figure 25.1C). Females reach approximately 2.2–3.6 mm in length. Developing larvae are easily visualized in gravid females (Figure 25.1B), with three days of development between fertilization and birth [10]. Newborn larvae migrate through the blood and lymphatics prior to host cell invasion [10,12].

The infective first-stage larvae are coiled up approximately two and a half times and are intracellular, embedded in muscle fibers (Figure 25.2). Larvae grow from approximately 100 um to 1 mm in length within 30 days [10]. The physiology of the infected muscle cell fiber changes in what is known as basophilic transformation, which includes the cell sarcoplasm becoming basophilic, sarcomere myofibrils disappearing, nuclei being displaced, and increasing size of nucleoli [12,13]. This

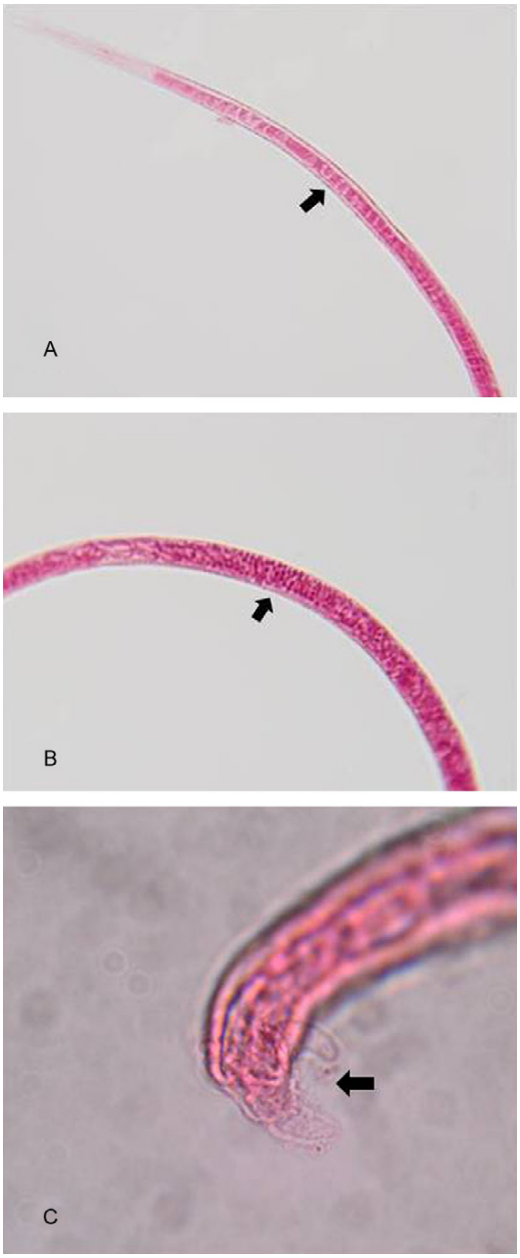

FIGURE 25.1

Adult *Trichinella spiralis*. A) Anterior end of adult *Trichinella spiralis* female. Note the large granular cells, or stichocytes, which comprise the esophagus. B) Gravid *Trichinella spiralis* female. Note the uterus full of developing larvae. C) Posterior end of adult *Trichinella spiralis* male. Note copulatory appendages.

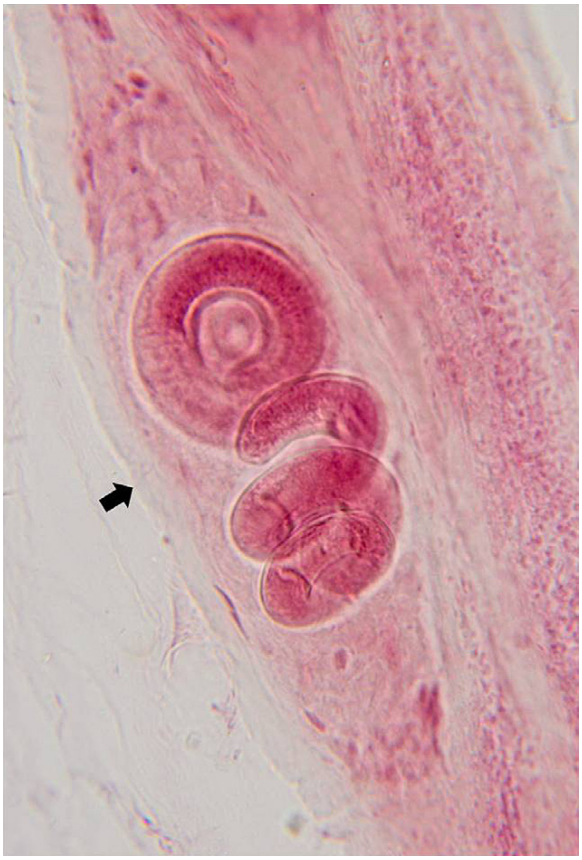

FIGURE 25.2

Two infective first-stage larvae of *Trichinella spiralis* coiled within a nurse cell in a rat muscle fiber. Note the thick wall of the surrounding collagen capsule.

transformation allows the larvae to develop and maintain viability for several years, and in some instances until ingested by a new host [12]. The infected muscle cell is now referred to as a nurse cell and formation of this cell is followed by an intense mixed inflammatory response [12,14].

During the migratory phase, larvae will invade many cell types, including those of the heart, brain, kidney, and eye. If larvae invade cells other than striated muscle, a nurse cell does not develop—rather the larvae kill the invaded cell, resulting in pathologic lesions throughout the body [12,13]. Depending on species or genotype, the coiled larvae within the nurse cell complex is then surrounded by a collagen capsule [3,7,10,15]. Encapsulated larvae do not move from their infected cell; however larvae that do not encapsulate are able to move to other muscle cells. Encapsulated larvae are also less susceptible to extreme temperatures that allow them to resist desiccation

due to freezing or prolonged exposure to heat. Host species also differ between the encapsulated and non-encapsulated larvae. Encapsulated larvae are, to date, found in mammals, while non-encapsulated larvae have been found in mammals, birds, and reptiles. The differences between encapsulated and non-encapsulated species and genotypes of *Trichinella* include morphological, biochemical, and molecular characteristics that suggest the possibility of two phyletic lines [15].

Diagnosis of trichinellosis can be grouped as either: (1) very unlikely, (2) suspected, (3) probable, (4) highly probable, or (5) confirmed. The grouping is based on diagnostic results compiled from history, clinical signs, gross identification, and serologic and molecular testing. Laboratory support for diagnosis is essential as many other parasitic and non-parasitic infections clinically mimic trichinellosis [11]. Serologic diagnosis is aimed at identifying key antigens and anti-*Trichinella* antibodies found in the host during infection [11]. Seroconversion occurs within 2–5 weeks post-infection but does not necessarily indicate acute infection, as antibody levels can remain detectable for years after the initial infection.

In most clinical cases, IgG antibodies are detectable by enzyme-linked immunosorbent assays (ELISA) approximately 12–14 days post-infection [16], reaching 100% sensitivity at day 50 post-infection [17]. At day 57 post-infection, the IgM ELISA had a peak sensitivity of 93.33%, and IgE had a peak sensitivity of 100% at day 85 post-infection. All three antibodies (IgG, IgM, and IgE) were still detectable after 2 years and 7 months post-infection at 88.24%, 11.76%, and 47.06% sensitivities, respectively [17]. It also has been found that antibody levels drop in patients that received treatment in the initial stages of infection [11]. Indirect ELISA using crude larval antigen are available for detection of *Trichinella* and have a sensitivity of 80–100% depending upon antigens used and stage of infection. However, care needs to be taken as cross-reactivity with other zoonotic nematode parasites is common, including *Ascaris, Trichuris*, and *Onchocerca* [5].

Capture ELISAs (cELISA) which use tyvelose-bearing epitopes (TSL-1 antigens), a highly specific immunodominant epitope, purified with specific monoclonal antibodies (MAb), demonstrated a 100% sensitivity and specificity during patent infections [18]. Polymerase chain reaction (PCR) assays are also available as a means of diagnosis on both the species and genotypic level, detecting as few as one larva [11].

Muscle biopsies, most commonly from the deltoid or gastrocnemius muscle [19], can be useful in identifying muscle-embedded larvae. This technique not only grossly verifies *Trichinella* infection, but can also estimate the level of infection by calculating larvae per gram (lpg) of tissue. Tissue is observed microscopically with or without muscle digestion using pepsin and hydrochloric acid (HCl). If digestion is used, larvae must be at least 10–12 days old prior to biopsy or they may be destroyed by the digestion [11]. Currently, the International *Trichinella* Reference Center (ITRC) offers services for *Trichinella* identification and trichinellosis diagnosis. Information regarding services can be accessed at the ITRC website (http://www.iss.it/site/Trichinella/index.asp). Additionally, indirect ELISA kits are also commercially available for serodiagnosis of *Trichinella* [19]. Confirmed trichinellosis cases should be reported to appropriate public health officials.

From farm to fork

Trichinellosis is a foodborne disease, acquired by the ingestion of *Trichinella* first-stage larvae in raw or undercooked meat [20]. The causative agent of trichinellosis is most commonly *Trichinella spiralis*, however eight genotypes including seven species have been documented to cause human infection (see Table 25.1). *Trichinella* infections in humans have been reported in 55 countries around the world with a mortality rate of 0.2%, out of an estimated 10,000 reported clinical cases each year [20]. *Trichinella* is found on every continent except Antarctica; however, its presence in a particular area does not guarantee human infection [20]. Sources and incidence of human *Trichinella* infection vary and are largely based on cultural practices regarding food preparation and consumption, as well as international travel and importation of contaminated meat [2,20]. Although wild and domestic pigs are the primary sources of *Trichinella* infection, sylvatic cycles maintain other reservoirs of infection which include, but are not limited to, badgers, horses, walruses, bears, cougars, foxes, dogs, and some species of birds and reptiles. As with pigs, infection from other sources is a direct result of ingesting raw or undercooked meat [2,4,6]. Epidemiologic data for trichinellosis in humans as well as reports in animals throughout the world can be found at both the ITRC and CDC websites.

Estimates of infective doses vary among cases worldwide. Dupouy-Camet and Bruschi [11] determined theoretically that a minimum infective dose of 100–300 larvae could cause clinical disease, with 1000–3000 larvae causing severe disease. These numbers were calculated based upon lpg in a given amount of ingested meat, and the number of larvae potentially shed by one female *T. spiralis*.

Control and prevention

As with most parasitic disease, early treatment is best. Treatment in the early stages of infection can help prevent larval stages from embedding in muscle cells, where they remain for years, or at least the development of acute trichinellosis. Unfortunately, treatment is usually delayed due to delayed diagnosis. Chemotherapies include broad-spectrum anthelmintics such as albendazole (Zentel®, GlaxoSmithKline) or mebendazole (Vermox®, Janssen) and glucocorticosteriods. Anthelmintics are most effective when used to treat adult stages in the intestine. They lose efficacy when treatment begins during the later stages of infection, as larvae are well established in the muscle cells and can survive for years. Glucocorticosteriods have been shown to alleviate myalgia associated with convalescent or chronic stages of the disease resulting from these larvae [2,11].

Industrialized farming and good veterinary practices have contributed to modern swine production systems that are more hygienic, keeping the environment free of raw garbage, rodents, and other wildlife that could serve as sources of *Trichinella* [8]. These production methods, along with the use of direct (trichinoscopy and artificial digestion of meat) and indirect (serological tests) methods of

detection before and after slaughter have contributed to an industry in which the risk of acquiring a *Trichinella* infection from commercially processed pork is relatively low in the United States and most other developed countries [8,21]. From 2000 to 2007, the CDC reported an average of 11 cases of trichinellosis in humans each year in the United States, a decrease in reports from an average of 400 per year in the 1940s. This dramatic decline can be attributed to good farm management practices, freezing processed pork, and awareness of the disease and potential risks associated with ingesting raw or undercooked pork [9].

Many species of *Trichinella* are resistant to conventional freezing methods. Studies have shown freezing at 5 °F (−15 °C) for 3 to 4 weeks will inactivate *T. spiralis,* however some *Trichinella* species are still viable after weeks or months of temperatures between −18 °C and −20 °C [2,4]. When cooking meat, core temperatures must reach a minimum of 159.8 °F (71 °C) and be sustained for a minimum of 1 minute, at which time the meat is no longer pink. Cooking via microwave oven and curing, drying, and smoking are recommended means of larval inactivation [2].

Gamble et al. [22] describe the certification of *Trichinella*-free pig production as recognized by the ICT. Certification involves controlled housing environments, feed and feed storage, rodent and wildlife control, garbage and carcass disposal, and acquisition of new animals. Established certification programs exist in Europe, detailed in the European Union Commission Regulation (EC) No 2075/2005 [23]. A pilot program exists in the United States, detailed in the National Trichinae Certification Program—Program Standards (http://www.aphis.usda.gov/vs/trichinae). Regardless of measures taken before, during, and after slaughter, the most certain way to prevent trichinellosis is to thoroughly cook meat that could potentially harbor the *Trichinella* nematode.

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CHAPTER

Food Safety Implications of Prion Disease

26

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Introduction

In 1996, a new neurodegenerative syndrome was identified in three young adults in Britain [1,2]. Although clinical signs and disease progression were similar to Creutzfeldt-Jakob disease (CJD), the occurrence in young individuals was different than previously described. It is now generally accepted that the disease called variant CJD (vCJD) was caused by ingestion of bovine products derived from cattle infected with a neurodegenerative disorder called bovine spongiform encephalopathy (BSE), better known as mad cow disease [3–5].

BSE represents a unique challenge to ensuring the safety of the food supply. Unlike other pathogens, the BSE agent (prion) appears to be an infectious protein devoid of nucleic acid yet capable of self-amplification within susceptible hosts [4,6]. Although the initiating event of the BSE outbreak remains unclear, it is generally accepted that the practice of feeding meat and bone meal (MBM) derived from prion-affected animals back to cattle amplified the disease significantly [7]. During the BSE outbreak within the United Kingdom, it had been estimated that approximately 750,000 BSE-affected cattle were slaughtered and entered the food supply, and as of April 2012 there were 222 patients with vCJD reported worldwide [2,8]. It is now known that BSE is part of a family of diseases found in several mammalian species, known as transmissible spongiform encephalopathies (TSEs), but is unique in its apparent ability to effectively cross species barriers, thereby affecting humans and multiple other animal species.

The first North American case of BSE was identified in May 2003 in Canada, and in December 2003 the first American BSE case was diagnosed [9,10]. Although the first American BSE case was a cow imported from Canada, later diagnosis of three additional cases in 2005, 2006, and 2012 demonstrated the presence of the disease within the United States [11]. Further analysis of these cases pointed to three distinct causes of cattle TSEs, similar to human CJD: infectious, genetic, and sporadic [10,11]. To date, there have only been three confirmed cases of variant CJD in the United States, all of which have been traced to individuals believed to

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have been infected outside the USA, in Europe or Saudi Arabia [12]. Nonetheless, the economic consequences to the American cattle industry resulting from BSE diagnosis have been severe. It has been estimated that the cost to the US cattle markets exceeded $3 billion, most of which could be traced to export losses and the implementation of the specified risk material removal [13]. In Canada, identification of BSE resulted in severe sociological tolls that continue to be felt, including loss of rural income and entire communities [14]. It is clear, therefore, that while foodborne prion diseases represent a very real threat to public health, the greatest impact of these diseases lies in potentially severe economic consequences associated with consumer confidence both domestically and internationally.

Clinical features

There are naturally occurring TSEs affecting a variety of mammalian species (Table 26.1). All are believed to be naturally transmitted by oral ingestion, although

Table 26.1 Known Transmissible Spongiform Encephalopathies (TSE) and Their Natural Hosts

TSE	Natural Hosts Reported	Human Health Risk
Scrapie	Sheep, goats	None reported
Bovine spongiform encephalopathy (BSE)	Cattle—transmits readily across species barriers	High—consumption of affected material
Chronic wasting disease (CWD)	Wild and farmed deer, elk, moose	None reported
Transmissible mink encephalopathy (TME)	Farmed mink	None reported
Variant Creutzfeldt-Jakob disease (vCJD)	Humans (derived from BSE)	High—transmission via blood products; nosocomial; acquired by consumption of BSE-contaminated food products
Creutzfeldt-Jakob disease	Humans	Sporadic, genetic, iatrogenic. High—transmission by exposure to contaminated CNS material; nosocomial
Kuru	Humans	Transmitted by ritualistic cannibalism
Fatal familial insomnia (FFI)	Humans	Autosomal dominant; pathogenic mutation with high penetrance
Gerstmann-Sträussler-Scheinker syndrome	Humans	Autosomal dominant; pathogenic mutation with high penetrance

other routes of transmission are also possible. Furthermore, all share an extended asymptomatic incubation period usually lasting years before onset of neurological symptoms [15]. Despite these similarities, there are a number of important differences between BSE and prion diseases of other human food species (Figure 26.1). Firstly, BSE is somewhat promiscuous in its host range, and is the only known zoonotic prion disease and the presumed causative agent of vCJD. In contrast, scrapie of sheep and goats, originally described in 1729, has never been shown to affect humans; also, to date there have been no instances of transmission of chronic wasting disease (CWD), a prion disease affecting a variety of North American cervid species, to humans [16–18]. Nonetheless, experimental data from transgenic mice expressing the human prion protein have shown that there is potential for cross-species conversion of the human prion protein by CWD prions [19]. Natural transmission of animal prion diseases also differs significantly. Whereas horizontal transmission of CWD is very efficient, there is no evidence for horizontal transmission of the BSE agent between cattle. Despite this, there remains the possibility of long-term environmental contamination with prions, which could lead to transmission to uninfected animals [20–23].

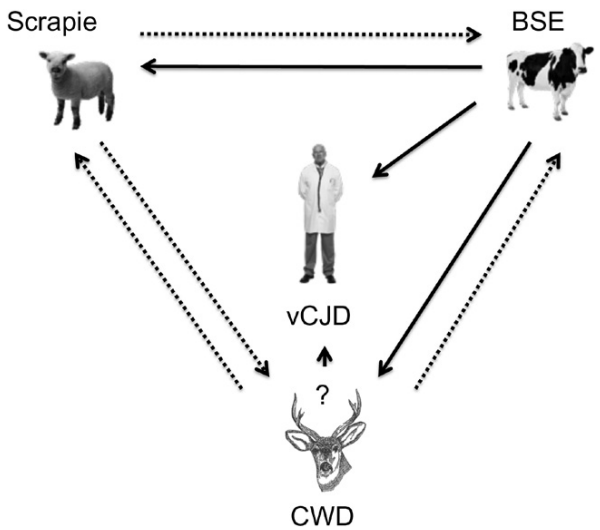

FIGURE 26.1 Interspecies Transmission of Prion Diseases

The three primary food animals susceptible to natural prion diseases are sheep (Scrapie), cattle (BSE), and cervids (Chronic Wasting Disease), and the ability of these diseases to transmit to humans (vCJD). Solid lines indicate demonstrated oral transmission between species and dashed lines represent experimental intracerebral transmission. There have been no proven cases of transmission of scrapie or CWD to humans, although *in vitro* studies may indicate a potential susceptibility of humans to CWD.

The incubation time of prion diseases following exposure varies between species but is generally measured in years, during which time detection of the conformationally altered prion protein (called PrP^d) within the affected individual can be difficult. Following ingestion, BSE infectivity and the BSE agent can be rapidly detected in lymphoid tissues of the alimentary canal [24–26]. Previous studies have clearly demonstrated that tonsils and lymphoid tissue of the ileum rapidly become infected and remain a source of infectious material for the duration of incubation. In most species except cattle, diverse lymphoid tissues then become infected as the disease progresses, presumably through blood-borne migratory B cells that carry the infectious agent throughout the immune system [27–29]. Despite this widespread lymphoid dissemination of the infectious agent, involvement of the central nervous system appears to be through direct infection of peripheral neurons [30–33]. It is assumed that the prion agent propagates along the neurons until it reaches the brain where local infection ultimately results in the formation of amyloid plaques, neurodegeneration, and ultimately death. For this reason, certain lymphoid tissues and central nervous system tissues of cattle have been designated as "specified risk material" (SRM) and must be removed from carcasses after slaughter (Table 26.2).

Microbiology

The infectious agent associated with TSEs is unique in that it is a conformationally altered, misfolded form (PrP^d) of the normal cellular prion protein called PrP^c [2,44,45]. The normal prion protein PrP^c is expressed ubiquitously but is particularly highly expressed by neuronal and lymphoid cells. Although the precise function of PrP^c remains elusive, it is clearly important in several cellular processes such as cell trafficking, copper uptake, protection against oxidative stress, cell adhesion and differentiation, and protection against apoptosis [46–48].

The consequences of conversion of PrP^c to PrP^d, however, appear to be independent of any loss of function of PrP^c. PrP^d has the unique capacity to catalyze the conversion of PrP^c into the diseased form PrP^d (Figure 26.2). During this process, the secondary structure of PrP^c is presumably altered from a preferentially alpha helical structure to one consisting of significant amounts of beta pleated sheets [49]. Since the primary amino acid sequence of PrP^c and PrP^d are identical, it seems clear that the pathological significance of conversion depends upon the unique conformation of the altered protein. The unique mechanism by which PrP^c is altered to the PrP^d structure remains unclear, however it seems that conversion may either occur at the cell surface or intracellularly after internalization of PrP^c [47]. Following conversion, PrP^d forms multimeric protein aggregates, ultimately resulting in the characteristic amyloid fibrils within germinal centers of lymph nodes and affected central nervous system tissues associated with disease progression [50]. As the amyloid plaques/fibrils accumulate, there is progressive cell death and the characteristic spongiform changes form in the brains of infected humans and animals.

Table 26.2 Distribution of PrP^d and Demonstrated Infectivity in Tissues from Domestic Animals Infected with Prion Diseases

BSE: Cattle			
Tissue	**PrP^d?**	**Infectious?**	
Neural tissue	+	+	[34]
Lymphoid tissue	IPP only	IPP only	[34–36]
Small intestine	+	+	[25]
Muscle	BASE*	BASE*	[26]
Tongue	–	+	[37]
Scrapie: Sheep and Goats			
Tissue	**PrP^d?**	**Infectious?**	
Neural tissue	+	+	[38]
Lymph nodes	+	+	[38]
Small intestine	+	+	[39]
Muscle	–	Atypical/Nor98	[40]
Tongue	+	N/A	[87]
CWD: Deer, Elk, and Moose			
Tissue	**PrP^d?**	**Infectious?**	
Neural tissue	+	+	[4]
Lymph nodes	+	+	[4]
Small intestine	+	N/A	[41]
Muscle	Cardiac	+	[42,43]
Tongue	–	N/A	[43]

**Atypical BSE only.*

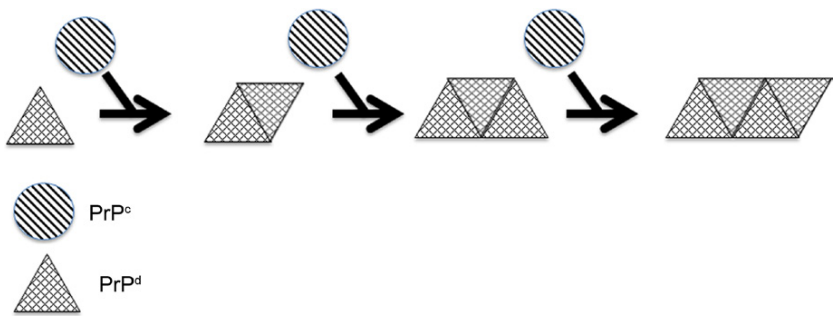

FIGURE 26.2 Schematic Representation of the Conversion of the PrP^c Structure to PrP^d

Amplification of PrP^d in affected individuals occurs when normal host PrP^c is refolded into the pathogenic structure PrP^d, which then polymerizes to form the characteristic amyloid fibrils associated with prion diseases.

A unique characteristic of the PrP^d isoform is its relative resistance to proteolytic degradation, which is the basis of most diagnostic techniques. To date, there are no approved tests which specifically differentiate between the altered PrP^d and normal PrP^c structures. As a result, diagnosis of prion disease is based on clinical signs, as well as the selective identification of protease-resistant PrP^d in lymphoid or nervous system tissues by immunohistochemistry, ELISA, or Western blot [51–53]. Briefly, test samples are digested with Proteinase K (PK) for a fixed period of time after which the PK-resistant residual protein is reacted with antibodies specific for PrP (Figure 26.3). By definition, residual PrP protein present in tissues that survives proteolytic digestion will be of the PrP^d isoform and can be identified in using standard immunological techniques. While effective on postmortem tissues containing significant concentrations of altered prion protein (PrP^d), the sensitivity of these assays appears to be a limiting factor in development of antemortem diagnostics.

There are a number of antemortem diagnostic tests for prion diseases currently under development, however most of these are focused on the identification of PrP^d in lymphoid cells and/or blood components [54]. It is important to note that cattle

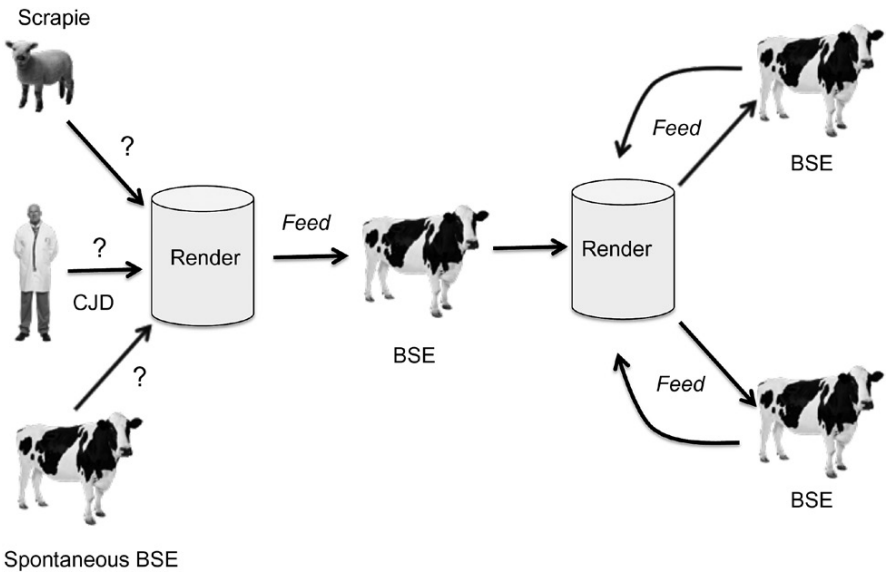

FIGURE 26.3 Representation of the Intitiation and Propagation of the BSE Epidemic

Although the precise initiating factor of the BSE epidemic remains unclear, it is clear that the rendering of animal tissue into meat and bone meal was the likely source of amplification for the epidemic. Proposed sources for the original PrP^d that resulted in BSE include an altered strain of sheep scrapie, potential contamination of meat and bone meal with human remains affected with CJD, and rendering of a spontaneous/genetic case of BSE which then amplified in the cattle population.

BSE, which is similar to human prion diseases (except vCJD) and is unlike those of other species, does not appear to extensively involve peripheral lymphoid tissues or blood but is instead restricted only to the Peyer's patches of the terminal ileum [34,55,56]. It is highly likely, therefore, that newer antemortem tests focused on detection of PrP^d in blood or blood cells will prove ineffective for BSE diagnosis in live cattle. Furthermore, recent studies clearly demonstrate that infectious BSE prions may exist in cattle tissues in the absence of detectable PrP^d as early as 4 months post-oral challenge, i.e., in calves younger than currently considered "at risk" for human consumption associated with BSE [25,37].

Current diagnosis is limited to complex immunohistochemical techniques, Western blots, or ELISA analyses of lymphoid and brain tissue obtained postmortem—techniques not suited for identification and diagnosis of TSEs in live animals [57]. As some leukocytes express the normal form of prion protein (PrP^c) and animals are capable of expressing PrP^d in lymph nodes, a number of tests are currently under development which focus on identification of PrP^d in blood cells and fluids [57]. B cells, follicular dendritic cells (FDCs), and dendritic cells (DCs) have all been shown to be associated with PrP^d in murine, cervid, and sheep prion diseases, although of all these cell types, only B cells have been associated with PrP^d *in vivo* [28,29]. However, data suggest that BSE, unlike other TSEs, does not exhibit blood-based infectivity, and as such, novel antemortem TSE tests based on blood may not be relevant, and alternative technologies focused on defining contamination of meat products with BSE or indirectly with SRM may be more practical.

A number of prion strains have been defined based more upon unique biological phenotype than on biochemical differences. In general, definition of strains is based upon: (1) incubation period; (2) distribution of PrP^d within neurological tissues; and (3) distribution of pathological lesions in affected individuals of any given species [58]. The use of defined mouse infection bioassays has proven to be the most reliable means to define prion strains and has been used to differentiate BSE from other prion diseases [59]. The nature of prion strains is not clear, however it is generally believed that unique conformational differences within PrP^d dictate the strain-specific characteristics of prion diseases. In BSE, typical and atypical BSE isolates have been reported. Typical BSE appears to be associated with a feed-borne etiology and seems to consist of a single strain, which retains its unique characteristics following infection in other species. In contrast, atypical BSE has been found as a high molecular weight (H-type) and low molecular weight (L-type) version mainly in older cattle (Casalone et al., 2004; Biacabe et al., 2004). Unlike typical BSE, H- and L-types of BSE may have a sporadic or genetic etiology which cannot be controlled by current feed-based mitigation measures.

Exposure pathways

TSEs have been identified in a wide range of mammalian species including deer, elk and moose (CWD), sheep and goats (scrapie), cattle (BSE),

mink (transmissible mink encephalopathy [TME]), and humans. Human prion diseases further include sporadic, genetic, and infectious forms of Creutzfeldt-Jakob disease, kuru, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia. Virtually all of these diseases, with the exception of BSE, are highly species-restricted. In contrast, BSE appears capable of infecting a broad host range of animal species across diverse species barriers, potentially leading to host reservoirs outside of cattle. For example, widespread infection of zoo cats and zoo ungulates was reported as a result of incorporating BSE-contaminated material in animal feed. Despite this fear, efforts to identify BSE within European sheep have proven futile, and there have been limited reports of BSE within a single goat [60]. It therefore seems likely that the primary concern for foodborne infection with TSEs lies in the consumption of contaminated beef products.

Meat products may be directly contaminated with prions through either pre-harvest infection or post-harvest contamination during slaughter. Although the primary targets of prion replication, and hence the presence of infectivity, lie in tissues of the central nervous and lymphoid systems, muscle tissue has been demonstrated to contain infectivity in cervids incubating CWD [42,61–63]. Firstly, the presence of local immune responses can lead to lymphoid follicles within muscle tissues, which may then serve as sites of prion amplification and deposition. Secondly, neuronal tissue found within muscle tissue may also contain PrP^d, and as such the infectivity of skeletal muscle has been estimated at 2000–5000-fold less than brain tissue. It is therefore possible that meat products may contain low levels of infectious PrP^d in cervids, but this has yet to be demonstrated for cattle BSE. Nonetheless, the hypothesized link between vCJD and BSE would suggest that certain meat products may in fact be infectious, and a source of contamination of the food supply. As a result, a great deal of work has focused on defining the presence of infectious material within specific tissues of food animals, and therefore their potential risk to the food supply (see Table 26.1).

Prevention and control

Although the precise initiating factor of the BSE epidemic in Europe remains unclear, hypotheses include: (1) contamination of meat and bone meal (MBM) with scrapie-infected sheep material; (2) contamination of MBM with a BSE-infected cattle material; and (3) contamination of ruminant feed with human remains infected with prions [7,64] (Figure 26.4). This contamination was then amplified by the further inclusion of affected cattle back into the ruminant feed supply. The most effective means of controlling BSE has been the implementation of a total ban on the use of ruminant-derived MBM in production of animal feed, combined with an effective surveillance program [7,65]. Unfortunately, current diagnostic techniques limit the sensitivity of any surveillance program, and as such the most powerful tool in restricting the spread of BSE in cattle was the ruminant feed ban [7].

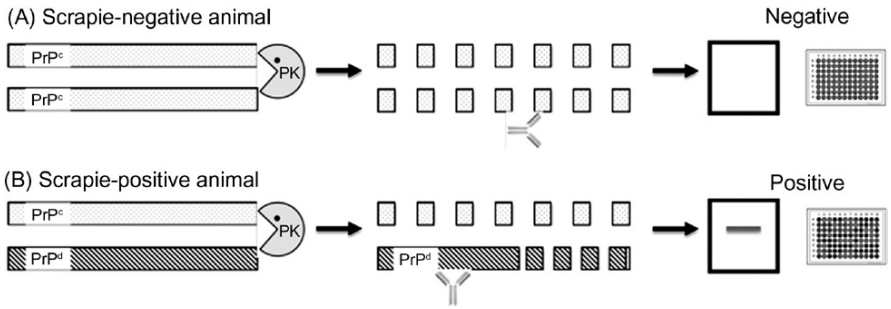

FIGURE 26.4 Current Prion Diagnostics Rely upon Selective Digestion of PrP^c but not PrP^d

Most traditional methods to detect PrP^d rely upon the selective degradation of PrP^c in test samples, which are then reacted with $PrP^{c/d}$ reactive antibodies. As all PrP^c is protease-sensitive, only PrP^d will remain to be detected by Western blot, immunohistochemistry, or ELISA techniques.

Initially, the ban of MBM in feeds was restricted to those intended for ruminants; however, in later years a total ban on the use of rendered ruminants in feed mills was instituted in the EU, which largely contained the epidemic. This ban proved highly successful but also affected an important protein source in animal feeds. As a result, there have been recent calls to return ruminant MBM to feed for use in non-ruminant species [65]. A survey of EU member states indicated that continuation of an active surveillance program to monitor epidemiology of disease in conjunction with relaxed regulations would be generally accepted [66].

While the feed ban has been effective at limiting the spread and amplification of so-called typical BSE, recent years have seen the development of atypical, apparently spontaneous or genetic form of BSE, termed L-type and H-type BSE, both of which are infectious; although its amplification in the cattle population can be controlled by ruminant feed bans, its incidence cannot. There is therefore a continued need for development of advanced surveillance techniques to limit the entry of SRM into the food supply, and active measures to prevent and treat prion diseases of humans. To date, these techniques have largely focused on the use of technologies designed to detect neurological contamination of meat products and animal feeds, irrespective of BSE status [67].

Approaches to the prevention and treatment of human prion diseases have been largely focused along similar lines as those applied to other amyloid diseases of humans, including Alzheimer disease. As the pathogenic protein shares a primary amino acid sequence with the natural host protein PrP^c, there does not appear to be an effective immune response to prion infection [68]. This self-tolerance not only prevents a reactive immune response to prion infections, but also inhibits approaches to develop active vaccines to combat infection. As a result, production of effective anti-prion responses can only be achieved in prion protein-knockout mice [69,70].

Despite these limitations, the identification of low-affinity antibodies in long-term prion-infected experimental mice may suggest that chronic infection may result in a low-grade immune response to the misfolded prion protein [71]. Furthermore, the presence of antibodies directed against PrP effectively inhibits prion replication *in vitro* [72–74]. Also, transgenic mice expressing anti-PrP antibodies were immune to prion infection and both passive and active vaccination against PrP^c delayed the onset of disease [75–77]. High concentration of antibodies effectively blocked disease, even when provided to mice up to one month after exposure to PrP^d-containing material [78]. This therapy, therefore, may have some therapeutic benefit when the date of exposure is known.

A number of techniques have been employed to develop active vaccination strategies against prion disease, effectively bypassing normal self-tolerance mechanisms. These strategies largely include DNA-based vaccines, vaccination with full-length recombinant PrP, dimeric PrP, synthetic PrP peptides, and polypeptides with a variety of adjuvants and various combinations of all of these [79–82]. More recently, specific approaches to target T-cell immunity have been used, including pre-loading dendritic cells with prion protein peptides prior to immunization of wild-type mice [83]. This approach resulted in measurable T-cell responses and may show promise for a technique to stimulate T-cells *ex vivo* in affected individuals, similar to several current approaches to anti-tumor therapies [69,84].

In an effort to overcome issues with systemic immunity to the ubiquitously expressed PrP molecule, a number of investigators have attempted to target regional immunity directed against the portal of entry. Delivery of attenuated *Salmonella* transfected with the PrP gene resulted in local mucosal immunity and significant delay in the onset of prion disease in mice [85]. In an alternative strategy, Cashman and colleagues attempted to produce PrP^d-specific antibodies that fail to recognize the native PrP^c by immunization with the Tyrosine-Tyrosine Arginine (YYR) epitope of PrP^c conjugated to a leukotoxin-carrier protein [86]. In mice, this vaccine strategy produced a strong, PrP^d-specific IgG response, suggesting potential for the development of a prion vaccine in the absence of autoimmune complications. There is clearly a need for more work; however, the potential for active protection against TSEs by immunological strategies remains a possible resolution to the food safety threat posed by prion agents.

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SECTION

Intoxications 5

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CHAPTER

Clostridium botulinum 27

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Introduction

Foodborne botulism was first described by the German poet and medical officer Justinus Kerner in the early nineteenth century when he published a monograph on numerous cases of "sausage poisoning." The microbiologist Emile Pierre Marie van Ermengem isolated an anaerobic bacillus, which he named *Bacillus botulinus*, from ham and spleen tissue samples obtained from the corpses of botulism victims after an 1895 outbreak. He demonstrated that the organism was capable of producing a toxic substance in the growth medium, and observed that cell-free extracts were capable of causing disease, establishing that foodborne botulism could be classified as an intoxication rather than an infection. *Bacillus botulinus* was eventually renamed to *Clostridium botulinum* to differentiate the anaerobic sporeformer from the aerobic spore-forming genus *Bacillus*.

Characteristics of the disease

Botulism

Botulism is a severe neuroparalytic disease caused by any of the seven serotypes (A–G) of botulinum neurotoxins (BoNTs) by *C. botulinum* and rare strains of *Clostridium baratii* and *Clostridium butyricum*. BoNTs/A, B, and E are responsible for the majority of human botulism cases, whereas BoNT/F is rarely implicated in cases of botulism. BoNT/C and BoNT/D are known to cause avian and non-human botulism, especially in cattle, dogs, mink, and horses.

Foodborne botulism occurs from the ingestion of preformed toxin and is characterized by an acute onset of descending, symmetrical flaccid paralysis that initially affects the muscles of the head and neck. Blurred vision, difficulty speaking and swallowing, slurred speech, dry mouth, and generalized weakness are classic symptoms of botulism; in severe cases, this may progress to respiratory paralysis, requiring mechanical ventilation. Gastrointestinal symptoms including nausea, vomiting,

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and diarrhea occur in one-third of the foodborne botulism cases and are absent in all cases of wound botulism.

The levels of toxin can vary from serving to serving within the same food, and in conjunction with differences in serving size can account for the range in severity of botulism symptoms displayed in patients who have consumed the same food. The time to onset of botulism symptoms depends upon the quantity of toxin ingested, with consumption of larger quantities of toxin resulting in a decreased incubation time. Symptoms typically manifest within 12–36 hours but can develop as early as after 2 hours in severe cases or as late as 14 days in milder cases. The severity and duration of disease also depends upon the BoNT serotype. BoNT/E presents the shortest duration of symptoms in human botulism cases, whereas BoNT/A causes the most severe forms of disease because of its persistence within the neuron [1]. With extensive supportive care and mechanical ventilation (for those with respiratory paralysis), patients with botulism recover slowly once regeneration of the nerve endings has begun. However, complications and long-term sequelae can arise in patients requiring mechanical ventilation and/or long-term hospitalization in intensive care units [1].

While foodborne botulism is an intoxication caused by the consumption of preformed botulinum toxin, infant and adult colonization botulism are classified as infections, resulting from the *in vivo* production of BoNTs. Unlike other clostridia, *C. botulinum* is not a natural inhabitant of the human gastrointestinal tract. The intestinal tracts of infants, and adults who have undergone substantial gastrointestinal surgery or extensive antibiotic treatment, are devoid of the natural gut microflora. In the absence of competitive microflora in these types of individuals, ingestion of BoNT-producing clostridial endospores can result in spore germination, outgrowth, colonization of the intestinal tract, and BoNT production.

Babies under the age of 1 year are the most susceptible to infant botulism, and in rare cases symptoms have been observed in children several hours after birth. Constipation is a classical symptom that occurs while the organism begins to colonize the intestine. Indications including lethargy, listlessness, weak cry, and difficulty swallowing manifest as the disease progresses until paralysis is fully evident. The prognosis for infant botulism is usually a complete and full recovery. Adult colonization botulism cases are infrequent and share similar clinical features as infant botulism.

Group I *C. botulinum* strains are most commonly associated with infant botulism. However, cases have been reported implicating BoNT/Bf and BoNT/F serotype strains. In addition, strains of *C. butyricum* and *C. baratii* producing type E and type F, respectively, have been isolated during infant botulism cases.

Wound botulism is also classified as an infection. The anaerobic environment of a wound can provide an optimal setting for *C. botulinum* spores to germinate, grow, and produce BoNT. The symptoms of wound botulism are similar to those of foodborne botulism, but gastrointestinal symptoms are absent. Wound botulism was the rarest form of botulism, but there has been a significant increase in the number

of cases associated with intravenous drug use due to contamination of the heroin or syringe needles with *C. botulinum* spores.

Diagnosis of botulism

While botulism cases are quite rare, botulism's very distinctive clinical presentation makes diagnosis likely, particularly in more severe cases requiring ICU admission and mechanical ventilation; likelihood of diagnosis is also increased in areas where cases are more common (such as among Native American tribes in Alaska). Diagnosis does require differentiation from other neuromuscular diseases including Guillain-Barré syndrome (which generally presents with an ascending pattern of paralysis, except for patients with the Miller-Fisher variant) [1], myasthenia gravis, and cerebral vascular accidents (CVAs). The time from earliest exposure to outbreak recognition has been estimated to range from 48 to 216 hours [2].

Botulism is confirmed by detection of BoNTs in the patient's serum and feces. Detection of the toxin and the organism in other clinical samples, including the gastrointestinal contents and wound tissue samples, supports the diagnosis [3]. In cases of infant and adult colonization botulism, detection of the organism in fecal samples may be sufficient for diagnosis.

Microbiology

Characteristics

C. botulinum is classified as an anaerobic, gram-positive bacillus that derives its energy for growth by fermentation. Its ability to produce highly resistant endospores permits its ubiquitous distribution throughout the environment. The most significant virulence mechanism, aside from sporulation, is the unique ability of the organism to produce an extraordinarily potent poison called botulinum neurotoxin (BoNT). During the several decades following the identification of the causative organism, investigation of several outbreaks resulted in the isolation of physiologically dissimilar strains, each producing one or more of seven serologically distinct BoNTs designated A–G. Strains of the heterogeneous polyphyletic species *C. botulinum* have also been organized into four groups (I–IV), based on physiological, metabolic, and genetic properties (Table 27.1). Several molecular techniques employed to categorize this species (including sequencing of genes encoding 16S rRNA, pulsed field gel electrophoresis, DNA hybridizations, and multilocus enzyme electrophoresis) further support the grouping of *C. botulinum* strains into the four physiological categories [5]. In addition, non-toxigenic but phenotypically similar species representing groups I, III, and IV have also been recognized (Table 27.1).

Proteolytic group I strains produce BoNT of serotypes A, B, and F and form heat-stable endospores. Non-proteolytic group II strains are psychrotrophs and

Table 27.1 Characteristics of the Six Physiologically and Phylogenetically Distinct Clostridia That Form the Botulinum Neurotoxin[a]

Neurotoxigenic Clostridia	***C. botulinum* Group I (proteolytic *C. botulinum*)**	***C. botulinum* Group II (non-proteolytic *C. botulinum*)**	***C. botulinum* Group III**	***C. botulinum* Group IV (*C. argentinense*)**	***C. baratii***	***C. butyricum***
Neurotoxins formed	A, B, F[b]	B, E, F	C, D	G	F	E
Non-neurotoxigenic equivalent clostridia	*C. sporogenes*	No species name given	*C. novyi*	*C. subterminale*	All typical *C. baratii* strains	All typical *C. butyricum* strains
Optimum growth temperature	37 °C	25 °C	40 °C	37 °C	30–45 °C	30–37 °C
Minimum growth temperature	10–12 °C	2.5–3.0 °C	15 °C		10–15 °C	12 °C
Minimum pH for growth	4.6	5.0	5.1			4.8
NaCl concentration preventing growth	10%	5%		6.5%		
Minimum water activity for growth, humectant: NaCl/glycerol	0.94/0.93	0.97/0.94				
Spore heat resistance[c]	$D_{121\,°C} = 0.21$ min	$D_{82.2\,°C} = 2.4$	$D_{104\,°C} = 0.9$ min	$D_{104\,°C} = 1.1$ min		$D_{100\,°C} < 0.1$ min

Adapted from [4].
[a]*Where values are not given, they are not readily available in the literature.*
[b]*In dual-toxin strains, more than one toxin is formed.*
[c]*Spore heat resistance determined in phosphate buffer, pH 7.0.*

differ from group I strains by their inability to digest protein, their requirement of neurotoxin activation by exogenous enzymes such as trypsin, and spores with lower heat resistance. Group III contains strains of *C. botulinum* serotypes C and D, while group IV is comprised of strains that only synthesize BoNT/G and have been renamed as *Clostridium argentinense.*

The majority of BoNT-producing clostridia synthesize only one serotype of toxin; however, group I strains can produce more than one toxin serotype and are called dual neurotoxin-producing strains. One neurotoxin serotype is typically produced in higher quantities than the other and is indicated by a capital letter (Ab, Ba, Af, and Bf). Furthermore, bivalent A(B) strains contain both type A and B neurotoxin genes, yet produce only BoNT/A due to a premature stop codon located within the gene-encoding BoNT/B [6].

Genetics and evolutionary considerations

Numerous neurotoxin subtypes within serotypes A (A1–A5), B (B1–B5), E (E1–E6), and F (F1–F7) were discovered through sequencing of the BoNT genes from multiple strains. These subtypes were defined based on significant sequence variation, ranging from 2.6% to 32%, within the amino acid sequences of the BoNT molecules [7–9].

Botulinum neurotoxins are covalently linked to other non-toxic proteins to form a progenitor toxin complex. The genes encoding the neurotoxin and associated proteins are organized in clusters that vary in composition among the different serotypes and strains. Two main types of gene clusters exist (Figure 27.1). All clusters contain the neurotoxin gene, *bont*, and the gene encoding the non-toxic non-hemagglutinin component, *ntnh*, which are co-transcribed as a single mRNA transcript. Strains producing toxin complexes with hemagglutinin activity possess genes (*ha70, ha17, ha33*) organized in one operon. This type of toxin gene cluster is called *ha* plus/*orf*X minus, and is typically associated with BoNT/A1, /B, /C, /D, and /G. The gene product of *botR* is an alternative sigma factor involved in the transcription of the neurotoxin gene. Certain *C. botulinum* strains have replaced the *ha* genes with genes *orf*X1, *orf*X2, and, in some strains, *orf*X3, which encode for uncharacterized proteins. These clusters are referred to as *ha* minus/*orf*X plus clusters and are affiliated with BoNT/A2, /A3, /A4, /E, and /F as well as the type A1 gene in A(B) strains [10]. Some of the toxin gene clusters also contain the gene *p47,* which encodes for a gene product of unknown function.

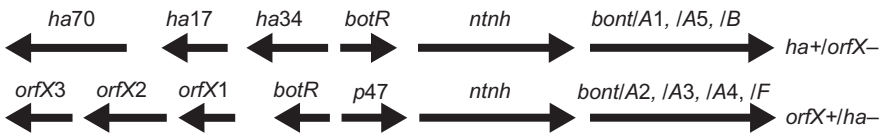

FIGURE 27.1

Gene content and arrangement of the *ha*+/*orf*X– and *ha*–/*orf*X+ botulinum neurotoxin gene clusters.

The genetic location of the neurotoxin gene clusters varies significantly among the serotypes and strains. In *C. botulinum* serotypes C and D, the BoNT/C and BoNT/D gene clusters are carried on pseudolysogenic bacteriophages which are maintained as circular megaplasmids within the cell. The BoNT/G cluster is also plasmid-encoded in serotype G strains. Most strains that produce BoNT/A, /B, /E, and /F carry the neurotoxin gene cluster on the chromosome. However, large plasmids harboring BoNT/A, BoNT/B, and/or BoNT/F gene clusters have been identified in several BoNT/A, BoNT/B, and dual neurotoxin-producing strains [10,11]. Conjugative transfer of BoNT-encoding plasmids in group I and group II strains has also been demonstrated [12].

Various molecular subtyping methods including 16S *rrn* sequencing, PFGE, MLST, and AFLP have allowed the analysis of numerous *C. botulinum* genomes, providing insight into the basic genetic properties and evolutionary history of the neurotoxin and the organism. Presently, GenBank (National Institute for Biotechnology Information) lists 24 genome sequencing projects for *C. botulinum*, and the genomes range in size (2.6–4.26 Mb) and %G + C content (27.3–29%). Comparative genomic indexing of 61 proteolytic strains using DNA microarrays based on the genome sequence of strain ATCC 3502 revealed a relatively stable genome among group I strains; however the neurotoxin gene cluster and the flagellar glycosylation island (FGI) were found to be regions of genetic heterogeneity [13]. It has been suggested that the neurotoxin genes and gene clusters have evolved independently of the organism, most likely the result of recombination [4]. The gene that encodes for NTNH has been shown to be chimeric in several *C. botulinum* strains and is considered to be a "hot spot" for recombination events to occur within the neurotoxin gene cluster [13,14].

The presence of putative insertion sequence elements flanking neurotoxin gene clusters, the evidence of partial IS elements within certain clusters, and the location of BoNT genes on conjugative plasmids and bacteriophages contribute to the horizontal transfer of neurotoxin genes among clostridial strains and provides an explanation for the diversity of organisms capable of producing BoNT.

BoNT structure and function

BoNTs are labile to high heat and acid conditions, and therefore are produced as progenitor toxin complexes. The auxiliary proteins of the complex protect the neurotoxin from harsh conditions such as low pH and proteolysis as the neurotoxin migrates through the stomach to the lumen of the gut upon ingestion by the host [15]. BoNTs are primarily absorbed in the upper small intestine but can be absorbed in the stomach [15].

BoNT is synthesized as a single inactive polypeptide chain of approximately 150 kDa that is proteolytically nicked to form an active dichain molecule consisting of a heavy chain (HC) and a light chain (LC). The HC binds the toxin to the target cell membrane and transports the toxin into the cholinergic neuron [16]. The LC is the catalytic domain and acts as a zinc-dependent endopeptidase, cleaving

neuronal substrates involved in synaptic vesicle trafficking and membrane fusion, called SNARE proteins. These SNARE proteins (VAMP/synaptobrevin, SNAP-25, and Syntaxin 1) are necessary for the fusion of synaptic vesicles at the nerve terminal, and once cleaved prevent the release of the neurotransmitter, acetylcholine, at the neuromuscular junction, resulting in muscle paralysis.

Isolation and identification of *C. botulinum* and botulinum neurotoxins

The method of isolating viable *C. botulinum* outlined in the FDA Bacteriological Analytical Manual [17] requires the anaerobic cultivation of the organism in broth culture with and without trypsin. The cultures are examined for turbidity, gas production, and digestion of meat particles for differentiation of proteolytic and non-proteolytic strains. The presence of typical clostridial tennis racket-like cell morphology and refractile endospores can be observed using phase contrast microscopy or by gram staining using bright-field illumination. Isolation of pure cultures of *C. botulinum* can be obtained by treatment of the enrichment culture with an equal volume of filter sterilized ethanol, or by a heat shock at 80 °C (proteolytic group I strains) or 60 °C (non-proteolytic group II strains) for 10 minutes to kill vegetative cells and select *C. botulinum* spores. Strains are lipase-positive and produce an iridescent sheen on and around the colonies on egg yolk agar (EYA). Fecal samples often contain high quantities of competitive organisms, and proteolytic *C. botulinum* can be selected by supplementing EYA with cycloserine, sulfamethoxazole, and trimethoprim. Purified colonies of *C. botulinum* can then be examined for toxin production and typing using the mouse bioassay.

Because multiple serotypes (BoNT/A, /B, /E, /F) may be responsible for illness, all toxin types must be assayed. The current standard method for detection of BoNT in clinical, food, and environmental samples is the mouse bioassay. Samples potentially containing BoNT are diluted in gel phosphate buffer and administered to mice through intraperitoneal injection. The mice are observed for symptoms of botulism, which include ruffled fur, respiratory distress characterized by "wasping" of the abdomen, and muscle weakness. The toxin serotype is determined through neutralization using type-specific antisera prior to sample injection. Despite the sensitivity of the mouse bioassay (detection limit of 20–30 pg/ml), it is laborious and expensive and poses ethical concerns due to the use of laboratory animals [3].

Extensive research has focused on the development of rapid and sensitive *in vitro* methods for the detection of BoNT in clinical, food, and/or environmental samples [3]. The most commonly used immunological technique often performed in parallel with the mouse bioassay is the ELISA method. Unlike the mouse bioassay which detects biologically active toxin, the ELISA assay detects both active and inactive toxin molecules, leading to false positive results. The genetic differences between the subtype neurotoxins within a given serotype may reduce the affinity toward specific monoclonal antibodies and presents a significant hurdle for immunological-based methods [9]. A study comparing the mouse bioassay and the

DIG-ELISA system indicated that DIG-ELISA can be used for screening but not for confirmatory identification of BoNT [18,19].

Endopeptidase assays based on the enzymatic cleavage of SNARE proteins by BoNTs detect active toxin molecules. This method can be more sensitive than the mouse bioassay and has the potential to eventually replace it for BoNT detection. However, this method requires sophisticated instrumentation and specialized skills to perform. An Endopep-MS assay has been developed that differentiates the toxin serotypes and is performed by incubating BoNT with a peptide substrate, which is cleaved at a location specific to each toxin serotype [20]. The reaction mixture is submitted into a mass spectrometer and the mass of the peptides generated is detected. Extraction of the toxin molecule from the sample matrix prior to the endopeptidase reaction is achieved using a multi-specific, cross-reactive, high affinity, monoclonal antibody that is capable of detecting BoNTs/A, /B, /E, and /F.

Exposure pathways

Reservoirs

C. botulinum spores are widespread in nature and frequently isolated from soils and sediments and the intestinal tracts of animals [21,22]. Subsequently, spores can enter the food chain through direct contamination of the animal or plant during production or through cross-contamination during harvest, handling, or processing. Distribution and prevalence of spore types vary by geographical region. The incidence of spores in various raw foods and the frequency of botulism often reflect the type and prevalence of the spores in soils and sediments of the region of origin of the food. In a review of botulism outbreaks occurring in the United States between 1950 and 1979, 87% of outbreaks occurring west of the Mississippi River were caused by type A strains, while type B strains predominated east of the Mississippi, accounting for 65% of outbreaks.

An average of 24% of inland soils tested in the United States was found to harbor *C. botulinum* spores. In analogous regions of Canada, Central and South America, Europe, and Asia the rate of isolation ranges from 1 to 30%, with the greatest prevalence near lakes, rivers, or coastal regions and areas prone to flooding [23]. In sediment samples obtained from the Great Lakes area, cooler coastal regions in Alaska, Washington, Northern Europe (particularly near the Baltic region), China, and Taiwan, 30–95% were found to harbor *C. botulinum* spores, primarily strains of serotype E [22].

Due to the pervasiveness of spores in coastal environments, marine and freshwater foods, such as fish, are often contaminated with type E *C. botulinum* spores. Type E spores were recovered from the intestines of 56%, 55%, and 31% of fish from Green Bay, the South Baltic Sea, and the Columbia River, respectively [24–26]. A survey conducted in France identified 7.8% of fish and shellfish used as ingredients for refrigerated foods as being positive for *C. botulinum* type A or B; when contaminated the average populations were 2–3 spores per kg [27].

Likewise, clostridial spores are known to survive in the intestine and ceca of clinically healthy land animals. *C. botulinum* type A and B serotypes are the primary strains isolated from cattle and pigs, and type B strains have been recovered from poultry [28,29]. *Clostridium botulinum*/*Clostridium* spp. have also been isolated from biowaste, such as manure, food refuse, and slaughterhouse waste, and retain viability in digested residues used for spread fertilizer [30]. The prevalence of *C. botulinum* spores in meat and meat products is less than 10%, and the spore load is often fewer than five spores per kg [21,27].

Vegetables, particularly root vegetables and those which are grown and harvested close to the soil surface, primarily contain type A or B spores, depending on the region of origin. In the UK, 50–100% of soil, potatoes and turnip skins, mushrooms, Brussels sprouts, and cauliflower samples contained strains of *C. butyricum* type E [31]. Canadian mushrooms were found to contain as many 41 *C. botulinum* spores per 100 g [32]. Surprisingly, several other surveys have failed to isolate *C. botulinum* from potatoes and mushrooms, even though these commodities have been identified as the vehicle of transmission in several botulism outbreaks [33–35].

In addition to the potential contamination of the raw commodity, spores can be inadvertently added to products during processing, particularly through the addition of dried vegetables or dry ingredients. One survey found that 16% of thickening agents, including starch, and 11.5% of dehydrated dairy ingredients were positive for *C. botulinum* using PCR–ELISA detection, with an estimate of 1–3 spores/kg [27]. A review of published surveys through 1996 reported that approximately 5% of honey samples worldwide contained detectable levels of botulinum spores [36]. When present, populations of botulinum spores were found at levels below 1/g of honey. However, honey samples implicated in cases of infant botulism in Canada and the USA were estimated to contain concentrations as high as 80,000 spores/kg [21,37,38].

Powdered infant formula (PIF) linked to a case of infant botulism in the UK was estimated to contain 13 spores in a 900 g can [39]. Processing conditions for PIF components seldom exceed 90–95 °C, allowing spores to survive. A survey conducted in the USA identified that 17% of 30 PIF samples ingested by patients with infant botulism, and 78% of nine market-purchased unopened powdered formula, contained clostridial spores [40]. Although none of the isolates in this survey were identified as neurotoxigenic clostridia, the *Clostridium* species recovered have similar environmental distribution as botulinum neurotoxigenic clostridial spores.

Dairy products have low rates of sporadic contamination and likewise few botulism outbreaks have been associated with these types of products. Less than 1 spore per liter was isolated from milk, but over 30% of mascarpone cheese samples associated with an outbreak was found to contain *C. botulinum* spores [41,42].

Infectious dose

Low populations of BoNT-producing clostridia spores can sporadically be found in most food commodities. Provided these foods are formulated, processed, or stored

to prevent botulinum growth and toxin production, they can be safely consumed by healthy children and adults. The minimum infective dose of *C. botulinum* spores required to cause infant botulism is unknown; however, it has been estimated to be as low as 10–100 spores [43].

Foodborne botulism can result from the consumption of food containing as little as 30–100 ng of preformed BoNT [4]. The quantity of BoNT in foods which were improperly canned, or held at improper storage temperatures during distribution or by the consumer, vary greatly. Bottles of carrot juice implicated in an international botulism outbreak contained as much as 6.6×10^5 mouse intraperitoneal lethal dose 50 per ml [44].

While it is generally accepted that conditions which support spore germination and growth of the vegetative cells will also support toxin production, there is no consensus on the minimum populations of *Clostridium botulinum* or the log increase which correlates with detectable toxin production. Several studies correlated detectable toxin production with a 100- or 1000-fold increase in cells when the initial inoculum was 2-log CFU/g [45,46]. However, other researchers demonstrated that BoNT production can be detected when population increases were no greater than 1-log or prior to any increase in plate counts [47,48]. Vegetative *C. botulinum* populations in samples have been shown to decrease by the time BoNT was detected, suggesting that cells may have lysed prior to plating [48]. Therefore, direct plate counts of *C. botulinum* may be an unreliable indicator of botulinum toxin production in food.

Food attribution

On average 80–100 cases of infant botulism are diagnosed in the United States each year [49]. Fewer cases have been identified globally, but the lower rate may be attributed to differences in active and passive surveillance [50]. Adult intestinal botulism is rare, with less than two dozen cases identified worldwide since 1980. Honey, infant milk formula, and dust are a few sources of *C. botulinum* spores implicated in many infant botulism cases, whereas medicinal herbs and teas used in Argentina are considered the sources of spores for infant botulism cases in that country. It is speculated that similarly contaminated foods and the environment may be the sources of spores for adult botulism, but no specific links have been made.

Comprehensive lists of foodborne botulism outbreaks and the factors contributing to the development of toxin in the food have been previously reviewed [4]. Requirements for commercial sterility of low-acid canned foods are responsible for the low incidence of foodborne botulism in commercially produced foods. In the second half of the twentieth century, only 7% of botulism outbreaks in the United States were linked to commercially processed foods, including foods served in restaurants [49]. Most foodborne botulism cases are attributed to home-preserved meats, fish, and vegetables, due to inadequate canning, drying/salting, or fermentation. In the USA, botulism outbreaks are commonly caused by group I strains, specifically serotype A strains. In Europe, group II strains are more commonly

associated with foodborne botulism cases. Globally, type E strains are responsible for 70% of outbreaks associated with fish, seafood, or marine mammals (particularly culturally fermented foods), whereas types A and B are more frequently associated with those associated with vegetables (beans, beets, asparagus, potatoes, and fermented bean products), herb pesto or sauces, olives, meat and poultry products, dairy products, and other vehicles.

Type E foodborne botulism associated with uncooked, improperly fermented aquatic animals (fish, marine mammals, and beaver) is endemic in the native human population of Alaska. The rate of foodborne botulism in this population is over 800 times greater than elsewhere in the United States [51]. The Republic of Georgia, Romania, and Poland also report high incidences of botulism cases (0.9, 0.18, and 0.20 cases per 100,000 inhabitants, respectively), which are substantially greater than the rate reported for the United States (0.0068 per 100,000 inhabitants). Foodborne botulism in these regions is typically associated with home-prepared meat products, mainly raw sausages and smoked-dried meat [52]. Homemade fermented bean products account for 63% of the botulism cases in China [53].

Prevention and control

The current good safety record for commercial foods is due, in large part, to the compliance of food manufacturers in processing, formulating, and controlling temperature during the distribution of foods. The presence of spores and a permissive environment allowing *C. botulinum* growth and metabolism are both required for certain threshold levels of toxin to be produced. Primary conditions associated with foodborne botulism include food formulations with pH and a_w within the growth range of *C. botulinum*, inadequate levels of antimicrobial food ingredients, reduced-oxygen environment, lack of competitive microflora, inadequate thermal processing, recontamination of commercially sterile products because of poor seals or after opening, and inadequate control of storage temperature-time.

Spore destruction

Inactivating spores in foods through thermal or alternate processing or by using sanitizing agents reduces the risk to acceptable levels to protect public health. Foods rendered commercially sterile (shelf-stable) by thermal processing are free of microorganisms capable of reproducing in the food under normal non-refrigerated (>10 °C) conditions of storage and distribution, and free of vegetative and spore-forming microorganisms of public health significance. The common method of preserving low-acid foods (pH >4.6 and water activity >0.85) for shelf stability is by thermal processing at 121 °C (retort processing, conventional canning) for a sufficient time to achieve commercial sterility. Under these conditions, no viable pathogenic microorganism can be detected by usual cultural isolation methods, or the occurrence of organisms is so low as to have no significance to safety or shelf life.

Thermal destruction of *C. botulinum* spores depends on many factors, particularly pH, water activity (a_w), the nutrient composition of the food, and the presence of added solutes or gases. For most low-acid canned foods, the minimal thermal process for a given food and size of container must be sufficient to produce a hypothetical 12-log decrease (12-D process) of *C. botulinum* spores, and must be validated by a competent process authority. F_0, the time equivalent at 121 °C of a process designed to inactivate spores demonstrating a z value of 10 °C, considers the integrated lethal value of all heat applied to the container during processing. Processing a food for 3 minutes at 121 °C, measured at the slowest heating point, achieves the commercial sterility which has ensured the remarkable record of the safety of canned foods.

Aseptic processing utilizes a process by which foods are rendered commercially sterile using ultra-high temperatures (such as 140 °C), continuous flow thermal processing for several seconds, and rapid cooling and then packed into a sterile container in a method that maintains sterility. Aseptic processing is frequently used for fluid foods, such as fruit juices, dairy products, and sauces.

Less severe heat treatment can be used in conjunction with other barrier technologies to inhibit growth and toxin production of *C. botulinum*. Storage at refrigeration temperatures less than 10 °C inhibits growth of group I (proteolytic) strains, but group II (non-proteolytic) strains have been shown to grow at temperatures as low as 3 °C under otherwise ideal conditions. However, group II spores are more heat-sensitive than group I strains and can be inactivated at temperatures less than boiling. For refrigerated process foods with an extended shelf life up to 6 weeks, heating to 90 °C for 10 minutes is recommended to provide a 6-log reduction of non-proteolytic *C. botulinum* spores. The storage temperature of the intact packages must be below 8 °C to prevent outgrowth of proteolytic strains.

Pressure-assisted thermal sterilization has recently been proposed as an alternative method to reduce total heat to render a food commercially sterile. Although spores can be resistant to pressures as high as 1500 MPa, combining pressure and thermal processing, such as 690 MPa and 110 °C for 4 minutes, has been shown to reduce clostridia spore populations by 4 to 5 logs [54]. In addition, the high temperature-high pressure treatment may sufficiently damage spores where survivors may require several months to recover and grow [55].

Another food which utilizes less stringent thermal processing conditions is shelf-stable canned cured meat products, which depends on the interactions of multiple factors, particularly the addition of nitrite, high salt levels, and low initial spore load [56]. Heating at a lower temperature for a short time, such as 113 °C for 15 minutes, will inactivate several logs of proteolytic spores, and any surviving spores are susceptible to the inhibitory effects of the salt and nitrites in the product.

Growth inhibition

Foods which are not treated to eliminate spores must be either stored or formulated under conditions which delay or prevent growth and toxin production during

storage (see Table 27.1). Low-acid foods, defined as having pH >4.6, may support growth of proteolytic *C. botulinum* under otherwise ideal conditions. Most non-proteolytic *C. botulinum* strains, especially type E strains, have the ability to grow at refrigeration temperatures, but the minimum pH requirement for growth is greater than 5.0. Although neurotoxigenic strains of *C. butyricum* produce BoNT/E, their minimum growth requirements are similar to group I strains because they are capable of growth at pH 4.8, but require temperatures greater than 12 °C for growth to occur. Deviation from the limits identified for control of *C. botulinum* for food formulations and storage conditions may shorten the time to toxin production and potentially lead to a greater concentration of toxin produced.

Refrigerated, processed foods of extended durability are heat-treated or processed using other treatments to reduce the presence of competitive microflora. As an alternative to thermal treatment to achieve a 6-log reduction of non-proteolytic spores, the safety of a food can be enhanced by increasing the acidity of a product to a pH below 5.0, adjusting the sodium chloride in aqueous phase to 3.5–5% or by using combinations of water activity, pH, and antimicrobials to inhibit growth of psychrotrophic group II strains.

Although strains of non-proteolytic *C. botulinum* can grow at or below refrigeration temperatures, the vast majority of foodborne botulism outbreaks have involved low-acid foods without secondary barriers and stored at temperatures greater than 10 °C. Growth of proteolytic *C. botulinum* can be inhibited by formulating foods to the limits of pH (<4.6) and a_w (<0.93), by less combinations of pH <5.6 and a_w <0.95, or by supplementing with traditional and clean label antimicrobial ingredients [57].

Food products can be naturally acidic, like citrus fruits, acidified by the direct addition of acids, or acidified by starter culture acid production such as in the manufacture of fermented sausages, cheeses, cultured dairy products, and other fermented vegetables and foods. While acid and acidified foods are considered to be inhibitory to *C. botulinum*, mold growth on the surface of the food can cause a localized increase in pH, which in turn can promote the growth of *C. botulinum*. This increase in pH under the mycelial mat may have contributed to a botulism outbreak involving tomato juice, and highlights the importance of controlling growth of other microbes in foods [58].

Efficacy of organic acid salts to inhibit *C. botulinum* growth is related to the concentration used and their respective pK_a. Organic acid salts with higher pK_a values, such as acetate and propionate, may be more effective than acids with lower pK_a values, such as citrate, in low-acid foods. Several investigations have demonstrated that sodium lactate and potassium sorbate delay BoNT production in culture media, beef, poultry, fish, refrigerated pasta, process cheese, and sauces. In general, the inhibitory effect of these compounds increases as the substrate pH decreases below pH 6 as a function of the concentration of undissociated acid and pH.

Sodium nitrite has potent antibotulinal activity in meat and poultry products, particularly in products like fermented sausage, which has a high salt concentration and a

reduced pH [56]. Although frequently debated, undissociated nitrous acid formed during the conversion of nitrite has been suggested to provide the antimicrobial activity by interfering with energy metabolism of the germinated cell and preventing outgrowth. Evidence suggests that the added nitrite concentration, rather than residual nitrite, is the greatest predictor of time to toxicity for the susceptible product. Isoascorbate enhances the antibotulinal effect of nitrite in freshly prepared cured meat, but may have an antagonistic effect in older product by accelerating nitrite depletion.

Antimicrobials derived from natural sources have been introduced as alternatives to conventional antimicrobial ingredients against *C. botulinum* and other pathogenic bacteria. These include phenolic compounds, terpenoids and essential oils, flavonoids, coumarins, alkaloids, lipid compounds, lectins and polypeptides, and other uncharacterized inhibitors derived from plant extracts or fermentation byproducts from lactic acid-producing bacteria.

Bacteriocins, including nisin, have been shown to delay botulinum toxin production in fresh fish stored at abuse temperatures and process cheese stored at ambient temperatures. Combinations of sodium nitrite and $\geq$100 ppm nisin were similarly effective by extending the time to toxicity in cooked cured chicken frankfurter emulsion. However, low solubility in bacon brine decreased nisin's antibotulinal effect and reports suggest that nisin may be denatured by proteolytic enzymes in raw meat. The efficacy of nisin may also be limited in the presence of high phospholipid or hydrophobic protein concentrations, and in products with a pH $\geq$ 6.0.

Certain green vegetables, such as celery, are high in nitrate. Although nitrate has no inhibitory effect on *C. botulinum*, it can be converted to nitrite using a nitrate-reducing starter culture (e.g., *Staphylococcus carnosus*). The extract can be dried and used as a flavoring agent but also serves as a source of nitrite. The final concentration of nitrite in the food is lower when added using this procedure than when directly adding sodium nitrite, and has a similar effect as that of comparable concentrations of traditional nitrite. Considering the concentration of residual nitrites is lower, additional barriers are recommended to provide equivalent protection to that of foods produced using conventional methods.

Fermentation byproducts which generate lactic acid can be buffered to provide equivalent composition and inhibition of sodium/potassium lactate; buffered vinegar can be used as an alternative to sodium diacetate. Blends of organic acids derived from natural sources can supplement the inhibitory activity of the low nitrite levels found in the plant-based extracts and extend the margin of safety in foods should they be temperature-abused.

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CHAPTER

Staphylococcal Food Poisoning

28

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Introduction

Staphylococcal foodborne poisoning (SFP) is of concern to public health authorities because it is one of the most common foodborne diseases worldwide. However, as clinical symptoms are self-limiting, reported outbreaks are usually confined to those involving a large number of people. Most staphylococcal food poisoning outbreaks are due to the ingestion of food containing one or more toxins formed by enterotoxigenic *Staphylococcus*: mainly *S. aureus* [1] and, less frequently, *S. intermedius* [2,3].

The first known outbreak of staphylococcal food poisoning occurred in Michigan, USA, more than a century ago, in 1884, due to consumption of cheddar cheese [4]. Other outbreaks were reported by J. Denys in 1894, and later on, in 1914, M. A. Barber reproduced in himself the symptoms of the disease after ingesting milk that had been contaminated with a culture of *Staphylococcus aureus* [5,6].

Biofilm production makes *S. aureus* an important microorganism in the food industry since it can be a source of persistent contamination leading to transmission of the microorganism to foods during processing. *S. aureus* is also of concern to physicians because of its virulence in healthy hosts and its role in nosocomial infections. Diseases caused by this microorganism range from infections of the skin and soft tissues to endocarditis, infection of the spleen and other organs, and infection of the lungs and the urinary tract [7].

Clinical features

Any food of animal origin can be contaminated by staphylococci. Foods that are directly manipulated by food service personnel are also important sources of these bacteria. Staphylococcal food poisoning results from the ingestion of enterotoxins synthesized during growth of staphylococci in food. So far, *S. aureus* and more rarely *S. epidermidis* have been involved in many SFP outbreaks. The most common symptoms are vomiting, nausea, abdominal cramps, and diarrhea. In more severe cases, headache, muscular cramping, and/or prostration may occur. Recovery usually occurs

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within 2 days [8]. Symptoms appear 1–7 (usually 2–4) hours after ingestion of food containing staphylococcal enterotoxin (SE). Death is rare but can occur (usually from severe dehydration or electrolyte imbalance), with fatality rates ranging from 0.03% for the general public to 4.4% for children and the elderly [9]. Although SEs are pyrogenic toxins, high fever is absent.

The amount of toxin necessary for causing illness in many outbreaks is in the range of 1 to 5 µg; however levels of less than 0.01 µg have been reported by Gilbert and Wieneke to cause illness [10]. Among the enterotoxins, staphylococcal enterotoxin type A (SEA) is the most frequently involved in staphylococcal food poisoning [1].

The microorganism

Members of the genus *Staphylococcus* belong to the family Staphylococcaceae with a G + C content of DNA varying from 30 to 39% depending on the species [11]. They are gram-positive cocci (0.5–1.5 µm in diameter) that occur singly and in pairs, tetrads, short chains, and grape-like clusters. They are non-spore-forming and non-motile chemoorganotrophs, and usually catalase-positive. *S. aureus* is a facultative anaerobe, as are most of the species in the genera [7,12].

Although the genus *Staphylococcus* includes over 40 species, those of interest in foods are listed in Table 28.1.

Table 28.1 Properties of Some Staphylococcal Species and Subspecies of Interest in Foods

Organism	Coagulase	Thermostable Nuclease	Enterotoxin	Mannitol Fermentation	Yellow Pigment	Hemolytic Activity
S. aureus subsp.						
aureus	+	TS	+	+	+	+
S. intermedius	+	TS	+	(+)	–	+
S. hycus	(+)	TS	+	–	–	–
S. schleiferi subsp.						
coagulans	+	TS		(+)	–	+
schleiferi	–	TS		–	–	+
S. caprae	–	TL	+	–	–	(+)
S. chromogens	–	–w	+	V	+	–
S. cohnii	–	–	+	V	–	–
S. epidermidis	–	–	+	–	–	V
S. haemolyticus	–	TL	+	V	v	+
S. lentus	–		+	+	v	–
S. saprophyticus	–	–	+	+	v	–
S. warneri	–	TL	+	+	v	–w
S. xylosus	–	–	+	v	v	+

(adapted from Bannerman and Peacock [7] and [5])
Note: + = positive; – = negative; –w = weakly negative; (+) = weak reaction; v = variable; TS = thermostable; TL = thermolabile.

Some substances produced by some species of *Staphylococcus* are associated with infectivity and disease. Among them there are cell wall components; exoenzymes such as proteases, for instance, coagulase; nucleases, like the thermostable deoxyribonuclease known as TNase; and hemolysins. They also produce several enterotoxins, known as staphylococcal enterotoxins (SEs).

There is a high correlation between the production of coagulase and SEs. This way, the detection of coagulase is an important tool in differentiating *S. aureus* associated with foodborne illness from other strains. However, it should be kept in mind that other species that are coagulase-negative can also produce SEs [1], as shown in Table 28.1.

When present in foods, *S. aureus* is not a good competitor. Most bacteria, especially *Pseudomonas* spp. and lactic acid bacteria (LAB), can make the growth of *S. aureus* difficult, or even inhibit it, depending on the type of food and the temperature of storage. LAB can inhibit the growth of *S. aureus* due to production of bacteriocins or hydrogen peroxide, competition for nutrients, and acidification [8].

Staphylococcal enterotoxins

Staphylococcal enterotoxins (SEs) are part of the pyrogenic exotoxin (PT) family, along with the toxic shock syndrome toxin (TSST-1) and streptococcal pyrogenic exotoxins (SPE). They are potent superantigens that activate T-cells up to 20% above antigen-specific activation, resulting in substantial cytokines release (see [13] for review). SEs also show potent emetic activity [14,15]. Summarizing, there are three ways for SEs to act: as enterotoxins, producing emesis and diarrhea in humans and other susceptible animals; as superantigens, inducing exacerbated T-cells stimulation; and as exotoxins, causing toxic shock.

SEs have been identified by letters as they have been discovered, and today there are 21 SEs or enterotoxin-like proteins (SE-*l*) described (from SEA to SEV, except SEF), excluding molecular variants [12]. SE-*l*s are proteins that, despite high homology with SEs, lack emetic properties or have not yet been tested. The new toxins presenting more than 90% amino acid similarity with pre-described SEs or SE-*l*s receive a number for subtype, according to the recommendations of the International Nomenclature Committee for Staphylococcal Superantigen Nomenclature (INCSSN) [16].

The first five SEs (SEA to SEE) are called classical. SEF was the original name of TSST-1, but when studies demonstrated that this protein lacked emetic properties [17] the SEF designation was abandoned.

SEs are short proteins of approximately 25–29 KDa and are rich in lysine, aspartic acid, glutamic acid, and tyrosine residues. They are extracellular, water-soluble proteins, and structurally stable, which are important characteristics in terms of food safety. They are resistant to most proteolytic enzymes, including those present in the digestive tract such as pepsin and trypsin. SEB can be destroyed by trypsin at pH 2, but is resistant to this enzyme at higher pH [18]. Partial neutralization of stomach

pH occurs during food digestion, providing a temporarily protective environment for ingested toxin. Recent studies have shown that levels of SEA and SED may decrease in boiled ham after a period of accumulation [19,20]. According to the authors the decrease in SE levels could have been caused by proteases from lactic acid bacteria present in the ham or by association of the toxins with *S. aureus.*

Heat resistance is another important feature presented by SEs. In food matrices SEs have shown to be more resistant to heat than in culture media, although they are inactivated by sterilization when present in low concentrations in canned foods [18]. Heat stability of the toxins is influenced by the environment encountered in the food, such as salt concentration, pH, and composition. Studies conducted with breaded chicken products showed that preformed SEA remained active after heat treatment of the cutlets, although no viable *S. aureus* cells were present [21]. It is also known that heat treatment of SEA and SED may interfere with the immunological activity in kittens, without affecting the emetic properties [22]. Thermostability of SEs varies and they have the ability to re-nature, regaining biological activity after the denaturing condition is removed [1]. Summarizing, the resistance of SEs to heat treatment and low pH is dependent on SE type, concentration, and food matrix.

Amino acid homology among SEs is generally high, with approximately 15% of the amino acid residues being completely conserved, and located on the central and C-terminal portions of the sequences [23]. SEs amino acid sequences can be grouped in four or five groups (Table 28.2), depending on the inclusion or not of SEH in Group 1 [24]. In spite of the divergences in the sequences, SEs present a very similar tertiary structure including an intramolecular disulfide bond (cysteine loop) required for proper conformation and probably involved in emetic activity. More detailed information on SE structural biology can be found in [23].

In contrast with the well-characterized superantigenic activity of SEs, the emetic activity is not completely understood. It is known that both activities are two separate functions located in two different regions of the toxin [23]. It is believed that cellular receptors specific for SEs may exist in the abdominal viscera which is the target for SEs responsible for SFP [25]. Hu et al. [26], using house musk shrew as a surrogate for primates, verified that the small intestine is the site for SEA emetic action. They also reported that serotonin (5-hydroxytryptamine

Table 28.2 Groups of SEs and SE-*l*s Based on Amino-Acid Sequence Comparison

Group	SEs and SE-*l*s
Group 1	SEA, SED, SEE, (SEH), SE-*l*J, SE-*l*N, SE-*l*O, SE-*l*P, SES
Group 2	SEB, SEC, SEG, SER, SE-*l*U, SE-*l*U2
Group 3	SEI, SE-*l*K, SE-*l*L, SE-*l*M, SE-*l*Q, SE-*l*V
Group 4	SET
Group 5	(SEH)

Source: Argudín et al. [24]

[5-HT]) is an important signaling molecule in the gastrointestinal tract in this process. They verified that 5-HT is released directly in the intestine, and not in the brain, and that the presence of 5-HT3 receptors on vagal afferent neurons is essential for SEA-stimulated emesis. In a recent study it was verified that the presence of aspartic acid in position 227 in SEA is important for both emetic and superantigenic activity of the toxin [27].

SEA is the most frequently reported toxin involved in staphylococcal food poisoning, followed by SEB, SEC, or SED, depending on the region of world. SEH was the causative agent, together with SEA, of the massive staphylococcal outbreak that occurred in Japan, in 2000, due to consumption of SEA-containing dairy products [28]. The importance of the novel SEs or SE-*l*s in SFP has yet to be determined. Although harboring *se* and *se-l* genes there is no confirmation of the expression of these genes. Emetic activity of SE-*l* has to be tested in primate feeding assay, the gold standard for estimating emetic activity according to the INCSSN [29].

Enterotoxin production is encoded by genes located in mobile genetic elements, i.e., pathogenicity islands (SaPIs), close to the staphylococcal cassette chromosome (SCC) elements, in prophages and plasmids, or in *v*Sa genomic islands [24]. *v*Sa is the name given to non-phage and non-SCC genomic islands that are exclusive to *S. aureus* and usually encode virulence determinants. It has been found in all *S. aureus* sequenced DNA, but not in other *Staphylococcus* species. The enterotoxin gene cluster (*egc*) that harbors a variable number of *se*/*se-l* genes is located in the *v*Saβ (see [24] and [30] for reviews on SE). Table 28.3 presents some information concerning SE gene location. The genetic elements can be horizontally transferred between staphylococci, modifying their ability to cause disease.

Expression of SE and other virulence genes is controlled by a complex regulation system, but there are no universally accepted rules for SE regulation. At least 16 two-component regulatory systems have been identified. SEA is constitutively expressed, and so far there are no data on mechanisms regulating its production [31]. SEA is produced throughout the log phase of growth, but SEB, SEC, and SED are produced during the transition from the exponential to the stationary phase indicating the participation of the accessory gene regulator (*agr*). It is known that *agr*, a gene conserved in staphylococci, is a quorum sensor and a global regulator with at least 24 genes under its control [1]. It is auto-induced by a post-translationally modified peptide [32]. The activation of *agr* inhibits the expression of certain genes encoding cell-wall associated proteins, and increases the expression of genes encoding exoproteins [33,34]. Loss of the *agr* signal transduction system directly affects the transcription of *seb, sec,* and *sed* and thus the corresponding toxins. Numerous transcriptional regulators affect the function of the *agr* system and may interfere with the transcription of *seb, sec,* and *sed.* The same environmental conditions and stresses that affect enterotoxin production will interfere with the regulators [35]. Other members of this complex regulation network are staphylococcal accessory regulator (*sar*) and the various homologous *sar*s; *S. aureus* exoprotein expression (*sae*); the repressor of toxins (*rot*), as well as by alternative sigma factor sig B (σ^B). Detailed information on *agr* and other regulatory factors can be found elsewhere [33,36].

Table 28.3 General Characteristics of SE and SE-l and Genomic Location of Encoding Genes

Toxin	Molecular Weight (kDa)	Emetic Activity	Gene	Genetic Backbone
SEA	27.1	Yes	*sea*	Prophage
SEB	28.4	Yes	*seb*	SaPI♦
SEC	27.5	Yes	*sec*	SaPI
SED	26.4	Yes	*sed*	Plasmid
SEE	26.4	Yes	*see*	Prophage
SEG	27.0	Yes	*seg*	*egc*♠
SEH	25.2	Yes	*seh*	scc♣
SEI	24.3	Weak	*sei*	*egc*
SE-*l*J	28.6	nd•	*selj*	Plasmid
SE-*l*K	25.5	nd	*selk*	SaPI
SE-*l*L	24.6	no*	*sell*	SaPI
SE-*l*M	24.8	nd	*selm*	*egc*
SE-*l*N	26.1	nd	*seln*	*egc*
SE-*l*O	26.8	nd	*selo*	*egc*
SE-*l*P	27.0	nd	*selp*	Prophage
SE-*l*Q	25.2	no	*selq*	SaPI
SER	27.1	Yes	*ser*	Plasmid
SES	26.2	Yes	*ses*	Plasmid
SET	22.6	Weak	*set*	Plasmid
SE-*l*U	27.1	nd	*selu*	*egc*
SE-*l*U_2 (SEW)	nd	nd	*selu2*	*egc*
SE-*l*V	nd	nd	*selv*	*egc*

(Modified from Argudín et al., [24] and [12])
♦*SaPI:* S. aureus *pathogenicity islands.*
♠egc*: enterotoxin gene cluster.*
♣*scc: staphylococcal cassette chromosome.*
•*nd: not determined.*
**no: emetic activity demonstrated in other animal models.*

Detection of *S. aureus* and enterotoxins

Microbiological methods are used for the detection or enumeration of *S. aureus*. Culture media have in their formulation selective agents and enrichment substances that will support the growth of *S. aureus* and inhibit or suppress the growth of other microorganisms. Baird-Parker agar is one of these media and is commonly used worldwide. Chromogenic media and alternative methods are now commercially available. Molecular methods based on polymerase chain reaction (PCR) have also been employed for detection of targeted *S. aureus*-specific DNA regions.

For the detection of enterotoxin in foods, the methods used are bioassays, molecular techniques such as PCR and multiplex PCR, and immunoassays. It is important to point out that in order to identify an outbreak, in addition to the isolation of the microorganism in large numbers (at least 10^5 *S. aureus* CFU per g of contaminated food in most outbreaks) from a suspected food or the enterotoxin produced by the organism, the use of genetic tools such as RAPD or pulsed field gel electrophoresis can be of help in determining the source of contamination of the food.

Exposure pathways

Factors influencing growth and survival

S. aureus is one of the most resistant non-spore-forming human pathogens when outside the body [1]. The extremes described here for each intrinsic or extrinsic parameter were derived from laboratory experiments in which all other tested conditions were ideal.

For growth, *S. aureus* requires B-group vitamins and aminoacids as a nitrogen source, as described for other gram-positive bacteria. *S. aureus* is capable of growing in the range of 7–48 °C. The optimum growth temperatures are between 35 and 40 °C. The production of enterotoxins occurs between 10 and 48 °C with the optimum in the range of 40–45 °C [8].

S. aureus cells are killed by pasteurization, but heat resistance can be increased depending on the composition of food, for instance, in high-fat food and in dry food.

The optimum pH for growth of *S. aureus* is 6.0–7.0, but most strains will grow in the range of 4.2–10. In a medium with HCl, *S. aureus* can grow in pH < 4.3, but if the acid is an organic acid the pH limits are much higher [8].

S. aureus is one of the most salt-tolerant pathogenic microorganisms in foods, growing at a water activity as low as 0.83, depending on the temperature, pH, type of humectants, and other parameters. The optimum water activity for growth is 0.98, in a range of 0.83–0.99 when in aerobic conditions and 0.90–0.99 when in anaerobiosis [8].

S. aureus grows in the range of −200 mV to +200 mV with the optimum oxidation-reduction potential being > +200 mV [37].

S. aureus shows high tolerance to different chemicals such as tellurite, polymixin, sodium azide, neomycin, and mercuric chloride. Resistance to these compounds has been explored as a tool for promoting selective growth of *S. aureus* in culture media for laboratory diagnostic purposes [6].

More recently, it has been shown that essential oils inhibit the growth of *S. aureus* [38,39]. The essential oil eugenol has been proved to repress the transcription of *sea, seb*, and other genes, suppressing, therefore, the production of SE besides inhibiting the growth of bacteria [38].

Reservoirs

The most important reservoirs of *Staphylococcus*, including *S. aureus*, are the skin and mucous membranes of humans. The most frequent site of colonization is the nares, although the microorganism may be found on other sites of the upper respiratory tract such as the throat. Mammary glands, axillae, and the inguinal and perineal areas are also sites of colonization [1,7]. Carriers can contaminate food through direct contact, or indirectly through skin fragments and also through respiratory tract droplets. Food industry equipment, such as knives, meat grinders, cutting blocks, and storage utensils, may be contaminated with *S. aureus* and thus become a source of food contamination.

The skin of humans is also the habitat of *S. epidermidis*, another species involved in staphylococcal food poisoning [7].

Animals are also reservoirs of *S. aureus*. They can be heavily colonized with staphylococci, especially in the setting of bovine mastitis—an infection of the mammary tissue that is a very serious problem for the dairy industry [6].

Outbreaks

Certain conditions are required for the development of staphylococcal food poisoning outbreaks: 1) a food that besides being a good substrate for growth of *S. aureus* also facilitates the formation of enterotoxins; 2) favorable temperature and sufficient time for bacterial growth and toxin production; and 3) ingestion of food containing sufficient amounts of preformed toxin to provoke symptoms. Poor hygiene practices during food processing [40], cooking, storage, and distribution [41] can also be associated with outbreaks.

Raw milk [42], noodle dishes [43], mashed potato made with raw milk [44], pasta and vegetable salad with mayonnaise sauce [45], pasteurized milk products [46], crepes [47], and cheeses made from unpasteurized milk [48] are examples of foods involved in staphylococcal food poisoning outbreaks in the last 10 years. Food handlers have also been directly implicated as the source of contamination in a number of staphylococcal food poisoning outbreaks [44,49,50].

An interesting example of an outbreak is the one that occurred in Japan in 2000 that affected 13,420 people due to the ingestion of dairy products. Laboratory investigation demonstrated the presence of SEA in leftover cartons of low-fat milk in concentrations in the range of 0.08–0.38 ng/ml [40]. Later on, using a PCR methodology Ikeda et al. [28] reported the presence of *sea* and *seh* genes in 10 samples of skim milk powder which was used as the raw material of the low-fat milk, and the *seg* and *sei* genes in seven samples of the same product. The quantity of SEH was almost identical to that of SEA in each sample of skim milk powder tested. The low-fat milk used as the raw material of the dairy product had high counts of viable bacteria due to a power cut in the plant.

Prevention and control

The factors that contribute to staphylococcal food poisoning are: inadequate refrigeration; inadequate cooking or heating processing; poor personal hygiene of infected handlers; and holding food at bacterial growth temperatures. If the contaminated food is kept below 4.4 °C or above 60 °C until consumed *S. aureus* will not multiply, resulting in the absence of enterotoxin production.

Other procedures that avoid or reduce contamination of food by *S. aureus* are the control of raw materials and the cleaning and disinfection of equipment from farm to fork.

Conclusions

Staphylococcal food poisoning is one of the most common foodborne diseases worldwide. It is caused by the ingestion of enterotoxins preformed in the food, mainly by strains of coagulase-positive staphylococci and, more rarely, by coagulase-negative strains. One important issue is the basis for staphylococci to produce an emetic toxin. Another important aspect is the involvement of SE other than classical ones in staphylococcal food poisoning outbreaks. The existing methods do not detect the newly reported toxins, which makes identification of staphylococcal food poisoning outbreaks caused by them difficult. The detection of genes by PCR that encode the new toxins is not sufficient since it does not show the expression of these genes.

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CHAPTER

Bacillus cereus 29

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Introduction

Bacillus species other than *B. anthracis* have long been suspected of causing foodborne diseases, since Steinar Hauge in 1955 demonstrated that *B. cereus* growing in vanilla pudding caused a diarrheal syndrome. The emetic syndrome caused by *B. cereus* growing in fried rice was discovered about 15 years later. *Bacillus cereus* is very widespread, but the foodborne diseases it causes are relatively mild and may occur at a much higher frequency than indicated by reports. However, the acute signs and symptoms of these diseases can be quite violent, making the victim incapable of operating complicated machinery. According to the Centers for Disease Control and Prevention's (CDC) Morbidity and Mortality Weekly Report (MMWR), the incidence of foodborne diseases caused by *B. cereus* seems likely to be under-reported because the organism is not included in the Foodborne Disease Outbreak Surveillance System [1,33]. In the USA, no causative agent is found in about 50% of investigated foodborne disease outbreaks, and a number of these may well have been caused by *B. cereus.* Its psychrotrophic properties and the resistance of its spores to heat enable *B. cereus* to survive at a broad range of temperatures, and make it more challenging to control, thus raising questions regarding the safety of pre-packed foods and ready-to-eat foods.

Clinical features of *Bacillus cereus* food poisoning

Two distinct types of illness have been attributed to the consumption of foods contaminated with *B. cereus*: diarrheal and emetic.

Bacillus cereus diarrheal syndrome

The disease

Diarrheal illness caused by *B. cereus* has an incubation time of approximately 4–16 hours and is manifested by abdominal pain and diarrhea that usually subsides within 12–24 hours. Nausea is sometimes observed, but vomiting is rare. In most

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cases, the disease is self-limiting and no treatment is necessary. In severe cases, fluid replacement therapy may be indicated. The infective dose is reported as being 10^5–10^7 cells.

Pathogenesis of the diarrheal syndrome

The virulence factors of the diarrheal syndrome are a complex of three toxins. The first is a hemolytic enterotoxin designated hemolysin BL (HBL) [2,3]. Positive results in ligated rabbit ileal-loop test (LRIL) and vascular permeability reaction (VPR) assays revealed that HBL consists of three proteins termed B, L_1, and L_2 [4,5,34]. A non-hemolytic enterotoxin (NHE) has been described [6], which also is composed of three protein components that are different from the HBL subunits. Sequencing and gene expression assays showed that both HBL and NHE are transcribed from one operon, with maximum enterotoxin activity produced during late exponential or early stationary phases of growth [7]. Also, a cytotoxic protein (CytK) implicated in necrotic enteritis has been isolated from a *B. cereus* strain involved in a severe outbreak (three deaths) of *B. cereus* diarrheal disease. The protein was very similar to β-barrel channel-forming toxins found in *S. aureus* and *C. perfringens* [8].

It has long been thought that the *B. cereus* diarrheal syndrome was a classical intoxication (i.e., ingestion of the toxin); however, it has been postulated that the disease is rather caused by ingested *B. cereus* cells that grow and produce enterotoxin within the intestinal tract of the patient and that it is a toxico-infection [9,10]. This claim is based on the level of contamination of food at the point of consumption being less than the concentration of cells generally necessary for enterotoxin production in many foodborne outbreaks associated with *B. cereus*; foods implicated in outbreaks frequently are found to be contaminated at 10^3–10^4 cells/ml or g, whereas most strains produce enterotoxin in significant amounts only after reaching cell concentrations of 10^7 cells/ml or g. Moreover, the activity of enterotoxin is dramatically affected or completely inhibited in low pH environments and following exposure to trypsin and chymotrypsin, suggesting that preformed toxin in food should be inactivated during transit through the stomach and duodenum.

However, the above studies were conducted using enterotoxin produced in brain heart infusion broth, and it should be noted that enterotoxin produced and ingested in a food matrix may be protected from damage by low pH and enzymes. It may well be possible that both modes of pathogenesis exist (intoxication and toxico-infection), especially in light of the existence of several diarrheagenic enterotoxins. In particular, a toxico-infection might be the mode of action in a newly observed, more severe form of *B. cereus* diarrheagenic syndrome [11].

Bacillus cereus emetic syndrome

The disease

A second type of *B. cereus* gastroenteritis, the emetic syndrome, was identified in the 1970s, associated with the consumption of fried rice [12]. The emetic illness

has an incubation time of 1–5 hours and is manifested by nausea and vomiting that lasts for 6–24 hours. Diarrhea is observed only occasionally. The symptoms are usually self-limiting, and treatment is seldom necessary, but in some severe cases it may cause death [13]. To transmit this type of *B. cereus* food poisoning, the food involved will typically contain 10^5–10^8 cells/g.

Pathogenesis of the emetic syndrome

The emetic syndrome of *B. cereus* is due to ingestion of preformed toxin in foods (i.e., intoxication). This was confirmed when cell-free supernatants were fed to monkeys and they produced the same symptoms as cultures in which the cells remained [12]. The toxin was found to be a peptide with a molecular weight of around 10,000 Da, stable at pH 2–11, and able to withstand heating at 121 °C for 90 minutes, as well as treatment with trypsin and pepsin. The toxin was named cereulide, and it is a dodecapepsipeptide, consisting of a ring structure, which is closely related to the potassium ionophore valinomycin. *In vitro* studies revealed that the toxin induces swelling of mitochondria with toxic effects due to potassium-ionophoretic properties [14–16], and also inhibits natural killer cells [17].

Characteristics of *Bacillus cereus*

Bacillus cereus is a gram-positive, catalase-positive, endospore-forming, rod-shaped bacterium. The *Bacillus cereus* group includes *B. cereus*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. thuringiensis*. These species are very closely related, and recent data on rRNA homology suggest that they be classified into one single species [18–20]. *Bacillus anthracis,* the causative agent of anthrax in mammals, is easily distinguished from the other members of the group based on its susceptibility to penicillin and the absence of hemolysis on sheep blood agar. The remaining species are identified based on motility and formation of crystalline parasporal inclusion bodies [12,21].

Growth and survival

Bacillus cereus has an optimum growth temperature of 30–40 °C; however, psychrotrophic members can grow in temperatures as low as 4 °C but will not grow above 43 °C. On the other hand, the mesophilic *B. cereus* can grow up to 55 °C. It can grow in a wide range of pH, from 5.0 to 8.8, with optimum of 6.0–7.0. The maximum generation time is approximately 23 minutes at 30 °C. In the presence of NaCl as a humectant, *B. cereus* will not grow at a_w of 0.93. However, when glycerol was used as a humectant, growth was possible at a_w of 0.93, but not 0.92. Spores of this microorganism form a major threat, impacting the safety and shelf life of foods. Spores are very heat-resistant; the $D_{121.1}$ value is 0.03–2.35 minutes, and the z value is 7.9–9.9 °C. Spores of psychrotrophic *B. cereus*, however, are less heat-resistant than are spores of mesophilic strains [22]. Germination of spores occurs when

conditions become more favorable, especially in the presence of purine ribosides and glycine or a neutral L-amino acid—L-alanine in particular [12].

Identification of *Bacillus cereus*

The identification of *B. cereus* is mainly based on the detection of the microorganism or its toxins. Cultural and non-cultural methods have been developed for this purpose. Cultural methods essentially involve direct plating onto selective agar media, such as mannitol-egg yolk-polymyxin (MYP) agar or polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA). Both rely on the presence of lecithinase (phospholipase C) [35]. Non-culture methods like PCR-based technologies that target specific genes in *B. cereus*, such as the *phospholipase* gene, *gyrase B* gene, and other genes, allow the detection of as low as <1 CFU/g [23–27]. Other immunological and biochemical assays have also been developed to detect spores and vegetative cells of the organism [28,29].

Detection of this pathogen may be done indirectly by detecting its toxins. Two commercial kits are available for the detection of *B. cereus* diarrheagenic toxins. Both kits are immunoassays, but they detect different antigens. The reverse passive latex agglutination (RPLA) enterotoxin assay produced by Oxoid reacts with a 43-kDa protein, the subunit L_2 of the tripartite enterotoxin complex (HBL) described by Beecher and Wong [4]. The second assay uses the enzyme-linked immunosorbent assay format (ELISA). This kit is marketed under the name *Bacillus* Diarrhoeal Enterotoxin (BDE) Visual Immunoassay kit (TECRA). It will detect a 41-kDa protein (NheA) that is part of the non-hemolytic enterotoxin complex (NHE) described by Lund and Granum [6].

Presence of *B. cereus* in foods

Members of the *B. cereus* group are ubiquitously distributed in the environment, mainly because of their spore-forming capabilities. Thus, *B. cereus* can easily contaminate various types of foods, especially products of plant origin. The organism is also frequently isolated from milk and dairy products, meat and meat products, pasteurized liquid egg, rice, ready-to-eat vegetables, and spices. Based on the ubiquitous distribution of the organism, it is virtually impossible to obtain raw products that are free from *B. cereus* spores.

The appearance of psychrotrophic strains in the dairy industry has added a new dimension to *B. cereus* surveillance in food. Studies indicate that both raw and pasteurized milk will harbor psychrotrophic *B. cereus,* with a prevalence of 9–37% in raw milk and 2–35% in pasteurized milk [30]. With an average generation time of 17 hours at 6 °C, *B. cereus* may produce enterotoxin during extended storage at slightly unfavorable temperatures [31,32].

Treatment and prevention

The symptoms of *B. cereus* infection are usually mild and self-limiting and do not normally require therapy. *B. cereus* can almost invariably be isolated from foods and can survive extended storage in dried food products, and it is not practical to eliminate low numbers of spores from foods. Control against food poisoning should be directed at preventing the germination of spores and minimizing the growth of vegetative cells. To accomplish this, foods should be rapidly and efficiently cooled to less than 7 °C or maintained above 60 °C, and be thoroughly reheated before serving.

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CHAPTER

Mycotoxins

30

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Introduction

Mycotoxins are fungal metabolites which when ingested, inhaled, or absorbed through the skin can cause disease or death in humans and domestic animals, including birds. By general agreement this definition excludes the toxins produced by macrofungi (the mushrooms), and compounds that cause disease only in plants or lower animals such as insects. Fungi produce a large number of metabolites, but only a few are classified as mycotoxins, i.e., they have been demonstrated to cause illness. Specific mycotoxins are produced only by specific fungi, usually by only a few species

Despite a few excellent studies of disease caused by fungi in feeds and foods in the first half of the twentieth century, the significance of mycotoxins in human and animal disease came only more recently. The term "mycotoxicosis" was first used in 1952, in a study of animal disease [1]. However the discovery of aflatoxins, following the deaths of 100,000 young turkeys in the UK in 1960, was the start of modern mycotoxin research. Over the next few years, laboratory and field experiments showed that many common fungi that cause both food spoilage and plant disease are able to produce a vast array of more or less toxic metabolites. Molecular structures of mycotoxins vary widely (Figure 30.1), so their effects on human and animal health also vary widely. The most commonly induced diseases include liver cancer, kidney failure, and effects on the brain or nervous system. Perhaps the most important point is that acute toxicity is rare: toxicity due to mycotoxins is almost always insidious, without any overt indication of effects on health in the short term. For this reason, the health effects of mycotoxins are among the most neglected areas of medical science.

It is generally agreed that the most important mycotoxins are: aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, and zearalenone [2]. Each of these will be dealt with below.

Aflatoxins

Health effects

Aflatoxins are the most important mycotoxins, both because they are of common occurrence and because aflatoxin B_1 is the most powerful liver carcinogen known.

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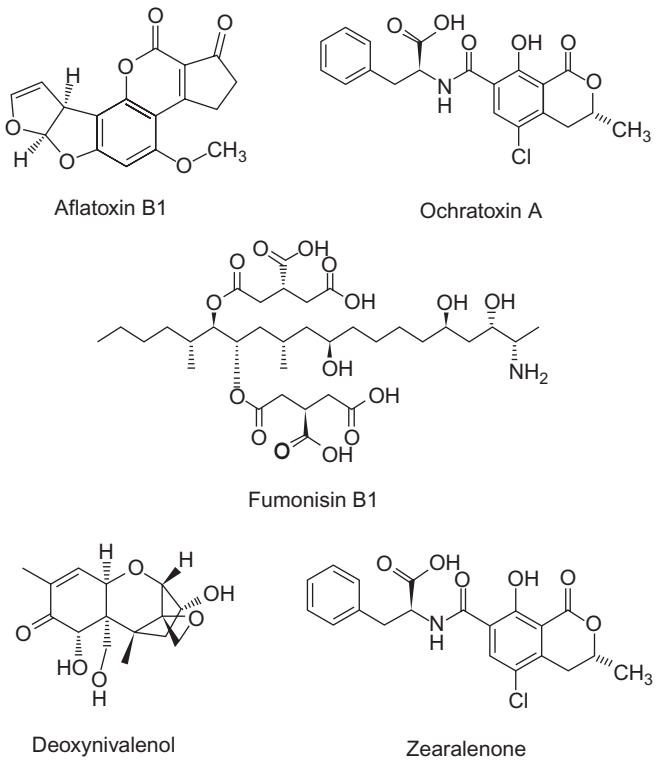

FIGURE 30.1

Structures of some major mycotoxins: aflatoxin B_1, ochratoxin A, fumonisin B_1, deoxynivalenol, and zearalenone.

The International Agency for Research on Cancer lists aflatoxin B_1 and naturally occurring mixtures of aflatoxins as Class 1 carcinogens, i.e., they are recognized as carcinogenic to humans. Hepatitis B virus also causes human liver cancer. In its risk assessment of aflatoxins, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has derived two potency factors for cancer formation by aflatoxins: for aflatoxin alone, 0.01 cases per 100,000 people per annum per ng kg^{-1} body weight per day, and for individuals carrying hepatitis B infection, 0.30 cases. Thus the two agents together are synergistic, indeed about 30 times as potent as aflatoxin alone [3]. Estimates of deaths due to aflatoxin ingestion range up to 100,000 or more per annum worldwide [4].

Aflatoxins are toxic in other ways as well. The acute disease (aflatoxicosis) is rare, with the most recent cases being from Kenya where several hundred deaths were reported in 2004 [5]. Evidence now exists that aflatoxin exposure before birth and in early childhood is associated with stunted growth—defined by WHO as height for age being more than two standard deviations below average height for

age in a given population. Aflatoxins have also been shown to suppress the cell-mediated immune response in both cell lines and domestic animals. Few studies have been reported in humans, but it is apparent that if the effects in humans mirror those in animals even approximately then the immunosuppressive effects of aflatoxins also have very wide implications for human health [6].

Fungal species producing aflatoxins

The major species producing aflatoxins are *Aspergillus flavus, A. parasiticus*, and *A. nomius*. *A. flavus* produces only B aflatoxins (B_1 and B_2), and some isolates also produce the lesser mycotoxin cyclopiazonic acid (CPA). About 40% of *A. flavus* isolates from nature produce aflatoxins. As well as B aflatoxins, *A. parasiticus* produces G aflatoxins (G_1 and G_2), but not CPA, and almost all isolates are toxigenic. *A. nomius* is morphologically similar to *A. flavus* but, like *A. parasiticus,* produces B and G aflatoxins, without CPA.

At least 10 other *Aspergillus* species are known to produce aflatoxins but have little or no significance in food crops, except *A. minisclerotigenes*. This species produces both B and G aflatoxins and also CPA but otherwise is distinguished from *A. flavus* with difficulty.

A. flavus is of universal occurrence in food crops in the tropical and warm temperate zones of the world but is especially associated with peanuts, maize, and cottonseed. Under conditions of drought stress, slow drying, or inadequate storage, aflatoxins are often produced at dangerously high levels in these commodities. *A. flavus* also infects tree nuts, usually as the result of insect damage or inadequate drying and storage, and less commonly rice, oilseeds, and other food commodities [7].

A. parasiticus has a more limited geographical range than *A. flavus*, being rare in Southeast Asia at least. It is associated with peanuts and is relatively uncommon in other food commodities [7]. *A. nomius* is not commonly found in foods but has been shown to be a major source of aflatoxin in Brazil nuts [8]. *A. minisclerotigenes* is common in peanuts in the Southern Hemisphere.

Aflatoxin formation in crops and its control

Unlike other mycotoxins discussed here, aflatoxins can be produced in nuts or kernels of susceptible crops throughout production—pre-harvest, during drying, and in transport and storage [7]. In peanuts, maize, and cottonseed, *A. flavus* is commensal, i.e., it grows in the plant and developing nut or kernel without any apparent damage to the plant.

Good agricultural practice (i.e., adequate moisture, weed control, and crop rotation) is usually considered to be the best method of aflatoxin control. Irrigation is effective; however peanuts are a drought-resistant crop and so are mostly grown under dry culture. Harvesting early can reduce contamination, while rapid mechanical drying of peanuts has a major effect in reducing the levels of aflatoxins. Insects are often involved in infection in maize, so the use of Bt maize cultivars is beneficial.

Entry of *A. flavus* into pistachio nuts depends when hull splitting occurs. Nuts in which hull splitting occurs early are more susceptible to *A. flavus* invasion. Some cultivars are more prone to early splitting than others, and this is especially important where nuts are harvested from the ground.

Figs are sometimes infected by *A. flavus* because of their unique structure developed for insect fertilization and because in some countries figs are harvested from the ground. Immature figs are not colonized by *A. flavus*, but once they are ripe, infection occurs readily and fungal growth continues during drying.

Biocontrol

Biocontrol of the type called competitive inhibition has performed well in cottonseed and peanuts in the USA, and is being extended to use in maize. In this technique, high numbers of spores of a non-toxigenic strain of *A. flavus* are introduced into fields where a susceptible crop is being grown, where they compete against the naturally occurring toxigenic spores for invasion sites on seeds, nuts, or kernels.

Control of aflatoxins in stored commodities

The prime consideration for storage of food commodities is the maintenance of sufficiently low water availability, i.e., water activity below 0.65, to prevent fungal growth during storage. That corresponds to 8% moisture for peanuts and other nuts, 12% for grains, and 22% for raisins, which contain a higher level of soluble carbohydrate [9].

Reducing aflatoxin by processing

Sorting is the main method used to reduce aflatoxins in most crops. Removal of discolored or damaged grains or nuts, mechanically or by hand, will decrease toxin content in the remaining sound commodity. In peanuts, color sorting was developed originally to reject discolored nuts, so as fungal growth is a cause of discoloration the process is an effective, non-destructive method for reducing aflatoxin levels. Blanching to remove skins and roasting to increase discoloration improves color sorting.

Maize samples are sorted by ultraviolet light, but the technique requires cracking of the grain, so it cannot be used for sorting individual kernels. Figs are sorted individually by UV light. Sieving of contaminated maize reduces both aflatoxin and fumonisin. No effective, non-chemical testing techniques exist for cottonseed or pistachios and, as with other commodities, non-destructive chemical assays are not available.

Aflatoxins are destroyed to some extent during heat processing. Destruction varies from less than 25% in boiling water, during extrusion or autoclaving, to up to 80% in dry roasting [10]. The alkali process used to produce refined table oil completely removes aflatoxin.

Ochratoxin A

Health effects

Ochratoxin A (OTA) is a chronic nephrotoxin, affecting kidney function. OTA has a long half-life in the bloodstream, so that in areas where OTA is common in

foods, the blood of healthy humans regularly contains detectable amounts of this toxin. OTA also has carcinogenic properties, but the mechanism of carcinogenicity remains unknown. The carcinogenic effects in animals are considered to be of less importance than the nephrotoxicity. Although OTA is demonstrably toxic to animals of all kinds, its effects in humans remain unclear and the subject of debate. Both genotoxic and non-genotoxic modes of action have been proposed [3]. The International Agency for Research on Cancer has classified OTA as a possible human carcinogen (Group 2B), based on sufficient evidence of carcinogenicity in experimental animal studies and inadequate evidence in humans.

Fungal species producing OTA

The ecology of OTA formation in foods and feeds is more complex than that of aflatoxin, because OTA is formed by both *Aspergillus* and *Penicillium* species, with differing—and quite specific—ecological niches. The major commodities susceptible to OTA production can be divided into warm and cool temperate crops. In warm climates, the foods most likely to contain OTA are coffee, dried vine fruits, and wines, while in cool climates cereals are the main crop affected.

In cool temperate climates, ranging across Northern and Central Europe, Canada, and Northern Asia, OTA is produced in cereal crops by *Penicillium verrucosum* [11]. As a consequence, in these regions OTA is found in products such as bread and flour-based foods, and in the meat of animals which are fed cereal grains. It should be noted that *P. verrucosum* is unable to grow above 30 °C and is not found in warmer climates, so small grains from tropical and subtropical areas rarely contain OTA [7].

One group of *Aspergillus* species producing OTA is centered on *A. ochraceus.* This species grows at low water activities and is quite common in stored food commodities. Until 2004, it was considered to be the major source of OTA in coffee [12]. Advances in molecular and chemical techniques resulted in the splitting of *A. ochraceus* into three species [13], and *A. westerdijkiae* is now recognized as the main OTA producer in coffee.

The second group of *Aspergillus* species making OTA centers on *A. carbonarius.* This species grows at quite high temperatures and has dark hyphae and spores, so it is resistant to UV light and sunlight. These characteristics provide a competitive advantage in vineyards and grape drying yards. Discovered to be a source of OTA only a decade ago, it is now recognized as the primary source of OTA contamination in grapes and grape products [14]. Grapes are dried in the sun without preservatives, so dried grapes (raisins, sultanas) may contain unacceptable levels of OTA [15]. *A. carbonarius* also produces OTA in coffee from some producing regions [12].

A low percentage of isolates of *A. niger*, a species closely related to *A. carbonarius*, are also able to produce OTA. However, in addition, Frisvad et al. [16] reported that *A. niger* can also produce fumonisins, previously regarded as produced exclusively by *Fusarium* species. Fumonisins produced by *A. niger* have been found in raisins and coffee.

Control of OTA formation in crops

OTA formation in foods is usually a post-harvest problem, associated with slow drying. However, *A. carbonarius* infects grapes before harvest as the result of damage by pathogenic fungi or rain [17]. OTA occurs in wines throughout the world, but levels are usually low, as the fermentation process positively stops growth of the fungus. Populations of *A. carbonarius* in vineyards can be reduced by irrigation and good agricultural practices [17].

Both *A. westerdijkiae* and *A. carbonarius* can infect coffee cherries if drying is too slow—a common problem in the misty upland areas where coffee is grown. Good sun drying or a combination of sun drying and mechanical dehydration provide effective control of OTA in coffee [12].

Reducing OTA by processing

OTA is largely removed during the winemaking process as it is bound to solid fractions, sediment, and some fining agents. The carryover from grapes into finished wine is between 1 and 8% [18].

OTA is partially destroyed during coffee roasting, instant coffee production, and decaffeination [19].

Fumonisins

All *Fusarium* species grow only at high water activities, above about 0.90 [7]. Production of *Fusarium* mycotoxins occurs only during growth of the fungus in the living plant and seed, or during early stages of drying.

Health effects

Fumonisins are remarkable for the wide range of effects they cause. Fumonisins inhibit the enzyme ceramide synthase, which causes accumulation of intermediates in the sphingolipid metabolism pathway and depletion of complex sphingolipids. These effects interfere with the binding of folate and some other proteins in cell membranes. The most dramatic effect occurs in horses, in which the disease called equine leucoencephalomalacia occurs. This is a rapidly progressing disease that causes equine brains to liquefy. In pigs, fumonisins cause pulmonary oedema, due to left ventricle heart failure, while in rats the primary effect is to cause liver cancer; programmed cell death (apoptosis) also occurs [20].

In humans, fumonisins produced by *F. verticillioides* cause none of these animal syndromes but are associated with esophageal cancer. Extensive studies in areas of low and high maize consumption in South Africa have suggested this connection. This disease is also prevalent in areas of China and occurs at significantly high levels in parts of Iran, northern Italy, Kenya, and a small area of the southern USA. In all of those areas consumption of maize and maize products is very high [20].

There is also some evidence that high intakes of fumonisins from maize are associated with neural tube defects such as spina bifida in areas of Guatemala, South Africa, and China and in a population along the Texas–Mexico border [20].

Fungal species producing fumonisins

Fumonisins are produced by *F. verticillioides* and the closely related species *F. proliferatum*. These species are endemic in maize worldwide and are endophytic in the plants [21]. Under conditions of water or insect stress, the symptomless endophytic relationship may convert to a disease and/or mycotoxin-producing interaction [22]. Fumonisins produced by these species are found only in maize and sorghum, as these species rarely infect other crops.

Control of fumonisin formation in crops

Good agricultural practice, the control of insects, development of resistance to ear diseases, and development of cultivars adapted to drought and temperature tolerance are all important in reducing the risk of fumonisin and other *Fusarium* toxin accumulation in maize. Some progress has been made in breeding cultivars resistant to ear rot [21,22].

Reducing fumonisins by processing

Sorting and cleaning reduce fumonisins in maize by removal of broken and damaged kernels. Milling does not destroy fumonisins, but they are concentrated in bran and germ rather than flour. Significant reductions of fumonisin levels occur during processes at temperatures >150 °C, as in maize meal production, frying chips, and extrusion processing, which is used extensively in the production of breakfast cereal and snack and textured foods [23].

Nixtamalization is a centuries-old process used in Central America, in which maize is soaked and then cooked with ash or lime high in alkali. It removes almost all fumonisins, resulting in tortillas and other maize-based foods being substantially free of these mycotoxins.

Deoxynivalenol (DON)

Health effects

The main risk from DON for humans is gastrointestinal poisoning. In one episode in India, DON levels in wheat ranged from 0.4 to 8.4 mg/kg, while in China, poisoning was linked to wheat contaminated with 0.3–100 mg/kg DON. Consequently, it has been suggested that acute toxicity may occur from exposures in the low mg/kg range. No other risks to human health from DON in foods have been identified.

Fungal species producing DON

Fusarium graminearum (often reported as *Gibberella zeae*—its sexual stage) and *F. culmorum* produce the trichothecene toxins deoxynivalenol and nivalenol and also the estrogenic mycotoxin zearalenone. These species are plant pathogens, invading maize, wheat, and barley plants, and causing diseases, known as Gibberella ear rot and Fusarium head blight, in developing grain. These diseases are prevalent in north temperate climates, especially in wet years, and are much less common in the tropics [21].

Control of DON formation in crops

Some success has been achieved in controlling DON formation in wheat by the use of azole fungicides at anthesis. Forecasting systems to advise farmers of the likelihood of DON formation have been developed in Canada and Europe [24]. Otherwise, control relies on reducing levels of *Fusarium* species in the field by good management and crop rotation.

Reducing trichothecenes in processing

Sorting and cleaning will reduce levels of trichothecenes in grains.

Zearalenone

Health effects

Zearalenone has a low acute toxicity, with oral 50% lethal doses in rats and chickens exceeding 2 g/kg body weight. However, much lower levels of zearalenone and its metabolites possess estrogenic activity in experimental and farm animals. The most obvious problems are seen in pigs: doses as low as 1–5 mg/kg can induce vulvovaginitis and vaginal and rectal prolapse in young female pigs. Sheep and cattle are more resistant, and it seems likely that rumen microorganisms are responsible for metabolizing zearalenone to compounds of lower toxicity. Chickens are also comparatively resistant, tolerating up to 30 mg/kg of zearalenone in feed.

Fungal species producing zearalenone and control

Zearalenone is produced by the same fungi that produce DON, in the same crops, and usually at the same time. The ecology of zearalenone production mirrors that of DON, at least in general terms. In consequence, occurrence and control are similar.

Methodology in mycotoxin detection

Mycotoxins constitute a very heterogeneous group of substances, so extraction and detection methods vary widely.

Mycotoxins are usually very unevenly distributed in commodities. For that reason, sampling is often the largest source of error in mycotoxin assays. Sampling plans have been developed for many commodities, especially for aflatoxins: for continuous lines, for 10 ton lots, and for bag stacks. Relevant papers should be consulted for more information. For example, the Codex Alimentarius Commission has developed sampling plans for various toxins and commodities.

It cannot be overemphasized that sample sizes should be as large as possible, because of the uneven distribution of toxins. For example, for aflatoxin in peanuts, entire samples of 8 kg or more should be comminuted in a vertical chopper or similar mill. Subsamples should then be further processed.

Extraction of mycotoxins from subsamples employs a variety of mixed polar and non-polar solvents, depending on the food matrix being analyzed. Methanol:water (80:20) is now the most commonly recommended, as it is non-toxic and does not interfere with immunological assays. For analysis, thin layer chromatography (TLC) is the oldest and most versatile method. It has been superseded in industrial countries by high performance liquid chromatography (with ultraviolet, mass spectrometry, or fluorescence detection), gas chromatography (with electron capture or flame ionization detection), or mass spectrometry and liquid chromatography tandem mass spectrometry [25]. However, TLC remains a cheap and versatile technique of great value in less industrialized nations.

Enzyme-linked immunoassay (ELISA) technology has found widespread application for the detection of mycotoxins in foods. ELISA has advantages of rapidity, accuracy, and sensitivity, and requires no complex equipment. However, immunoassays require antibody preparations, which are relatively unstable and usually require refrigeration.

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CHAPTER

Seafood Intoxications 31

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Introduction

Seafood consumption is the leading cause of foodborne illness with known etiology. It is responsible for 10–20% of outbreaks among all food types and about 5% of all individual illnesses ([1]; CSPI, 2007). The annual acute care costs of seafood-borne disease are estimated to be up to two-thirds of a billion dollars [2]. Persistent symptoms are seen in about 2–3% of cases and the costs of medical care, lost productivity, and functional disability associated with chronic sequelae are thought to exceed those of acute care. These are conservative estimates as there is considerable diagnostic uncertainty and under-reporting with respect to seafood-related illnesses [3]. It is anticipated that the number of cases will continue to rise over the next decade with shifting preferences to heart-healthy diets, increased travel to coastal destinations, increased consumption of imported fish, the growth of coastal urban communities, and growing segments of the population involved in marine recreation [2,4]. Future vulnerabilities also include: the increased frequency and distribution of harmful algal blooms (HABs) and related toxins in many regions of the world as a result of natural environmental factors (hurricanes and earthquakes); anthropomorphic activity (increased eutrophication, marine transport, and aquaculture); and climate change.

This chapter is organized around seven commonly recognized seafood poisonings. Five are due to consuming shellfish, coral reef fish, or finfish tainted by naturally occurring algal toxins: ciguatoxin, brevetoxin, saxitoxin, okadaic acid, and domoic acid. None of these toxins is detectable in fish or shellfish by visual inspection, taste, or smell. These marine toxins are also heat-stable and unaffected by cooking, freezing, drying, or smoking. Finally, diagnostic tests are not yet available to detect the presence of these toxins in people, and there are no available antidotes. Hence, the prevention, diagnosis, and management of seafood poisonings can be challenging. The seventh, and most commonly occurring seafood poisoning, scombroid poisoning, is the result of eating fish that have been inadequately preserved or handled. Finally, tetrodotoxin (puffer fish) poisoning as a result of toxins found naturally within the species of fish itself will be discussed. The geographical

Foodborne Infections and Intoxications.

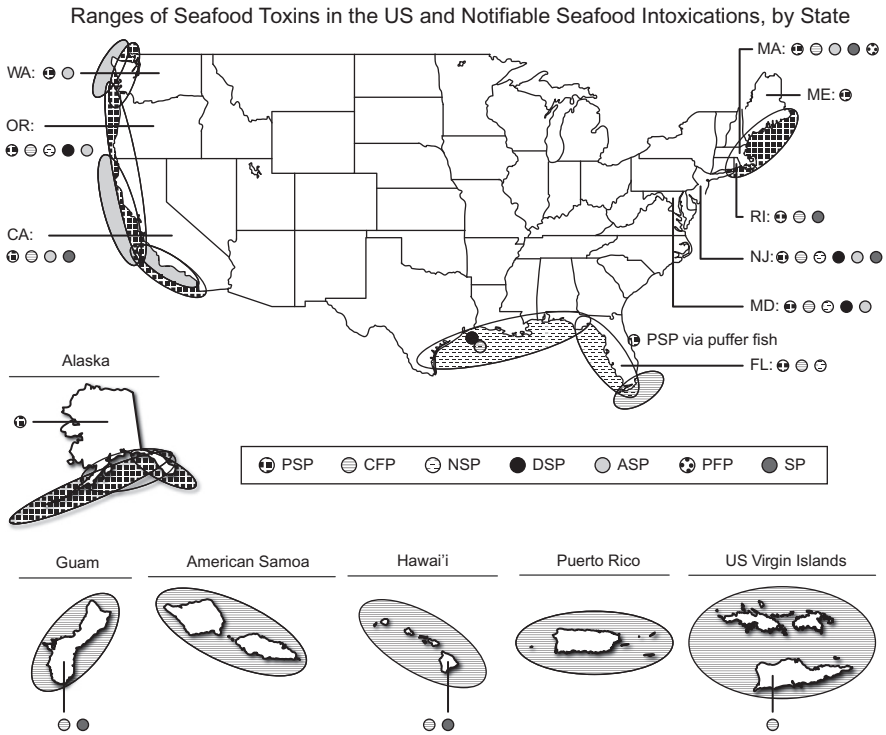

FIGURE 31.1

All foodborne disease outbreaks are nationally notifiable (http://www.cdc.gov); however, some states require that individual cases of seafood intoxication be reported by physicians to the state health departments, as noted above (current as of January 2012).

Adapted from an illustration by the US National Office for Harmful Algal Blooms, Woods Hole Oceanographic Institution (http://www.whoi.edu/redtide/page.do?pid=14898).

distribution of the toxic fish and shellfish in the United States may be found in Figure 31.1. Current knowledge regarding the clinical features, diagnosis, treatment, and mechanisms of illness will be reviewed (see Table 31.1 for summary) followed by important elements of prevention.

Ciguatera fish poisoning (CFP)

Ciguatera is the most frequently reported seafood-related disease in the United States and the most common foodborne illness related to finfish consumption in the world [18,19]. It is endemic in areas where consumption of reef fish is common, including the Caribbean, southern Florida, Hawaii, the South Pacific, and Australia. However, recent reports suggest expansion of the biogeographical range of ciguatoxic fish [9,20]. CFP is caused by consuming reef fish that have accumulated

Table 31.1 Seafood Intoxications

Syndrome (Major Toxin)	Vectors (Known and Potential)	Onset Time and Duration	Major Symptoms	Treatment	Prevention
Ciguatera Fish Poisoning[a] (Ciguatoxin)	Large, predatory tropical reef fish (barracuda, grouper, red snapper, amberjack); some types of eels; farm-raised fish that feed on contaminated fish[b]	12 to 24 hours; neurological symptoms can last months to years	**n**, **v**, **d**, **ab**, **p** (especially hands and feet), **t**, **bp**. Also: metallic taste, itching, dizziness. Possible recurrence of neurological symptoms during times of stress, after ingesting alcohol or low level fish. Low mortality in the USA.[c,d,e]	Supportive. Mannitol therapy is recommended for neurological symptoms.[f] Brevenal has also been indicated.[g]	Avoid consuming risky fish; monitoring,illness surveillance
Diarrhetic Shellfish Poisoning[h] (Okadaic Acid)	Mussels, oysters, scallops, clams, cockles, some species of crabs[i,j,k]	30 minutes to 15 hours; full recovery, within 3 days[i]	**d** (incapacitating), **n**, **v**, **ab**. Headache, fever. No reported mortality.	Supportive. Most people do not seek medical treatment.	Monitoring seafood and water; regulated in European countries, though outbreaks still occur[l]
Neurotoxic Shellfish Poisoning[m] (Brevetoxins)	Mussels, clams, whelks, conch, coquinas, oysters, scallops; liver and stomach contents of some planktivarous fish; inhalation of toxin aerosolized by coastal wind and waves[m]	*Consumption*: A few minutes up to 18 hours (often within 3 to 4 hours) *Inhalation*: Minutes to hours (<24 hours)	*Consumption*: **p** (perioral, face, extremities), **ab**, **t**, **d**, **b**, **r** (most severe cases). May appear disorientated or intoxicated (slurred speech, pupil dilation, overall fatigue, involuntary muscle spasms). *Inhalation*: **a**, **b**, **r**. Throat irritation, sneezing, coughing, itchy and watery eyes, burning of upper respiratory tract. No reported mortality for either pathway.	*Consumption*: Supportive. *Inhalation*: Leave the beach and go to an air-conditioned area.	Coastal and seafood monitoring and quarantine; closures to recreational harvesters[n]; persons with asthma or respiratory problems should avoid beaches during "red tides"

(*Continued*)

Table 31.1 (Continued)

Syndrome (Major Toxin)	Vectors (Known and Potential)	Onset Time and Duration	Major Symptoms	Treatment	Prevention
Paralytic Shellfish Poisoning[o] (Saxitoxins)	Scallops, mussels, clams, geoducks, cockles, puffer fish, some fish, gastropods, crustaceans[p]	30 minutes to 3 hours; a few hours to a few days	**p** (perioral, often spreading to neck and extremities), **n**, **v**, **r** (severe doses: respiratory paralysis and death). Muscular weakness, drowsiness, incoherent speech. No mortalities in recent US and European outbreaks.	Supportive. Artificial ventilation in severe cases.	Coastal monitoring; quarantine of seafood and region; rapid case reporting; beach closures to recreational harvesters[q]
Amnesic Shellfish Poisoning[r] (Domoic Acid)	Razor clams, mussels, oysters, squid. Viscera (not muscle) of scallops, sardines, anchovies, crab, and lobster[s]	Within 48 hours; months to years with permanent amnesia.	**ab**, **n**, **v**, **r**, disorientation, seizures, permanent short-term memory loss, possible neurodevelopmental delay. Excessive respiratory secretions.[t] Coma and death only among most severe cases[r] or elderly.[s]	Supportive.	Coastal monitoring of water and shellfish; harvesting beach closures; rapid illness reporting
Puffer Fish Poisoning[u] (Tetrodotoxin)	Puffer fish ("fugu"), marine snails, sea slugs[v]	Usually within 30 minutes; within a few days	**p** (perioral), **n**, **v**, **d**, **r**, **bp**. Feelings of doom, lightheadedness, dizziness.	Supportive. Gastric lavage and activated charcoal have been used in emergency settings.	Avoid consumption; regulated food source (only one legal Japanese importer of puffer fish in US)[v]; preparation; rapid reporting

Scombroid Fish Poisoning[w] (Histamines and Other Products of Decomposition)	Fast swimming and migratory finfish with red colored meat and high levels of free histidine (tuna, skipjack, bonito, mackerel)[x]	10 minutes to 1 hour; usually one day	**a**, **p** (oral), **n**, **v**, **ab**, **d**, **bp**. Peppery or metallic taste, palpitations. Cardiac and pulmonary complications occur rarely (among those with pre-existing conditions).	Antihistamines.	Regulated food handling (temperature); surveillance of fish species with naturally high levels of histidine; educate recreational fishers about time–temperature handling[y]

Abbreviated symptoms: **a**, *allergic-like;* **ab**, *abdominal cramps;* **b**, *bronchoconstriction;* **bp**, *decrease in blood pressure;* **d**, *diarrhea;* **n**, *nausea;* **p**, *parathesias;* **r**, *respiratory distress;* **t**, *reversal of temperature sensation;* **v**, *vomiting*[z]

[a] *[5]*
[b] *DiNubile and Hokama, 1995*
[c] *[6]*
[d] *[7]*
[e] *[8]*
[f] *[9]; see also for treatment for specific symptoms*
[g] *Nguyen-Huu et al., 2010*
[h] *Hossen et al., 2011*
[i] *[4]*
[j] *[10]*
[k] *Vale and Sampayo, 2008*
[l] *see [h] and [11]*
[m] *see for examples: [h, k] and Hinder et al., 2011*
[n] *Plakas and Dickey, 2010. See Terzagian, 2006, for examples*
[o] *Etheridge, 2009*
[p] *Deeds et al., 2008*
[q] *For examples, see [c] and [12]*
[r] *Grant et al., 2010*
[s] *Lefebvre and Robertson, 2010*
[t] *[13]*
[u] *[14]*
[v] *[15]*
[w] *[16]*
[x] *See [x] for a complete listing of species*
[y] *[17]*

potent neurotoxins (ciguatoxin) in their flesh and viscera. The toxins are produced by the marine dinoflagellate, *Gambierdiscus*, that live on various, sometimes harmful, microalgae in coral reef ecosystems. Herbivorous fish consume these dinoflagellates and, through the process of bioaccumulation and magnification, the toxin advances through the food web via carnivorous species. More than 400 fish species are thought to have the potential for ciguatera toxicity [21], including farm-raised fish (DiNubile and Hokama, 1995). However, the risk is greatest for carnivorous, predatory fish, such as barracuda (of which >70% may be toxic), snapper, grouper, and amberjack [22].

Diagnosis, clinical symptoms, and treatment

Diagnosis of ciguatera fish poisoning (CFP) or "ciguatera" is based upon clinical symptoms within the context of a carefully elicited history of recent predatory reef fish consumption. The initial symptoms begin within 12 hours of eating toxic reef fish, with severe gastrointestinal problems (nausea, vomiting, diarrhea, and abdominal pain) which usually abate within 24 hours [6]. Cardiovascular problems (generally a combination of bradycardia with hypotension) or neurologic symptoms may also be present during this acute episode. In the Caribbean and southern Florida cardiovascular disorders typically reverse within 48 to 72 hours [6,23]. However, in Pacific regions there have been reports of rapid progression to respiratory distress, coma, and death [24,25].

From a few hours to 2 weeks after exposure, a diverse range of subjective neurological complaints emerge in about 70% of cases. These may include pain and weakness in the lower extremities; painful tingling around the mouth, teeth, nose, and throat; and peripheral paresthesias, headache, metallic taste, hyporeflexia, and/or dysphagia. The hallmark of CFP neurological symptoms is an unusual paradoxical disturbance of thermal sensation, i.e., cold objects feeling hot and sometimes hot feeling cold. Recovery from acute neurologic symptoms is longer and less predictable than gastrointestinal or cardiac symptoms, ranging from 1 week to 6 months or longer. A chronic ciguatera syndrome may occur and is typically characterized by intractable fatigue, weakness, and/or paresthesias and is often accompanied by depression. Ciguatera symptoms may also reappear after a period of presumed recovery. This recurrence may be triggered by alcohol use or repeated consumption of fish with low levels of ciguatoxin, suggesting that persons who have had one episode of ciguatera are at increased risk for repeated illness [8].

Ciguatoxins (CTXs) are potent neurotoxins that are predominantly responsible for CFP. The variable signs and symptoms of ciguatera suggest the involvement of multiple toxins (and toxin precursors), most likely associated with an increase in marine diversity and ecosystems over time. As noted above, symptoms are more severe following ingestion of ciguatoxic fish from Pacific regions compared with tropical Atlantic or Caribbean regions. It is well recognized that different toxin assemblages may explain this distinction. The primary CTX is a heat-stable, lipid-soluble polyether that is resistant to gastric acid [26]. The gastrointestinal

symptoms (diarrhea) seen in patients with ciguatera appear to result from direct stimulation of mucosal ion transport, without accompanying damage to the intestinal mucosa [27]. The neurologic symptoms are thought to represent the direct interaction of CTX with voltage-gated sodium channels along the peripheral nerves (see [28] or [29] for review).

Treatment for CFP involves non-specific, supportive symptom management. In most cases the illness is self-limiting. To prevent relapses, patients are typically instructed to avoid risky fish, nuts, or alcohol for 6 months after the poisoning episode. In severe cases (most often a result of Pacific ciguatoxins) administration of intravenous D-mannitol within 48 hours of symptom onset is accepted as the most effective way to manage neurological symptoms [30]. Although the efficacy of this intervention was not supported in one double-blind trial where D-mannitol was compared with normal saline [31], infusion of D-mannitol remains the treatment of choice for severe ciguatera intoxications (Kumar-Roine et al., 2010), though brevenal may also be effective (Nguyen-Huu et al., 2010). People who suffer chronic symptoms for months or years benefit from reassurance, ongoing symptom management, and reminders to avoid high-risk fish.

Paralytic shellfish poisoning (PSP)

Paralytic shellfish poisoning (PSP) is a potentially lethal clinical syndrome caused by ingesting bivalve mollusks (mussels, scallops, and clams) contaminated with a group of structurally related marine toxins collectively referred to as saxitoxins or STX (Etheridge, 2009; [4,32]). PSP toxins are concentrated in the shellfish as a result of the continuous filtration of toxic algae produced by several dinoflagellates (including *Alexandrium*, *Gymnodinium*, and *Pyrodinium*) during "red tide" blooms. Predators of bivalve shellfish (scavenging shellfish, lobsters, crabs, and fish) may also be vectors for saxitoxins, thus expanding the potential for human exposure (Deeds et al., 2008; [33]). They exert their effect by binding directly on the voltage-dependent sodium channels in nerve and muscle cell membranes, interrupting nerve signal transmission and leading to paralysis [4,34]. Geographically, the most risky regions for PSP are cold water marine coasts. In North America, this includes Alaska, the Pacific Northwest, and the St Lawrence region of Canada. Toxic shellfish have also been found in cold water regions of southern Chile, England, Japan, and the North Sea.

Diagnosis, clinical symptoms, and treatment

The initial symptoms of PSP are numbness or tingling around the mouth and lips after shellfish consumption, the onset of which (10 minutes to 2 hours) appears to be dose-dependent [12,35]. The numbness and tingling may then spread to the neck and face and be accompanied by headache, abdominal pain, nausea, vomiting, diarrhea, and a wide range of neurological symptoms (e.g., weakness, dizziness,

dysarthria, paresthesias, double vision, loss of coordination, vertigo or dizziness, and/or a "floating" sensation). In most cases, recovery is rapid and complete, with most symptoms resolving within 24–72 hours and a maximum duration of 14 days [35,36]. However, in the most severe cases, symptoms may rapidly progress to respiratory arrest in someone who otherwise exhibited no evidence of respiratory distress and death may result. With this in mind, symptomatic individuals should seek medical attention immediately and preferably in an urgent care setting with ongoing monitoring for the potential loss of airway patency.

Neurotoxic shellfish poisoning (NSP)

Neurotoxic shellfish poisoning (NSP) is typically caused by ingesting bivalve shellfish (clams, oysters, and mussels) contaminated with brevetoxins. The brevetoxins represent a group of more than 10 natural neurotoxins produced by the marine dinoflagellate, *Karenia brevis*, formerly *Gymnodinium breve* [37]. Similar to ciguatoxins, brevetoxins are lipophilic polyethers and are regarded as depolarizing substances, which open voltage-gated sodium ion channels in cell walls, leading to uncontrolled Na+ influx into the cell [29,34,38]. Sensory symptoms may result from the transformation of fast sodium channels into slower ones, resulting in persistent activation and repetitive firing [39].

The risk for NSP toxins in shellfish is associated with harmful algal blooms (HABS) or "red tides" along the Gulf of Mexico. The greatest number of cases appear to come from the west coast of Florida, although this may be due to differences in surveillance rather than actual differences in occurrence. Similar to other HAB-related illnesses, there is an ongoing threat of new NSP cases as harmful algal blooms may be transported to new regions. In fact, the largest number of reported US cases came from a single outbreak of 48 persons in North Carolina whereby brevetoxin-producing organisms were transported up the eastern seaboard [40].

Diagnosis, clinical symptoms, and treatment

The diagnosis of NSP is based upon clinical presentation and history of bivalve shellfish consumption from an endemic area. Symptom onset may begin from a few minutes to 18 hours after consuming contaminated shellfish; however, in most cases time to illness is about 3–4 hours [40,41]. The most frequently reported symptoms are nausea, vomiting, abdominal pain, and diarrhea. However, these are often not the primary presenting complaints. Of greater concern to most individuals are the neurological symptoms which may include paresthesias of the mouth, lips, tongue; peripheral tingling; partial limb paralysis; slurred speech; dizziness; ataxia; and a general loss of coordination. Hospitalization is sometimes necessary; however, no fatalities have been reported as a result of NSP [42]. Treatment for NSP typically involves supportive care which may include fluid replacement, monitoring of respiratory functions, the administration of sedatives, and pain management.

Recent studies suggest that aerosolization of brevetoxins from sea water produces an additional, transient, self-resolving inhalation syndrome characterized by respiratory problems and eye irritation [43]. Exposure is thought to be due to wave action and aerosolized sprays along Florida beaches during "red tide" events. Adverse respiratory effects include upper airway irritation and discomfort, decreases in pulmonary function, and exacerbation of asthma symptoms.

Amnesic shellfish poisoning (ASP)

The potential risk of domoic acid (DA) to human health was discovered in 1987 in Montreal, Canada ([44,45]; Teitlbaum et al., 1990; see Lefebvre and Robertson [2010] for review). Persons who ate affected blue mussels harvested from the Prince Edward Island region suffered serious medical illnesses, and in some cases death. Survivors were left with a permanent and profound memory disorder: amnesic shellfish poisoning (ASP). Aggressive monitoring by national and state health fisheries and food and drug agencies appears to have been effective in preventing further deaths by closing shellfish beds if DA levels exceeded 20 ppm. Within the past 15 years, measured DA levels have been significantly elevated on the US Pacific coast. Persistent low levels in some coastal areas have been interspersed with dangerously high levels. The extent to which chronic low level exposure impacts human health remains to be determined (Grant et al., 2010).

DA is a naturally occurring toxin produced by blooms of *Pseudo-nitzschia*. Shellfish and other marine organisms feed on *Pseuto-nitzschia* and concentrate the toxin within them. Hence, the shellfish become toxic to people who consume them. Although DA has been found in the viscera of Dungeness crab and other organisms, razor clams are the most significant vector as they retain the toxin for up to 1 year in the natural environment, or several years after being processed, canned, or frozen [46].

Diagnosis, clinical symptoms, and treatment

Clinical diagnosis is largely based upon symptom complaints and eliciting a detailed history of recent shellfish consumption. Acute symptomatology of high level exposures include vomiting, abdominal cramps, diarrhea, headache, seizures, respiratory excretions, confusion, coma, and, in some cases, death [13,44]. During the Prince Edward Island outbreak, the most severe neurological sequelae were found in males over 60 years of age, with symptom onset within 48 hours of ingestion. In the younger age groups, the most vulnerable individuals were those with pre-existing illnesses such as renal disease, hypertension, or diabetes. After physical symptoms abate, most patients are left with a permanent and in one case the delayed onset of temporal lobe epilepsy was observed [47].

DA is a water-soluble amino acid that activates the AMPA/kainite subtype of glutamate receptors [48]. Considered a chemical analogue to kainic acid, DA binds at the same receptor cites in the central nervous system, producing a pattern of cerebral damage similar to kainic acid neurotoxicity. Based upon human autopsy and

cross-species research, DA neuropathology in adults occurs primarily in the hippocampus, a cerebral region with a high density of kainite receptors and critical to memory functions.

Treatment is symptomatic with close observation for symptom progression. Hospitalization is usually required and patients should be followed for at least 1 year post-ingestion as there is the potential for the delayed onset of temporal lobe epilepsy [47]. Neuropsychological assessment and follow-up will be needed to document the nature and extent of memory deficits and develop a plan for the cognitive rehabilitation therapies to follow.

Diarrheic shellfish poisoning (DSP)

Diarrheic shellfish poisoning (DSP) is an acute gastrointestinal illness triggered by the ingestion of shellfish contaminated with okadaic acid (OA) and related toxins. Mussels, clams, scallops, and oysters are the most common vectors for the DSP toxins, which are produced by a community of dinoflagellates, most notably *Dinophysis* and *Prorocentrum* [49]. Outbreaks of DSP have been reported in Japan, France (c.f. Hossen et al., 2011), other parts of Europe, Canada, New Zealand, the United Kingdom (c.f. Hinder et al., 2011), and South America. There have not been any confirmed cases of DSP in the United States, although the responsible organisms (*Dinophysis*) have been identified in Texas Gulf coastal waters and oysters from that region concurrently tested positive for OA [5,50].

Diagnosis, clinical symptoms, and treatment

Similar to the diagnosis of other shellfish illnesses, the diagnosis of diarrheic shellfish poisoning is largely determined by dietary history and symptoms. Symptom onset typically occurs within 30 minutes to 4 hours after ingesting contaminated shellfish. The main symptom is incapacitating diarrhea, followed by nausea, vomiting, and abdominal cramps [4]. The symptoms may be severe and lead to dehydration but are usually self-limiting and continue for about 3 days [5]. Treatment for DSP is largely supportive. No fatalities have been reported for DSP.

OA is a lipophyphilic polyether that inhibits eukaryotic protein phosphatases and plays an important role in many regulatory processes in cells [51]. It is thought to cause acute diarrhea by stimulating phosphorylation of proteins that control sodium secretion in intestinal cells ([3]; Cohen et al., 1990). DSP toxins are also thought to be tumor promoters and may increase risk for colorectal or other digestive cancers [10,11].

Tetrodotoxin poisoning/puffer fish poisoning

Puffer fish is a lethal source of food poisoning with a high rate of mortality. It is estimated that the responsible neurotoxin (tetrodotoxin, TTX) can cause death in

60% of individuals who ingest it [14]. Species of puffer fish that carry the toxin include blowfish, sea squab, toadfish, balloon fish, globe fish, swellfish, and fugu (or bok). These tropical and subtropical fish are found throughout shallow waters of the Atlantic, Pacific, and Indian Oceans, and likely obtain the toxin through microorganisms in their diet. Puffer fish can be exported from China, Japan, Mexico, the Philippines, and Taiwan.

Diagnosis, clinical symptoms, and treatment

TTX is a heat-stable, powerful sodium channel blocker that inhibits neuronal transmission in skeletal muscles. After ingestion, initial symptoms emerge within minutes and death may occur within 6 hours (Lange, 1990). The clinical stages of poisoning have been described as:

1. perioral and lingual numbness or paresthesia (within 10–45 minutes after ingestion), nausea, and vomiting;
2. numbness progresses with motor paralysis of extremities;
3. progressive motor and bulbar muscle paralysis; and, in severe poisonings,
4. respiratory failure, hypoxia, unconsciousness, hypotension, and fixed, dilated pupils [52].

Other common symptoms include lightheadedness, dizziness, feelings of doom, weakness, salivation, muscle twitching, diaphoresis, pleuritic chest pain, dysphagia, aphonia, and convulsions [14]. Diagnosis is based on clinical symptoms and history of consumption of toxic organisms, and can be confirmed by the presence of TTX in urine or serum [53]. Supportive treatment includes rapid gastric lavage [14] and ventilatory support [53].

Scombroid poisoning

Scombroid poisoning (aka histamine poisoning) is typically a mild, but often distressing, seafood intoxication associated with the ingestion of fish that has been improperly stored or handled. Accounting for 38% of all seafood-associated outbreaks in the USA in the 1990s [54], the majority of cases are associated with fish species with naturally high levels of free histidine belonging to the Scombridae family (e.g., tuna, mackerel, and skipjack), although other species have been implicated (e.g., mahi-mahi, sardines, marlin, and bluefish). Scombroid poisoning has also been reported after consuming canned tuna fish. However, this was attributed to mishandling of the open can of fish rather than contamination of the fish prior to canning [55,56].

Since scombroid poisoning is largely attributable to the time-temperature mishandling of fish products, many cases are associated with harvests from recreational fishers with inadequate refrigeration in Japan, the UK, and the United States. Historically, the highest rates of scombroid poisoning in the USA have been reported in Hawaii. This may be explained by the state's large sports fishing

industry combined with the fact that recreational fishers may offset expenses by selling excess fish from their catches directly to restaurants, markets, and distributors, bypassing commercial fishing regulations [17,60]. Overall, reported incidences of scombroid poisoning are most likely underestimates as the illness is mild, self-limiting, passes rapidly, and has no long-term sequelae, so individuals do not seek medical treatment; those who do are often misdiagnosed with a seafood allergy.

Histamine is considered the primary active toxin in scombroid poisoning ([57]; Chegini and Metcalfe, 2011). However, since no clear dose–response relationship exists between histamine and illness severity, the mechanisms of toxicity are most likely complicated by the involvement of multiple other substances that may suppress or enhance the process [16]. Essentially, under conditions of improper temperature storage, the action of bacterial histamine decarboxylase (HDC) triggers the production of histamine from free histadine within the dark flesh of scombroid fish during decomposition (see [16] for review).

Diagnosis, clinical symptoms, and treatment

The non-specific but characteristic symptomatology of histamine food poisoning that emerge 10 minutes to 1 hour after eating a scombrotoxic fish should alert physicians to the possibility of scombroid poisoning. The total range of symptoms is variable and may include peppery or metallic taste, oral numbness, headache, dizziness, palpitations, rapid and weak pulse (low blood pressure), difficulty in swallowing, nausea, vomiting, abdominal cramps, diarrhea, and thirst, in addition to allergy-like symptoms such as hives, rash, flushing, and facial swelling. The symptoms are usually self-limiting and last for a day or less. There have been reports of more extreme cases with tachycardia and respiratory complications [16]. However, these are rare and usually involve individuals with pre-existing conditions. No deaths have been reported as a result of scombroid poisoning.

Differential diagnosis between scombroid poisoning and a seafood allergy is important. This is best facilitated by eliciting a detailed history of fish consumption, as well as the effects of the fish on others who shared the same meal. When multiple people share the same meal, most of them will be sickened by scombrotoxic fish. However, this is not the case with seafood allergies. Treatment for scombroid poisoning is usually supportive, with antihistamines, and, unless complicated with shock or respiratory distress, typically concludes with a good prognosis.

Prevention of seafood intoxication

Prevention is the best way to manage the risk of potentially serious seafood poisonings. For the harmful algal bloom or “red tide”-related illnesses (ciguatera and the shellfish poisonings), this necessitates a science-based management approach. This includes routine monitoring of risky coastal waters, seafood, and shellfish with

rapid and sensitive detection methods, and closing shellfish harvesting beds and posting warnings as needed. For treatment providers, the early diagnosis and immediate reporting of sentinel patients to local and state health departments is also critical so that sources of illness can be identified, harvesting beds can be closed, and the risk of secondary cases in cluster outbreaks can be reduced.

To prevent ciguatera fish poisoning, patients should be instructed to avoid consumption of large predatory fish in or from endemic areas. This is particularly important if the individual had prior episodes of ciguatera. Prevention of tetrodotoxin poisoning rests on avoiding consumption of puffer fish and other TTX-carrying organisms. Post-harvest rapid identification of histamine-forming bacteria is one of the best ways to minimize the risk of scombroid fish poisoning. Another important preventive measure is educating sports fishers about the importance of maintaining refrigeration on board their boats and rapidly chilling their harvests. Ongoing research is also needed to identify health risk factors, rapid toxin detection methods, and human biomarkers to prevent illnesses from some of the most potent naturally occurring toxins known to date.

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CHAPTER

Plant Toxins

32

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Introduction

Food poisoning caused by plant toxins has been thoroughly documented for many decades. Intoxication due to food contamination by poisonous plants has been associated with several different classes of plant constituents. Furthermore, the upsurge in usage of a number of dietary supplements and traditional herbal medicines has been associated with health risks due to the intrinsic or extrinsic presence of phytotoxins, in addition to their possible adverse interactions with prescription drugs. The majority of plant species in the world are not edible, largely owing to the existence of toxins they produce. The process of domestication has diminished the levels of these toxic compounds over time. As a result, the plant foods we consume today are far less toxic than their wild parents. Plant toxins are usually secondary metabolites that are produced and secreted by plants. These metabolites are either accumulated in the tissues or deposited on the plant surface. Levels of toxic substances vary considerably in plants for several reasons, including ontogenic, ecotypic, genotypic, and chemotypic factors.

Phytotoxic food poisoning occurs for the following reasons:

1. *Inedible plants*: Certain wild plants, such as wild mushrooms and giant elephant ears, contain potent toxins that are not easily destroyed by cooking. These wild plants may be inadvertently ingested as edible plants.
2. *Edible plants without proper processing or cooking*: Certain food plants such as green beans and the cyanogenic plants, namely bitter apricot seeds, need sufficient heating to destroy the toxic substances. For other plants such as cassava and bamboo shoots, toxic cyanide can be removed more effectively by soaking in water or by cutting the plant into small pieces before cooking.
3. *Heat-resistant toxins*: Consumption of food plants containing toxins that are resistant to heat of cooking, for instance green potatoes or their sprouts.

The symptoms of poisoning extend from mild gastrointestinal disorders to severe effects on the central nervous system. The degree of intoxication is contingent on the amount of the plant consumed, the concentration of toxins present, and the susceptibility of the individual.

Foodborne Infections and Intoxications.

One of the ecological functions of phytotoxins is the defense against potential enemies, such as insects, pathogenic microorganisms, and herbivorous animals. In addition, phytotoxins may, as their primary role, promote the survival of the plant against abiotic environmental stresses such as ultraviolet radiation [1].

Chemically, plant toxins display a diverse range of structures from small organic molecules to large peptides and proteins. This chapter will provide an overview of the plant toxin classes, including alkaloids, cyanogenic glycosides, glucosinolates, isothiocyanates, and furanocoumarins that are most frequently encountered in food poisoning. The discussion will focus on their natural occurrence, chemistry, adverse effects, implications on health, and clinical relevance. Particular emphasis will be placed on pyrrolizidine alkaloids as they are the most common and geographically widespread cause of phytotoxic food poisoning.

Alkaloids

Naturally occurring alkaloids characteristically contain one or more nitrogen atoms in heterocyclic rings. Alkaloid-containing plants are, to some extent, an intrinsic part of the regular human diet. Humans have used alkaloid-containing plants since ancient times as poisons, stimulants, narcotics, insecticides, aphrodisiacs, and medicines. Alkaloids are mainly derived from amino acids, but terpenoids can also serve as precursors for certain classes of alkaloids.

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) have been the principal plant metabolites responsible for plant intoxication [2] and diseases in humans. Such intoxications occur largely due to consumption of grains and breads contaminated with PAs [3]. Contamination of agricultural grains with seeds containing PAs poses a worldwide health risk [3]. This threat is due primarily to co-harvesting of grain crops with seeds of toxic weeds that grow with the grains [3]. In addition, PAs may act synergistically with aflatoxins and hepatitis viruses to cause human liver cancers [4]. The content of PAs in plants varies according to the species, plant part, stage of growth, and climatic conditions [5].

Toxic PAs in *Jacobaea vulgaris* (*Senecio jacobaea*) and *Ageratum conyzoides* have been the main cause of human poisoning episodes in Europe and Ethiopia, respectively, due to food contamination [3]. One of the deadliest incidents involving pyrrolizidine intoxication occurred in Gulran Province, Afghanistan, from 1974 through 1976, where 1600 people died and 7800 others became ill but survived. Many people were diagnosed with hepatic veno-occlusive disease (VOD), based on liver biopsies [6]. The suspected weed, which commonly grows in wheat fields, was identified as *Heliotropium popovii*. The toxic alkaloids were transferred from the plant to wheat flour that was subsequently used to make bread. The second major outbreak in Afghanistan, which occurred from 1999 through 2001, affected over 400 individuals and caused more than 100 deaths. In additional

incidents in Afghanistan and India, consumption of bread contaminated by seeds from *Heliotropium lasiocarpum* and *Crotalaria* species led to an outbreak of VOD in Afghanistan between 1974 and 1976 [6].

PA-contaminated food ingredients have been widely reported in underdeveloped countries due to catastrophes or natural disasters such as famine. Such contamination has occurred repeatedly with deleterious consequences [7,8,9]. These contaminated food ingredients include bread and grain [7,8,9] and, to a lesser extent, milk [10–12], honey [13,14,15], eggs [16], and salad greens [17]. In general, however, PA intoxication occurs uncommonly and only sporadically (i.e., not in large outbreaks) in developed Western countries. The World Health Organization (WHO) has considered PAs to be a threat to human health because of their carcinogenic potential [18] and because they have been implicated in a number of food poisoning episodes [19]. The maximum level of PAs allowed by different European countries is set at 0.1 µg/day, orally administered, and 0.1 µg per 100 g of food [20].

PAs are seen in more than 12 unrelated higher plant families of angiosperm and occur in many geographical regions worldwide [15]. It has been reported that approximately 3% of the world's flowering plants contain toxic PAs [21,22,23] and they are particularly abundant in three plant families, namely Asteraceae, Fabaceae, and Boraginaceae. More than 660 PAs and pyrrolizidine alkaloid N-oxides have been recognized in over 6000 plants and more than half of them exhibit hepatotoxicity [24,7,25,8,9]. Sources of contamination include the human consumption of food products of animal or plant origin, such as milk, meat, eggs, honey, and salads.

Milk

Animals grazing on fodder mixed with toxic weeds containing PAs were found to produce PA-contaminated milk. PAs have been detected in cow and goat milk [18]. The toxic alkaloids identified in milk are water-soluble PA N-oxides, and this finding indicates that skimmed milk is more likely to be contaminated with PAs at toxic levels than whole milk [18]. PA-containing human milk was also shown to cause liver diseases in neonates and infants [26]. Nevertheless, human health risk due to transfer of PAs to milk remains controversial [27], and in some reports milk is not considered as a major source of health risk and safety concerns [28].

Meat and eggs

Residues of PAs have not been detected in meat of grazing animals fed on PA-contaminated pastures [27], as the majority of PAs are metabolized in the liver before entering the bloodstream, whereas eggs represent a poultry product-based food which may deliver PAs to the human consumers if grains contaminated with PAs are used for feeding poultry.

Salads

A number of PA-producing plants have been implicated in human intoxication due to their use as components in salads. One of these plants is *Senecio vulgaris,* which is a common weed of field crops. This plant was identified in salad greens as a source of

exposure to PAs. Another plant, *Borago officinalis* (borage), is used also as an ingredient in salads, and has been found to contain up to 10 mg of PAs/kg of herb [17]. In addition, the leaves or roots of comfrey (*Symphytum officinale*) are often used as ingredients of salads, and hence possibly contribute to comfrey PA intoxication.

Honey

PA-contaminated honey also is a possible source of foodborne PA exposure and may pose a threat to human health [29]. Kempf and colleagues (2008) reported that 19 of 55 analyzed samples of honeys were contaminated with PAs. In these samples, the levels of PAs were estimated, based on GC/MS analysis, to be in the range of 0.02–0.12 µg/g. Such concentrations of PA might be sufficient to cause chronic liver disease or liver tumors [4].

The source of the toxic alkaloids in honey is PA-contaminated pollen. Many PA-containing plant genera contribute to the potential toxicity of honey, including *Echium, Ageratum*, *Heliotropium*, *Eupatorium*, *Senecio*, *Borago*, *Petasites*, *Myosotis*, *Crotalaria*, *Tussilago*, *Cynoglossum*, and *Chromolaena* [20]. The most common plants associated with PA poisoning [18] are S*enecio vulgaris* (Common groundsel, a common field weed that has been involved in salad contamination with PA), *S. aquaticus* (March ragwort), *S. squalidus* (Oxford ragwort), and *S. erucifolius* (horny ragwort). All of these species are known to contain toxic PAs. *Crotalaria retusa* is a highly toxic plant for humans and animals [30] because its seeds contain high concentrations of the pyrrolizidine alkaloid monocrotaline, which is reported to be hepatotoxic and genotoxic. Monocrotaline and its metabolites have the capacity to cross the blood–brain barriers [31]. In addition to *Crotalaria retusa*, other plants such as *Heliotropium lasiocarpum, Symphytum officinale*, and *Tussilago farfara* are also known to contain toxic PAs.

Chemistry of pyrrolizidine alkaloids

PAs comprise a large group of alkaloids possessing the pyrrolizidine nucleus (5-membered ring with nitrogen atom). Several hundred of these alkaloids have been isolated since the first two were identified in *Senecio latifolius*.

PAs are chemically stable with a range of water solubility. The basic structure of PAs is made up of two parts, i.e., an amino alcohol termed necine and an acid named necic acid. The necine base is either saturated or has a 1,2-unsaturation. PAs are classified into three groups [32]: i) macrocyclic diesters (connecting C_7 with C_9) of the necine base; ii) monoesters (at C_9), and iii) nonmacrocyclic diesters (connecting C_7 and C_9).

The largest subclass of the PAs consists of macrocyclic esters, and they occur at high concentration in the genus *Senecio,* reaching up to 18% of the plant dry weight [16]. The saturated necine-based PAs are non-toxic, whereas PA esterified with the 1,2-unsaturated bases are regarded as toxic and carcinogenic [24]. Some types of these PAs are abundant, highly toxic, including hepatotoxicity, and carcinogenicity [33].

Representatives of these toxic PAs are: i) retronecine type (retrorsine, symphytine); ii) heliotridine type (heliotrine); and iii) otonecine type (senkirkine). Three structural features are essential to induce hepatotoxicity: a 1,2-unsaturation in the necine base; esters of the hydroxyl group in one or more positions; and a branched carbon chain in at least one of the esters (Figure 32.1).

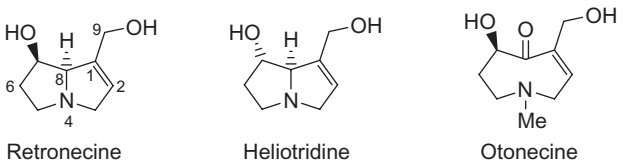

FIGURE 32.1

Structures of some representative pyrrolizidine alkaloids (PAs).

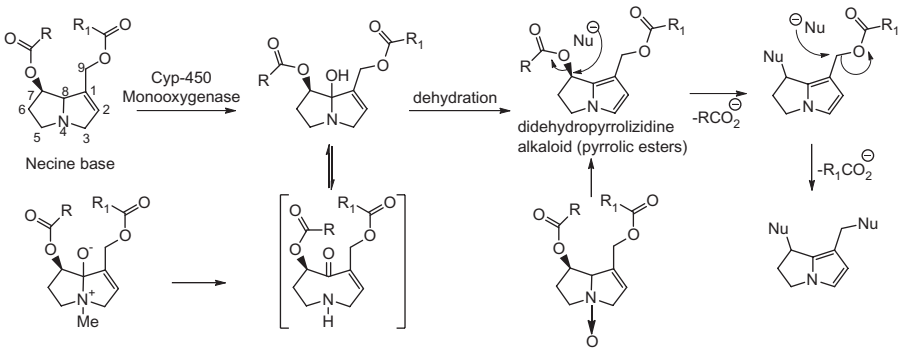

FIGURE 32.2

Formation of the metabolite didehydropyrrolizidine, cabocation-carrying bases (electrophiles), and their reactions with nucleophiles.

Otonecine-type PAs, which have a methyl group at the nitrogen atom and a keto function at position 8 (Figure 32.1) undergo sequential metabolization that ends up in the formation of didehydropyrrolizidine (Figure 32.2) [2,34,35]. The latter metabolite is further metabolized *in vitro* and *in vivo,* to carbocation-carrying quaternary bases (see also Figure 32.2). These carbocations have the capacity to react with nucleophiles such as mercapto, hydroxyl, and amino groups of proteins and amino groups of purine and pyrimidine bases in nucleosides like DNA and RNA to form alkylated biomolecules (see also Figure 32.2) [36]. PAs are activated by cytochrome P-450s, primarily of the 3A4 family. Certain cytochrome P450s convert PAs to less toxic forms, and animals such as sheep that possess this pathway suffer less severe toxic effects than other animals (such as rats and horses) that metabolically convert PAs to the toxic pyrrole form.

PA poisoning may lead to acute, subacute, or chronic intoxication. In addition to dose and duration of contact (exposure) to PAs, other factors also may contribute to the severity of poisoning. These factors include gender, age, and species [37]. PAs undergo metabolic activation in the liver. Therefore, the liver is the first organ to be affected by pyrrolizidine poisoning. Hepatotoxicity and genotoxicity of PAs are determined by factors that modulate two competing biochemical reactions: the

metabolic activation for the formation of the toxic pyrrole esters, and the detoxification reactions *via* GSH conjugation, hydrolysis of the parent PAs, or hydrolysis of the formed pyrrole esters (Figure 32.2) [37,38].

Acute poisoning causes hepatotoxicity with hemorrhagic necrosis, while chronic intoxication occurs largely in the liver, lungs, and blood vessels, and sometimes in the kidneys, pancreas, gastrointestinal tract, bone marrow, and brain. Exposure to PAs over a longer period can result in veno-occlusion in the liver and lungs, fatty degeneration, loss of metabolic function, inhibition of mitosis, proliferation of biliary tract epithelium, liver cirrhosis, nodular hyperplasia, and adenomas or carcinomas [24,33,39].

Death caused by exposure to PAs is attributed largely to the development of cerebral edema that occurs as a consequence of hepatic encephalopathy [40]. PAs have been isolated/detected in plants predominantly as their N-oxides [3]. On oral ingestion, however, they undergo reduction in the gastrointestinal tract or in the liver by NADH or NADPH to free bases and thus become fully toxic [41–44]. On the other hand, some of the absorbed PAs undergo cleavage by the ubiquitous nonspecific esterases in the human body to produce necins and necic acids, which are not toxic [2]. The alkylated biomolecules (PAs bound to proteins, DNA or RNA) show abnormal functions, and in the case of alkylated DNA, this may lead to mutations. This toxic effect takes place in the liver which undergoes damage, leading to VOD in humans. The disease in humans is considered specific to PA poisoning [2]. The lowest intake of PAs (total) necessary to cause VOD was estimated by WHO to be 1 mg/day for a 70 kg adult [33]. The clinical symptoms of VOD occur abruptly and may include vomiting and enlargement of the liver, bleeding, and diarrhea [2]. Children are more sensitive to PA poisoning than adults [45,46,47].

Half of the characterized PAs are genotoxic, and a large number of these plant metabolites are tumorigenic. In general, PAs are mutagenic *in vitro* and *in vivo*, and apparently their carcinogenicity is ascribed to their mutagenicity [2]. This genotoxicity originates from the metabolic activation of PAs, which is followed by production of DNA adducts, DNA cross-linking, DNA breaks, sister chromatid exchange, micronuclei, chromosomal aberrations, gene mutations, and chromosome mutations *in vitro* and *in vivo* [2].

Tropane alkaloids

Tropane alkaloids are widely distributed in nature and to date more than 200 tropane alkaloids have been identified. Some important tropane alkaloids such as (+)-hyoscyamine, (−)-hyoscyamine, racemic hyoscyamine (also known as atropine), (−)-scopolamine (hyoscine), and cocaine are shown in Figure 32.3. Even though plants that contain tropane alkaloids have been used for traditional medicine, these alkaloids can also be contaminants in crops like soybeans and linseed and cause intoxication through inadvertent consumption. *Datura stramonium* L. (family Solanaceae), which also is called Apple of Peru, Devil's Apple, Jamestown Weed, Mad-apple, and Stinkweed, contains high concentrations of hyoscyamine and scopolamine. The toxicity of *Datura* species

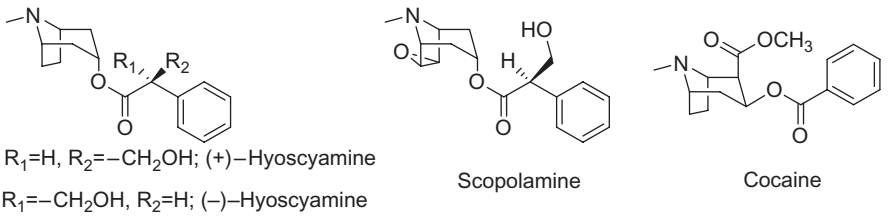

FIGURE 32.3

Structures of some representative tropane alkaloids.

is well documented and has been linked to deaths and poisonings for centuries [86]. Pharmacologically, atropine and other tropane alkaloids prevent binding of the neurotransmitter acetylcholine to its receptor and, as a result, they cause blurred vision, suppressed salivation, vasodilation, increased heart rate, and delirium [48].

Some tropane alkaloids, such as atropine and scopolamine, have medical uses because of their biological activity. Atropine reduces rigidity in parkinsonism and is used as an antidote to poisoning with parasympathomimetic agents such as nerve gases and organophosphorous insecticides. Scopolamine is an antimuscarinic agent used as an analgesic and a smooth muscle relaxant, and it is used extensively in the treatment of motion sickness and as a pre-operative medication [48]. Cocaine has local anesthetic properties but its therapeutic use is very limited, and it is a drug of abuse in many countries [49]. According to the German Federal Institute for Risk Assessment, the cocaine content of a coca leaf extract-containing soft drinks does not pose any serious adverse health effects from consumption because such drinks contain very low concentrations of cocaine.

A recent report [50] by the RIKLIT Institute of Food Safety concluded that buckwheat for human consumption, soybeans, and linseed in animal feed are products that should be rigorously monitored to prevent accidental exposure of humans to tropane alkaloids. In modern food chains, however, the levels of these tropane alkaloids in food and beverages appear to be low and such alkaloids are unlikely to cause health risks to consumers. Nonetheless, the public should be aware of the risks of abuse and accidental poisoning due to plants that contain tropane alkaloids.

Quinolizidine alkaloids

Lupin proteins are replacing animal proteins and other plant ingredients in several foods, such as bakery products, imitation dairy and meat products, and beverages [51]. One of the major safety issues [52] of lupin-based foods is the presence of quinolizidine alkaloids (QAs), which are bitter compounds produced by lupin plants, most likely as a defense mechanism to protect against predators. There are almost 70 different QAs found in various *Lupinus* species and several members of the pea family, of which sparteine, anagyrine, and lupanine and its isomers (Figure 32.4) are the most common.

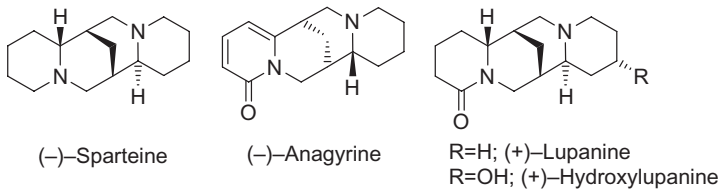

FIGURE 32.4

Structures of some representative quinolizidine alkaloids.

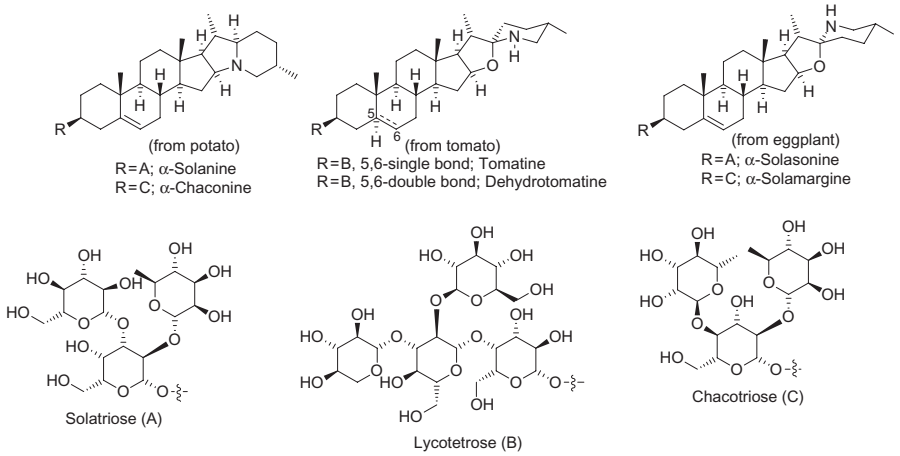

FIGURE 32.5

Some of the representative structures of glycoalkaloids.

Bitter lupins or European lupins are predominantly consumed in southern Europe as seeds (lupini beans). They often have a high QA content (10–20 g/kg), which can be reduced to 0.5 g/kg by a "de-bittering" process involving soaking or washing the seeds with water. Sparteine and lupanine both display moderate acute toxicity, and the general toxic symptoms include malaise, nausea, respiratory arrest, visual disturbances, ataxia, progressive weakness, and coma. On the basis of the data available, the acute lethal dose for humans is approximately 30 mg/kg of body weight, which is considerably lower than the lethal dose levels reported in rodents [53].

Glycoalkaloids

Glycoalkaloids, a class of nitrogen-containing compounds with a steroid scaffold coupled to one or more monosaccharaides, are biologically active secondary metabolites commonly found in plants of the *Solanum* genus (Figure 32.5). They occur in some species of nightshade, among which are potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), and eggplant (*Solanum melongena*). These alkaloids are

mainly produced by the plant for resistance against pests and pathogens. They have been shown to exhibit a concentration-dependent toxicity for a wide range of organisms from fungi to humans [54].

Glycoalkaloid levels in potatoes are increased by a number of factors, e.g., unfavorable climatic conditions such as extreme temperatures [55], and exposure to artificial light or sunlight [56,57] can cause a 300-fold increase in glycoalkaloid content of potatoes. Simply peeling a 1.5 mm layer of skin from the potato can remove up to 50–95% of the glycoalkaloids. The concentration of glycoalkaloids is influenced by the type of cultivar more than the type of food processing (baking, cooking, or frying).

Generally, these glycosides are less toxic than corresponding non-glycosylated alkaloids (aglycons) due to their poor bioavailability. However, when consumed, these glycoalkaloids can readily undergo hydrolysis in the stomach or enzymatically by bacterial glycosidases in the large intestine. There have been no reports of the ill effects of glycoalkaloids from eggplants or tomatoes, but a number of reports have correlated potato glycoalkaloids with toxic effects in humans. When α-solanine and α-chaconine are consumed at low doses, these glycoalkaloids may cause gastrointestinal disturbances, e.g., vomiting, diarrhea, and severe abdominal pain. When such alkaloids were consumed at high doses, neurological disorders such as drowsiness, confusion, weakness and visual disturbances had been reported [58,59]. In general, however, normal consumption of moderate amounts of potatoes does not lead to adverse effects in humans. The glycoalkaloid level in potatoes for human consumption should be sufficiently low to produce a consumed dose lower than 100 mg/kg of body weight to avoid intoxication, and new cultivars of potatoes must be analyzed for glycoalkaloid concentration to make certain that standard can be achieved before commercialization [60].

Although glycoalkaloids are perceived as potentially toxic, studies during the past 10 years suggest that they may also possess beneficial effects depending on dose and conditions of use. These potential applications include anticancer, anti-inflammatory, antinociceptive, antipyretic, anticholesterol, antifungal, and antibacterial effects [54].

Pyrimidine alkaloids

Favism is a disease that results from a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD). Favism usually is due to a genetic disorder, but also can result from consumption of broad beans [61]. G6PD is a metabolic enzyme involved in the pentose phosphate pathway, which is essential in red blood cell metabolism. G6PD deficiency, the most common human enzyme defect [62], is associated with symptoms that include hemolytic anemia (red blood cells break down prematurely), dark urine, fatigue, shortness of breath, and a rapid heart rate. In addition to being due to consumption of broad beans, which may or may not cause symptoms and the genetic disorder, the symptoms of favism are triggered by a number of other factors, including infection or exposure to certain medications or chemicals [63].

At the biochemical level, the G6PD enzyme converts glucose-6-phosphate into 6-phosphoglucono-δ-lactone and is the rate-limiting enzyme that maintains the supply of NADPH. This cofactor, in turn, maintains the level of reduced glutathione in

the cells that scavenges free radicals to prevent oxidative damage to the cell. The G6PD/NADPH pathway is the only source of reduced glutathione (GSH) in erythrocytes. Lack of adequate GSH in erythrocytes puts them at high risk of damage by oxidative stress, which may lead to hemolytic anemia [64].

Oxidative stress factors include infection, chemicals, medicines, and certain foods such as fava/broad beans (*Vicia faba*) [65], which contain a high level of the glycoalkaloids vicine and 6-hyroxy vicine (covicine) (Figure 32.6) and the corresponding aglycons, divicine and isouramil. All these compounds are harmful oxidative agents. Heating fava/broad beans to relatively high temperatures is not effective in reducing the concentrations of these glycosides. However, the concentration of vicine and covicine and their corresponding aglycons can be greatly reduced in fava/broad bean food preparations by enzymatic hydrolysis [66].

Cyanogenic glycosides

Cyanogenic glycosides (Figure 32.7) are plant secondary metabolites that release HCN gas when exposed to the hydrolyzing enzymes β-glycosidases [67]. The glycosides degrade into cyanohydrin aglycone plus a sugar part. Subsequently, the aglycone may decompose and release hydrogen cyanide gas. The sugar in most cases is D-glucose.

Many plants that are economically and nutritionally important from a dietary perspective contain cyanogenic glycosides at varying levels and thus pose a potential health risk [68]. Among these plants are *Prunus* spp., bitter almond (amygdalin), prunasin;

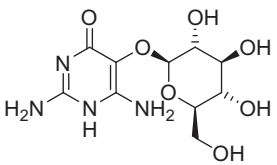

FIGURE 32.6

Structure of pyrimidine alkaloid vicine.

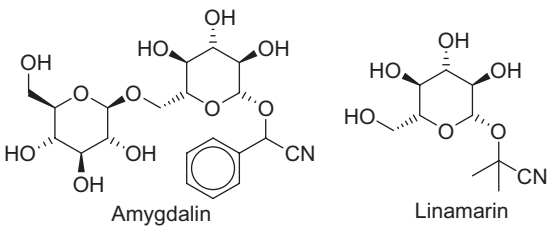

FIGURE 32.7

Some of the representative structures of cyanogenic glycosides.

Trifolium repens, *Linum usitatissimum* (linseed); *Manihot esculenta* (Cassava roots), linamarin; *Vicia* spp. (vicianin); *Sorghum* species (dhurrin), and *Lotus corniculatus* (lotaustralin). These toxic species are widely distributed geographically, and it is estimated that around 2500 plant species exist that contain cyanogenic glycosides [68].

Of these, cassava root is a significant food source for about 500 million people in Africa, Asia, and Latin America [67]. It ranks fourth after rice, wheat, and corn as a food crop to provide carbohydrates for humans [69]. The poisonous effects of cassava roots (*Manihot esculenta*) have been attributed to their content of the cyanogenic glucosides linamarin and lotaustratalin [70]. Different cultivars of cassava show variable cyanogenic glucoside contents, ranging from <50 to >100 μg CNeq/g [69]. WHO set a value of 10 μg/g as a safe level of cyanogenic glucosides in cassava-based foods; currently food poisoning due to exposure to cyanoglucosides in cassava is uncommon, accounting for illness in <1% of the population using cassava as a staple food [71].

Raw lima bean or butter bean (*Phaseolus lunatus*) may sometimes contain poisonous quantities of linamarin. When consumed, these glycosides are often converted to less toxic thiocyanates, which display biological actions similar to that of iodine deficiency [72]. Intoxication with cyanogenic glucosides inflicts cellular edema, peripheral vacuolation of the liver, swelling, and proteinurea. Clinical signs include ataxic neuropathy and paralytic "Konzo" [73].

Glucosinolates and isothiocyanates

Glucosinolates are a large group of secondary metabolites which are widely present in the plant kingdom. Their degradation products have a variety of chemical and biological functions [74]. They are found mainly in the family Brassicaceae, which includes several important food crops. More than 120 different glucosinolates are known and they share a common chemical backbone consisting of an S-glycosylated thiohydroximate sulfate ester with variable side chains attached (Figure 32.8). Glucosinolates and their hydrolytic enzymes, myrosinases, are stored

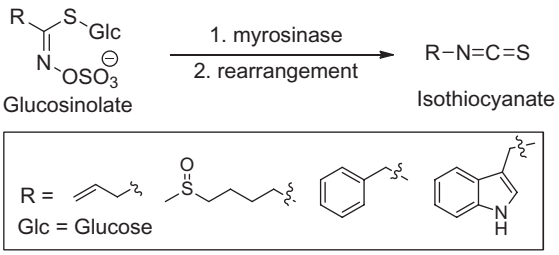

FIGURE 32.8

Some of the representative structures of glucosinolates and isothiocyanates.

in separate compartments in the intact plant tissue. Upon tissue disruption, bioactivation of glucosinolates is initiated, i.e., myrosinases get access to their glucosinolate substrates, and glucosinolate hydrolysis results in the formation of toxic isothiocyanates and other biologically active products [75]. Glucosinolates exert their toxic effects (mainly as goitrogens) in both humans and animals at high doses [76]. The ingestion of large amounts of food containing glucosinolates may reduce intake, cause thyroid gland hypertrophy, and reduce levels of circulating thyroid hormones, mainly by inhibiting the iodine uptake by the gland.

Allyl isothiocyanates are mainly responsible for the pungent flavor of certain foods including mustard and horseradish, where they are present at 50–150 ppm levels. Isothiocyanates have been implicated in causing hyperthyroidism (goiter), particularly in geographical regions like Asia and Africa where the consumption of minimally processed food occurs together with iodine deficiency. Similar to other constituents of foods that can be nutrients at one level, toxic at another, and therapeutic agents at yet another, recent studies indicate that isothiocyanates from cruciferous vegetables can prevent various cancers by inducing cell cycle arrest, apoptosis, and antiangiogenic effects [77,78].

Furanocoumarins

Furanocoumarins are a group of natural constituents of common vegetables and fruits such as citrus, parsnips, parsley, celery, figs, carrots, and spices, including ammi and pimpinella. Structurally, these furanocoumarins are divided into linear and angular coumarins depending on the attachment of furan group to the coumarin scaffold (Figure 32.9). Psoralen, imperatorin, isoimperatorin, and xanthotoxin are typical examples of linear furanocoumarins, whereas isopsoralen is representative of the angular type. These furanocoumarins are phototoxic and photogenotoxic in combination with UV radiation in a dose- and time-dependent manner [79]. In addition to their phototoxicity, recent clinical evidence has shown that intake of excessive amounts of furanocoumarins may lead to kidney and liver toxicity [80]. Cases of consumption of furocoumarin-rich food combined with UV exposure have been associated with phototoxic skin reactions, while long-term oral exposure to high doses of certain pure furocoumarins in PUVA (psoralen + UVA treatment for eczema, psoriasis, etc.) therapy may lead to some types of skin tumors in humans and experimental animals [81].

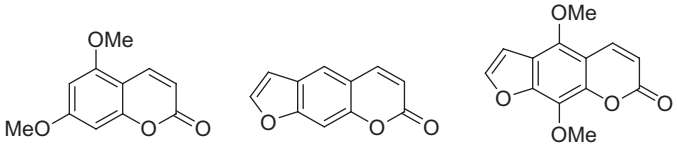

FIGURE 32.9

Some of the representative structures of furanocoumarins.

Risk assessment of natural furocoumarins in the diet is currently based on a threshold approach and on estimations of 1.2–1.45 mg for the average daily exposure to furocoumarins for adults via the diet in Western populations [82]. At high concentrations, coumarins induce liver damage in test animals, and its use as a food additive has been banned by the US Food and Drug Administration. However, recent literature suggests that when used in limited quantities, these agents can be safely used in cosmetics [83] and as photo pesticides [84]. In addition, it has been recently concluded that consumption of regular quantities of vegetables, fruits, juices, and citrus-flavored soft drinks that contain furanocoumarins does not initiate phototoxicity if the food products are appropriately stored or processed. However, microbial contamination due to improper storage and stress factors due to processing/production of celery and parsnips may result in considerable increase in the content of furanocoumarins, which could pose a health risk [85].

Conclusion

In conclusion, pyrrolizidine alkaloids (PAs) have been recognized as the principal plant metabolites that pose a serious health threat to humans via foodborne plant intoxication. The main source of exposure to PAs is consumption of grains and bread. Other sources such as milk, eggs, honey, and salad greens are less commonly implicated in human disease. PAs are particularly abundant in three plant families, namely Asteraceae, Fabaceae, and Boraginaceae. The major health risk of PA intoxication is the development of hepatic veno-occlusive disease (VOD). PAs may also be associated with severe adverse effects including carcinogenic, genotoxic, teratogenic, and pneumotoxic reactions. Because of naturally limited concentrations, low potency, and advanced processing techniques in detoxification, plant toxins other than PAs do not at present pose a major threat to human health.

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SECTION

6 Policy and Prevention of Foodborne Diseases

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CHAPTER

Effects of Food Processing on Disease Agents

33

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Introduction

This chapter presents the effects of processing on food safety. The great importance of food safety is discussed elsewhere in this book in detail. Suffice to say that microbiological safety is critical to the success of a product and to the health of the consumer.

The concepts and procedures presented here apply to other fields such as the processing of sterile drugs, medical devices, and biologics once the differences among the requirements for the corresponding processes are taken into consideration. Success and corresponding probability of failure of commercial sterilization, terminal sterilization, and high level disinfection defined in the appropriate sections of the Code of Federal Regulations (CFR) and the International Organization for Standardization (ISO) documents can be addressed using the technical approach presented. Indeed, the sterilization, cleaning, and disinfection of medical devices has led to technological developments of great interest and potential applicability for food processing. In particular:

- Radiation sterilization of medical devices is strongly influenced by the initial microbial population or bioburden. Development and validation of many industrial radiation sterilization processes for medical devices are based on rigorously determined and controlled device bioburden. Once bioburden has been established, the sterilization and verification radiation doses may be obtained from tables available for that purpose. This approach is approved by the regulatory authorities and used extensively by industry. Accordingly, industry and regulatory authorities have developed corresponding standards that support this technology, e.g., ANSI/AAMI/ISO 11737–1 [1] deals with determination of microorganisms on product.
- Cleaning of reusable medical devices is supported by AAMI TIR30 [2]. The safety of reusable medical devices that enter in direct contact with normally sterile regions of the patient's body require a high level of assurance of the effectiveness and consistency of the corresponding processes. This technical information report (TIR) is a compendium of very valuable related information, and is potentially applicable to food processing.

Foodborne Infections and Intoxications.

The discussion begins with defining the general approach to evaluating the safety of a process and/or product, measurement of the microbial population (potential contamination), and the relationship of that population to the probability of failure of a process. The corresponding quantitative aspects are presented within the frame of the food safety objectives (FSO) approach that defines the main components of the probability of failure as related to the initial microbial population density, i.e., the bioburden, the reduction in the population density due to process, and the potential for microbial growth [14]. The FSO approach includes the time period that lapses until the product is consumed. Therefore, this chapter covers the concepts, transformations, and systems that define the effect of process on the microbiological safety of a food product, including detailed examples that illustrate the application of the corresponding statistical and mathematical tools to provide some insight into the main related conceptual aspects and capability to evaluate the effect of process on food safety.

Food processing

Food processing has an important effect on microbial population type and numbers. Processing operations may physically remove microbes, inactivate them, or provide conditions that restrict their growth. For instance, trimming, filtration, and cleaning will physically remove some of the microorganisms; pasteurization, commercial sterilization (i.e., canning), and disinfection will reduce their numbers; and drying, freezing, refrigeration, and pickling will generate conditions that restrict the growth of significant fractions of the microbial population of foods. The two main outcomes achieved by processing foods are enhancing their stability (increasing their shelf life) and diminishing the probability that pathogenic microorganisms or their toxins (or both) reach the final consumer to the extent that the corresponding food may be considered safe for human consumption.

Food processing is a dynamic field. The variety and quality of processed foods available to us are consequences of this evolution. We benefit from new technologies, such as microwave heating and high pressure pasteurization and sterilization; from strategies that assist the control of potential risks inherent to foods, such as the hazard analysis and critical control point (HACCP) approach; from the use of quality engineering procedures; and from the establishment of food safety objectives (FSO), among other advances in food processing. For example, when the space program was developing the technology that enabled astronauts to go beyond our planet's atmosphere, assuring that the food eaten by the astronauts would not make them sick was a major concern. It is well known that in the absence of gravity some of our physiological functions become more complicated in the best of cases. Definitely, a foodborne illness or intoxication had to be prevented at all costs. Thus, the space program joined efforts with the Pillsbury Company, and their efforts led to the HACCP approach that has assisted the modern food industry to improve significantly the safety of our food in general. In this approach, careful risk analysis is performed to identify the potential for microbial survival and growth during food processing, and to assign control measures that control the corresponding hazards appropriately.

Hazard analysis and critical control point (HACCP)

Information related to the HACCP approach may be found at the FDA web page www.fda.gov/Food/FoodSafety/HazardAnalysisCriticalControlPointsHACCP/ucm114868.atm#app-d and in Chapter 35 of this book. The preliminary tasks include assembling a team; description of food and its distribution; description of the intended use and consumers for the food; development of a flow diagram; and, finally, verification of the flow diagram. Once the main spectrum of action has been thus defined, a series of principles apply:

1. Conduct a hazard analysis.
2. Determine critical control points (CCPs). These are steps at which control can be applied and are essential to prevent or eliminate a food safety hazard, or reduce it to an acceptable level.
3. Establish critical limits: maximum or minimum values to which a parameter must be controlled.
4. Establish monitoring principles.
5. Establish corrective actions.
6. Establish verification procedures.
7. Establish record-keeping and documentation procedures.

The information provided by the FDA is detailed and includes appropriate illustrative examples. Adoption of the HACCP approach by industry has provided an excellent frame of reference to minimize the risk of foodborne disease.

Microorganisms found in foods are bacteria, viruses, molds, yeasts, protozoa, and some parasites of microscopic dimensions. Some of these microorganisms are capable of causing disease if ingested, and these are termed pathogenic. In addition, some microorganisms may, when the conditions are appropriate, produce toxins that may in some cases persist even if the original microorganisms are inactivated. Most of the microorganisms in foods are not pathogens but may, when conditions are appropriate for their growth, produce alterations in foods such as odors and acidity, and their colonies may become visible when large numbers are reached.

Many food processing operations are related to modifying foods to make them inhospitable to growth and survival of microorganisms (drying, freezing, refrigeration, preservation, etc.) or to the removal or inactivation of microorganisms (trimming, cleaning, radiation, canning, pasteurization, etc.). Indeed, sterilization and pasteurization are defined with respect to the required levels of inactivation of microorganisms of interest.

Most of the food processing operations have a significant impact on the corresponding microbiological population dynamics. This effect is reflected in the safety and stability of related food products. Some of the most relevant operations and their main principles of action relevant to food safety follow.

Drying

Microbial growth is dependent on the "water activity" (Aw) of foods. Aw is the ratio of the vapor pressure of water in the product to the vapor pressure of pure

water at a certain temperature. It ranges from 0–1. Microbial growth is considered insignificant for practical purposes when Aw is lower than 0.7. Different types of product may have a heuristic threshold. For instance, commercially sterilized products may be related to a 0.85 Aw value.

Drying of foods is a complicated process that may involve the study of heat and mass transfer in capillary porous bodies and the use of sublimation (in freeze-drying). The relationships between water and the components of foods such as carbohydrates, proteins, fats, and oils are also complicated because water in foods has different degrees of "freedom" or physicochemical linkage to the food components. Some of the water is "free water" and behaves like a simple dilute solution and is relatively easy to remove by evaporation. Once the "free water" has been removed, mass and heat transfer dominate the drying process and define the characteristics of the drying curves and corresponding processes.

Refrigeration

Refrigeration consists of the forced reduction of food temperature. When the value of food temperature is in the refrigeration range, microbial growth is significantly slowed and the growth of pathogens or formation of toxins is reduced. *Food Code 2009* from the FDA prescribes that in order to be considered safe, the temperature of cooked foods that will support the growth of pathogens (potentially hazardous foods) must be reduced from "135 °F to 70 °F within 2 hours, and within a total of 6 hours from 135 °F to 41 °F or less." In addition, potentially hazardous food must be cooled within 4 hours to 41 °F or less if prepared from ingredients at ambient temperature (e.g., tuna salad). There are additional considerations, such as immediately placing raw eggs in a refrigerated environment maintained at 45 °F or less and performing a careful professional review of the official source as a prerequisite to taking related safety and process-related decisions.

Freezing

Freezing of foods virtually stops microbial growth due to the absence of liquid water needed to support it. The interaction between water and the other food components during freezing is quite complicated. A certain fraction of the available water will often remain in the liquid state at temperatures that are low enough to completely solidify pure water. The use of the thermodynamic diagrams developed by Reidel (some of them are presented in Loncin and Merson [3]) is recommended as a source of valuable data needed to evaluate freezing operations of foods.

Canning—sterilization and pasteurization

The effect of thermal treatments on the microbiological population of foods is probably the most relevant operation to render them safe and stable. The technology is nicely presented in [27], and the inactivation processes are presented in detail in the section of this chapter dedicated to the reduction of the population density.

Trimming and cleaning

Separation of the microorganisms from foods through trimming and cleaning will have a corresponding effect on food safety. The removal of damaged or contaminated parts will reduce the probability of an unsafe condition, as described later, due to the correspondence between the values of the population density of microbial pathogens and the corresponding probability of process failure. Cleaning of foods is a challenging field and substantial progress is being made to describe and understand the mechanisms of adhesion of microorganisms to food materials and the corresponding effects on the population and respective safety levels.

Fermentation

Most pathogenic microorganisms are at a disadvantage with respect to the natural microbial population of foods because pathogens thrive under the conditions found in the human body. Their optimum pH, temperature, and ionic strength conditions are, therefore, often significantly different from those prevailing in foods. Thus, the dynamics of the corresponding populations most often favor the growth of microorganisms that are more capable than pathogens of growing in the microenvironment provided by food products. Fermented food products, such as pickles, yogurt, kefir, aged cheeses, beer, and wine, take advantage of these dynamics to preserve foods. In addition, fermentation can be used to reduce anti-nutritional factors, as it does for tofu and similar fermented products of soybeans.

Finally, a significant favorable factor of many fermented foods is the production of vitamins that enhance the nutritional value of the corresponding products. Most processing operations diminish the nutritional value to some extent but fermentation is an exception to the rule.

Nixtamalization

This is a hot-alkaline process used in the preparation of "masa," the dough used to produce tortillas and related corn products. This process may reduce the concentration of mycotoxins present in the grain and enhances the nutritional value of the corn. The corresponding heat treatment is severe enough to inactivate most pathogenic microorganisms.

Measurement of the microbial population density

The measurement of the microbial population density is often used to represent the effect(s) of processing and the initial and final contamination conditions of food products and their ingredients. The unit of measurement most often used to describe the population density is the colony forming unit (CFU). It is very important to be aware of the characteristics and limitations of this type of unit, especially its potential to underestimate the microbial population density. For instance, a group of microorganisms (clump) may produce a single colony and be counted as one. On the other hand,

comparison between CFU results and microscopic observations has shown that many microorganisms will not produce colonies, depending on the incubation conditions and the growth medium used. In addition, dormant spores may not germinate (for instance, often, less than 10% of dormant *B. stearothermophilus* spores—commonly used as biological indicators of commercial sterilization processes—will germinate after heat-shock activation). Finally, modern DNA techniques have shown that the fraction of microorganisms that can be measured as CFUs is often small: Many species are not successfully grown under the current state of art.

Relationship between the microbial population density and the probability of failure

The methods presented here are aimed at the estimation of the values of population density of subpopulations of critical interest for food safety. These methods use mathematical and statistical tools to describe the population density and its variability. Thus, a rigorous link between the values of the population density and the corresponding probability of finding survivors to a process is required. The food safety requirement is that the food products have a small enough probability of inducing injury through survival and/or growth of pathogenic microorganisms to be considered safe enough for consumption.

The exponential nature of the processes used to reduce the population of pathogens implies that a zero probability of process failure is not attainable in practical terms, so values that are low enough to achieve safety have to be defined. Such is the case of the 10^{-6} sterility assurance level (SAL) used in terminal sterilization of drugs, medical devices, and biologics; the 10^{-6} probability of failure for shelf-stable high pH canned food products recommended by Stumbo; the 12 D_{250} (12 orders of magnitude reduction in the population density) for *Clostridium botulinum* often mentioned by food process authorities (originated apparently in the work by Esty and Meyer [4]); and, more recently, the FSO to be defined for food products. The connection between values of the population density of microorganisms of interest (due to their resistance and/or pathogenicity) and the corresponding probability of finding survivors to a process is again the basis for a practical understanding of the effect of processing on food safety.

Halvorson and Ziegler [5] presented a classical approach to deal with the relationship between the population density and the probability of finding a colony forming unit (CFU). They divided the volume of interest into a large number of fractions just large enough to contain one of the microorganisms. Some of these fractions would contain microorganisms and the rest would not. Based on this assumption, they demonstrated that the probability of finding no microorganisms in a 1 cc. aliquot when the population density (number of microorganisms per cc.) is x is

$$P = e^{-x}$$

and the corresponding probability of finding a CFU when an aliquot of a cc. is removed is

$$Q = 1 - e^{-a\,x}$$

They used these expressions to develop the most probable number (MPN) method of estimating the microbial population used in microbiology.

A traditional interpretation of a fractional value for the probability of finding a CFU is that a proportionally larger number of aliquots need to be removed to find a CFU (and not that we would find a fraction of a bacterium or spore!). Therefore, we may say that a 0.01 value for Q may be interpreted as the need for approximately 100 test units or an aliquot correspondingly larger to have a high probability of finding a CFU. Remember that the correspondence is not directly proportional but exponential.

The connection between the probability of finding a CFU in a sample of food and the corresponding probability of a product reaching the consumer with a risk of inducing injury through foodborne infection or intoxication because of a process failure is best understood through a summary analysis of how the population density (x) and the probability of failure (Q) behave when the population density is close to 1 or smaller. Two important considerations between the probability of finding a CFU and the population density (Figure 33.1) are first that the parallelism

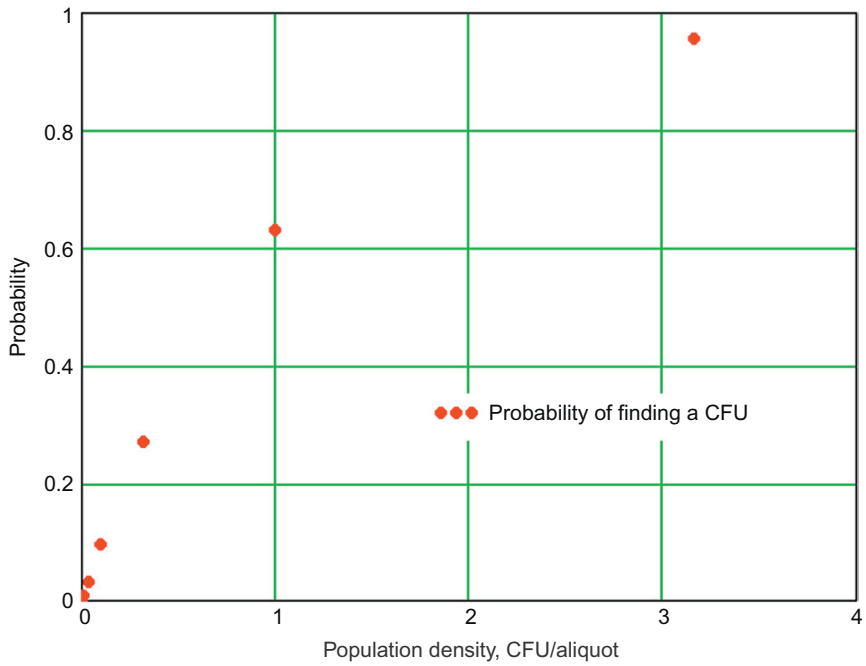

FIGURE 33.1

Relationship between the probability of finding a CFU and the population density.

between the two variables will not exist for values of the population density that are significantly larger than 1, because the value of the probability can never exceed unity, and second that numerical values of both variables converge for small values of the population density. Most of the practical applications in food processing will require low probabilities of failure, and in this region the relative numerical difference diminishes (Figure 33.2). Therefore, we can use both variables to represent the probability of failure of a food process, and take advantage of powerful mathematical methods that are available to describe the population density. These methods may be implemented using modern computerized tools to efficiently understand the risk(s) related to food processing.

Initial population density or bioburden

The measurement of the value of the initial population density or bioburden has been explored intensively because industrial processes of radiation sterilization of medical devices use bioburden-based procedures in their development and validation work. Applicable mandatory concerns have been developed and published in the standards currently in use by the medical device industry throughout the world

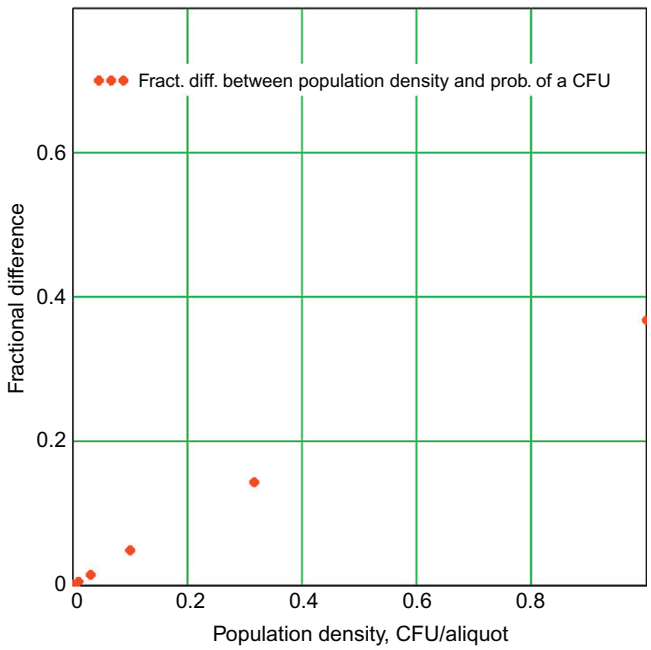

FIGURE 33.2

Fractional difference between the value of the population density and the probability of finding a CFU.

(Table 33.1). The word "shall" in these documents implies that the corresponding concern is mandatory if the standard is used as the frame of reference for a regulatory submission. Availability of this information may assist the food industry in improving related work to prevent the need to "rediscover" the corresponding technology.

Table 33.1 Mandatory Concerns Presented in the International Standards for Measurement of Bioburden

Mandatory Requirement	Document	Section
"Procedures for determination of bioburden shall be specified."	ANSI/AAMI/ISO 11737–1	4.1.1
"Records shall include all original observations."	ANSI/AAMI/ISO 11737	4.1.3
"Records shall include all calculations."	ANSI/AAMI/ISO 11737	4.1.3
"Records shall include all derived data."	ANSI/AAMI/ISO 11737	4.1.3
"Records shall include all final reports."	ANSI/AAMI/ISO 11737	4.1.3
"Records shall include the identity of all personnel involved in sampling, preparation, and testing."	ANSI/AAMI/ISO 11737	4.1.3
"Calculations and data transfers shall be subject to appropriate checks."	ANSI/AAMI/ISO 11737	4.1.4
"All items of equipment required for correct performance of the specified tests and measurements shall be available."	ANSI/AAMI/ISO 11737	4.2.3
"A documented system complying with ISO 13485, ISO/IEC 17025, or ISO 10012 shall be specified for the calibration of all equipment, including instrumentation for test purposes, used in meeting the requirements of this part of ISO 11737."	ANSI/AAMI/ISO 11737	4.3.2
"Methods shall be specified for the preparation and sterilization of materials used in the determination of bioburden, including appropriate quality tests."	ANSI/AAMI/ISO 11737	4.3.3
"Procedures for investigation of out-of-specification results and for correction, corrective action, and preventive action shall be specified. These procedures shall comply with ISO 13485 or ISO/IEC 17025."	ANSI/AAMI/ISO 11737	4.4
"The procedures for selection and handling of product for the determination of bioburden shall ensure that selected product is representative of routine production including packaging materials and processes."	ANSI/AAMI/ISO 11737	5.1.5
"Consideration shall be given to the timing of the performance of the determination of bioburden relative to taking samples."	ANSI/AAMI/ISO 11737	5.1.3

(*Continued*)

Table 33.1 (Continued)

Mandatory Requirement	Document	Section
"If the bioburden is demonstrated as being evenly distributed on and in the product item, the sample item portion (SIP) may be selected from any portion of the item. Otherwise, the SIP shall consist of portion(s) of product, selected at random, which proportionally represent each of the materials from which product is made. If the bioburden distribution is known, the SIP may be selected from the portion of the product that is considered to be the most severe challenge to the sterilization process. The SIP can be calculated on the basis of length, mass, volume or surface area (see Table 1, page 4 for examples)."	ANSI/AAMI/ISO 11737	5.2
"An appropriate method shall be selected for the determination of bioburden. The method shall comprise techniques for removal of microorganisms if appropriate."	ANSI/AAMI/ISO 11737	6.1.1, a
"An appropriate method shall be selected for the determination of bioburden. The method shall comprise techniques for culturing of microorganisms."	ANSI/AAMI/ISO 11737	6.1.1, b
"An appropriate method shall be selected for the determination of bioburden. The method shall comprise techniques for enumeration of microorganisms."	ANSI/AAMI/ISO 11737	6.1.1, c
"The precision (of the techniques listed above) shall be appropriate for the purpose for which data are to be used."	ANSI/AAMI/ISO 11737	6.1.1
"For an identified product where removal of viable microorganisms is part of the method, the efficiency of removal shall be considered and the outcomes of this consideration recorded."	ANSI/AAMI/ISO 11737	6.1.2.1
"Consideration shall be given to the ability of the technique to remove microorganisms."	ANSI/AAMI/ISO 11737	6.1.2.1, a
"Consideration shall be given to possible types of microorganisms and their location in the product."	ANSI/AAMI/ISO 11737	6.1.2.1, b
"Consideration shall be given to effects of the removal technique on the viability of microorganisms."	ANSI/AAMI/ISO 11737	6.1.2.1, c

(*Continued*)

Table 33.1 (Continued)

Mandatory Requirement	Document	Section
"Consideration shall be given to the physical or chemical nature of product under test."	ANSI/AAMI/ISO 11737	6.1.2.1, d
"For an identified product where removal of viable microorganisms is not part of the method, the efficiency of enumeration of microorganisms shall be considered and the outcomes of this consideration recorded."	ANSI/AAMI/ISO 11737	6.1.2.2
"Consideration shall be given to possible types of microorganisms and their location in the product."	ANSI/AAMI/ISO 11737	6.1.2.2, a
"Consideration shall be given to the physical or chemical nature of product under test."	ANSI/AAMI/ISO 11737	6.1.2.2, b
"Consideration shall be given to aggregates of cells forming single colonies due to *in-situ* culturing."	ANSI/AAMI/ISO 11737	6.1.2.2, c
"If the physical or chemical nature of product is such that substances can be released that adversely affect the number or types of microorganisms found, then a system shall be used to neutralize, remove, or if this is not possible, minimize the effect of any such released substance. The effectiveness of such a system shall be demonstrated."	ANSI/AAMI/ISO 11737	6.1.2.3
"Culture conditions shall be selected after consideration of the types of microorganisms likely to be present. The results of this consideration and the rationale for the decisions reached shall be recorded."	ANSI/AAMI/ISO 11737	6.1.3
"Appropriate techniques for microbial characterization of bioburden shall be selected."	ANSI/AAMI/ISO 11737	6.2.1
"Microbial characterization shall be accomplished using one or more of the following: **a)** Staining properties **b)** Cell morphology **c)** Colony morphology **d)** Use of selective culturing **e)** Biochemical properties **f)** Genetic sequence for which there is an adequate data base."	ANSI/AAMI/ISO 11737	6.2.2

(*Continued*)

Table 33.1 (Continued)

Mandatory Requirement	Document	Section
"The method for determining of bioburden shall be validated and documented."	ANSI/AAMI/ISO 11737	7.1
"Validation shall consist of the following: **a)** Assessment of the adequacy of the technique for the removal of microorganisms from product, if removal is part of the method. **b)** Determination of the recovery efficiency in order that a correction factor be derived. **c)** Assessment of the adequacy of the enumeration of microorganisms including culture conditions and microbiological counting techniques. **d)** Assessment of the suitability of the techniques of microbial characterization."	ANSI/AAMI/ISO 11737	7.2
"If bioburden data are to be used to establish the extent of treatment of a sterilization process, any requirements applicable to the use of bioburden data specified in the appropriate standard for the development, validation, and routine control of the sterilization process shall be met."	ANSI/AAMI/ISO 11737	8.4
"Data derived from determination of bioburden, obtained over a period of time, shall be used to identify trends. Acceptable limits shall be reviewed and revised as necessary."	ANSI/AAMI/ISO 11737	8.6
"The application of statistical methods to define sample size, sampling frequency and/or acceptable limits shall conform to ISO 13485."	ANSI/AAMI/ISO 11737	8.7
"Changes to product or manufacturing process shall be reviewed to determine whether they are likely to alter bioburden. The results of the review shall be recorded (see 4.1.2). If there is potential for alteration of bioburden, specific determination of bioburden shall be performed to evaluate the extent and nature of any change."	ANSI/AAMI/ISO 11737	9.1
"Any change to a routine method of bioburden determination shall be assessed. The assessment shall include: **a)** Evaluation of the effect of the change on the outcome of determination. **b)** Establishment of the recovery efficiency of the method following the change."	ANSI/AAMI/ISO 11737	9.2

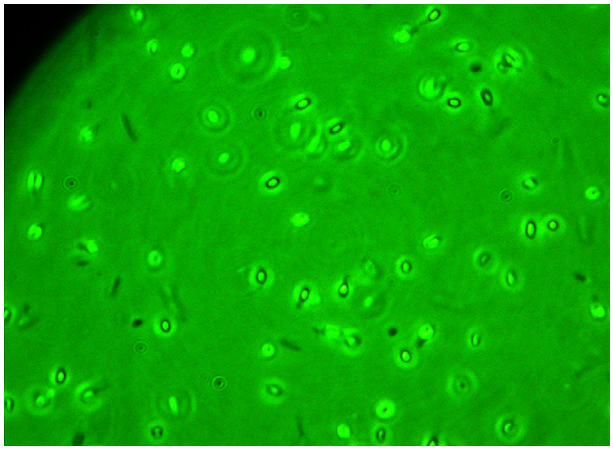

FIGURE 33.3

Dormant, reasonably clean spores of *Bacillus atrophaeus* v. 5230 viewed using phase contrast microscopy.

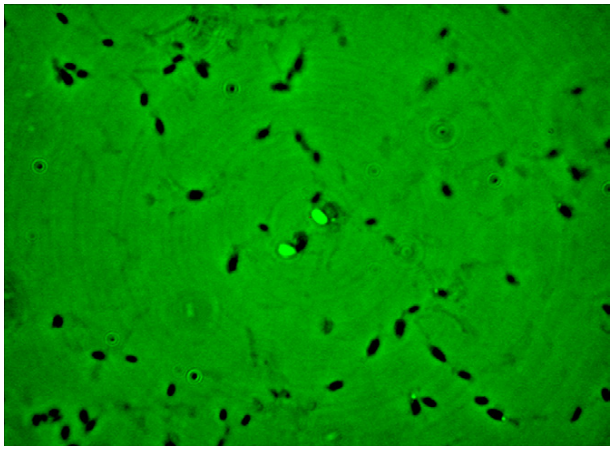

FIGURE 33.4

Opaque spores of *Bacillus atrophaeus* v. 5230 (dormancy broken, no longer resistant) viewed using phase contrast microscopy.

Use of bacterial spores as biologic indicators of industrial sterilization processes merits particular attention to the quality of the spore suspensions used, as the spore suspensions used in regulatory submissions are not uniform and this can have a strong influence on outcome (Figures 33.3–33.5). Figure 33.3 presents dormant spores that are reasonably free of debris, clumping, and vegetative cells. Dormant

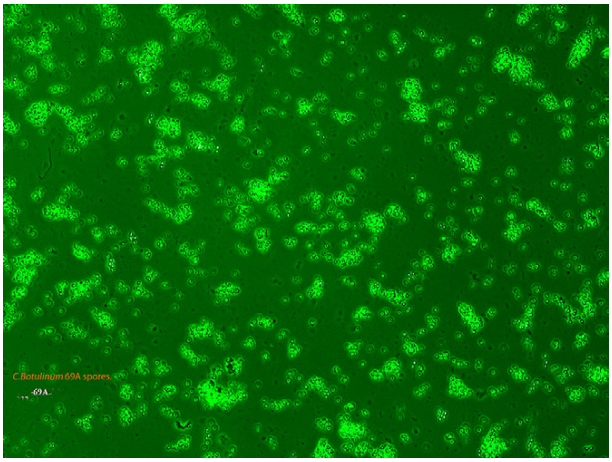

FIGURE 33.5

Spores of *Clostridium botulinum* v. 69A viewed using phase contrast microscopy. Clumping, opaque spores, and debris are evident.

spores are birefringent under polarized light and also very resistant to moist heat and other lethal agents. Thus, this is the type of spore suspension that we should use as indicators. Figure 33.4 shows the same suspension after allowing some of the spores to break dormancy (lose birefringency and become opaque). These spores are capable of germinating as CFUs; however, they have lost the extreme resistance characteristic of dormant spores. If we used this suspension to judge a process we would be using spores with only a fraction of the intended resistance and we would overestimate the effectiveness of the process. In addition, survival curves prepared using a poor-quality mixed population of spores likely will lead to broken curves that may be found puzzling or motivate the use of curve-fitting software tools to describe them in an attempt to legitimize the corresponding work. The practical message is clear: Use clean dormant spores.

Figure 33.5 presents a suspension of spores of *Clostridium botulinum* (used in a professional inoculated pack study) that contains many clumps of spores. Inasmuch as each clump will lead to a single CFU, the estimated population density will be in error and will lead to unexpected aberrations and deviations from log-linearity (which are commonly reported in the literature). Quality control and microscopic examination of spore suspensions used in research and industrial settings are extremely important.

Reduction of the population density

Convection is a transformation in which microorganisms are transported by flowing air, water, or fluids. Convection plays a major role in processes that reduce the microbial population density of food materials. Examples include cleaning,

elimination of altered parts, and rinsing. Inactivation of microbial populations renders microorganisms incapable of reproduction and developing into CFUs, and is most often related to kinetics of the corresponding transformations.

Inactivation

Inactivation of microorganisms driven by the inactivation transformation of a single population is well described by a pseudo-first order mathematical model (Chick's model [5,9, 16–18]). In the case of steam sterilization it would be:

$$\frac{dN}{dt} = -kN$$

where N is the number of survivors, k is the rate constant, and t is time.

If the effect of concentration of the lethal agent must be taken into consideration, Chick-Watson's model can be used:

$$\frac{dN}{dt} = -kC^n N$$

where C is the concentration of the lethal agent and n represents the effect of changes in the concentration in the reaction rate.

For instance, for n = 1 (ethylene oxide), if the concentration doubles, the rate also doubles; for n = 3 (some phenolic compounds), if the concentration doubles, the reaction rate increases by a factor of 8.

In the area of sterilization science and technology, the rate constant has been traditionally replaced by the decimal reduction time (D) which is the time it takes the curve of the decimal logarithm of the survivors vs. time to go through an order of magnitude (or a log cycle). In the case of steam sterilization, D is inversely proportional to the rate constant. In the case of the Chick-Watson's model, D is inversely proportional to the rate constant times the concentration to the n power [7].

For constant lethal conditions, such as temperature, concentration, and pressure, as long as the system consists of a single transformation (inactivation) and a single population (normally dormant, clean, clump-free genetically pure bacterial spores), the graphical representation of the logarithm of the survivors vs. time will resemble a straight line. The intercept of the curve corresponds to the initial number of survivors (N_0), and the negative-reciprocal of its slope corresponds to the decimal reduction time for the set of conditions used in the test. Experimental and biological variability induce random deviations from a perfect straight line. Systematic or excessive deviations (a process authority should define these when conditions are deemed important) often must be investigated before the model can be applied to practical problems.

For transient lethal conditions, where temperature is a function of time, the function that describes the effect of temperature on the rate constant or D must be

known or developed. For that purpose, the rate constant is appropriately described by the activation energy in Arrhenius' equation, and the D value is described by parameter z, which is the number of degrees it takes the log (D) vs. temperature curve to go through one order of magnitude. Both models work reasonably well in the temperature ranges of practical importance for the food industry. Arrhenius' model is a better descriptor for temperatures that are significantly different from the reference temperature [6].

This model leads to some formulae of interest for steam sterilization. Similar formulae are available for chemical and high pressure sterilization [7,8].

Survivors of a transient steam sterilization cycle:

$$N(t) = \frac{N_0}{10^{\left[\frac{1}{D_{250}}\int_0^t 10^{\frac{T(t)-250}{z}}dt\right]}}$$

where D_{250} is the value of D at 250 °F, t is time, and T is temperature.

Accumulated lethality when the reference temperature is 250 °F (121.1 °C), and z is 18 °F (10 °C):

$$Fo = \int_0^{time} 10^{\frac{T(t)-250}{18}}dt$$

where T is temperature and t is time.

Survivors as a function of time when the value of D is known as a function of any significant parameters (i.e., temperature, pressure, pH, etc.):

$$SLR = \log\left(\frac{N_0}{N}\right) = \int_0^{time} \frac{dt}{D(T(t), C(t), P(t), pH, ...)}$$

where SLR is the "spore log reduction" and N_0 is the number of survivors when t=0 [9,20,23,24,26].

This equation, coupled with the availability of mathematical software to perform the numerical integration, makes the calculation of the survivors of a lethal process straightforward regardless of the complexity of the function that describes D. This makes possible the estimation of survivors to combinations of lethal factors such as temperature, pressure, and concentration.

The inactivation process may be defined by the changes in the values of the microbial population density due to exposure of the population to a lethal agent. A series of variables have been identified experimentally to have a significant effect on the dynamics of the inactivation process. The decimal reduction time (D) provides information on how fast the inactivation (reduction in the value of the

population density with respect to time) processes happen. Its natural logarithm—(ln [D])—is a function of a series of variables:

$$\ln(D) = f(Temperature, Pressure, Concentration, Aw, pH, dose, ...)$$

Calculation of the population density

Calculation of the population density is straightforward once the value of D is known. Therefore, understanding the variation of D using the formula presented above will provide an understanding of the variation of the population density as the inactivation processes affect it. Using Gauss' Law of the propagation of error, we can write an expression for the variance of D as related to the variances of the significant parameters:

$$\sigma^2(\ln(D)) \approx \frac{\partial f}{\partial T}\sigma^2 T + \frac{\partial f}{\partial P}\sigma^2 P + \frac{\partial f}{\partial C}\sigma^2 C + \frac{\partial f}{\partial A_w}\sigma^2 A_w + \frac{\partial f}{\partial pH}\sigma^2 pH + ...$$

This equation may be used to develop more detailed expressions depending on each particular application. It provides a linkage between kinetics and statistics as it relates the variances to the kinetic parameters and formulae. Indeed, if the assumption of orthogonality holds (minimum interaction among the effects of the different parameters), the corresponding numerical estimation is quite straightforward.

The mathematical form of the partial derivatives has been determined for the most significant variables. For instance, the partial derivative with respect to temperature may be found from Eyring's, Arrhenius', or Bigelow's models. The partial derivative with respect to pressure is a simple expression involving the activation volume [8]. The partial derivative with respect to Aw (in the range 0.5<Aw<1.0) may be described by the following expression [9]:

$$\frac{\partial(\ln(D))}{\partial A_w} = \frac{m}{A_w}$$

where m is the slope of the curve found plotting ln(Aw) vs. ln(D).

The partial derivative with respect to the concentration of disinfectants can be found using the Chick-Watson's model [7]. Care should be taken when disinfectants that are very reactive with organic matter such as chlorine and ozone are used. One of the assumptions used to justify the use of a pseudo-first order model is that the disinfectant is always in excess. This assumption must be true, or the inactivation reactions may

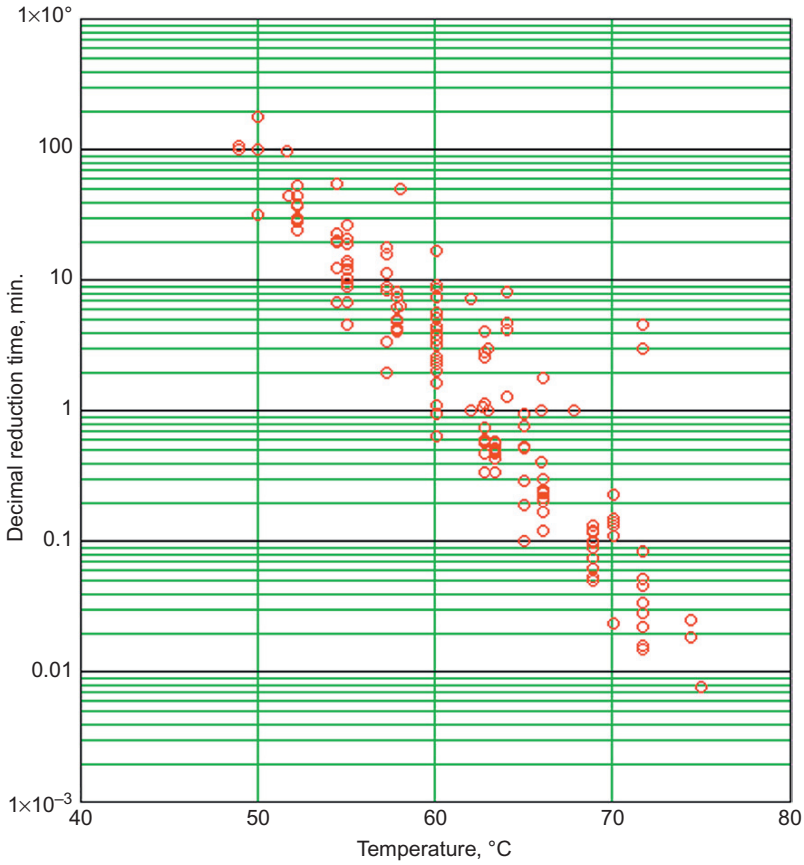

FIGURE 33.6

Decimal reduction time vs. temperature for *Listeria monocytogenes*.

shift to second order and the apparent reaction rate diminishes significantly. This change in the reaction order is a potential cause of apparent tailing in inactivation processes. Therefore, the estimation of the lethal effect of an inactivation process on the microbiological population consists of defining the significant parameters for the application of interest, defining mathematically their effect on the value of the rate constant or decimal reduction time, and calculating the effect of the selected regime of lethal conditions on the SLR. Temperature, concentration, pressure, and other factors may need to be simulated to provide the required inputs to the estimation of the lethal effect of the conditions under study.

A significant factor to be considered is the biological variability in the decimal reduction time and its corresponding relationship with temperature and other factors such as pH and pressure. It is evident from reported values for the decimal reduction time at different temperatures for *Listeria monocytogenes* (Figure 33.6) and pathogenic *Escherichia coli* (Figure 33.7) that in practical applications the idea

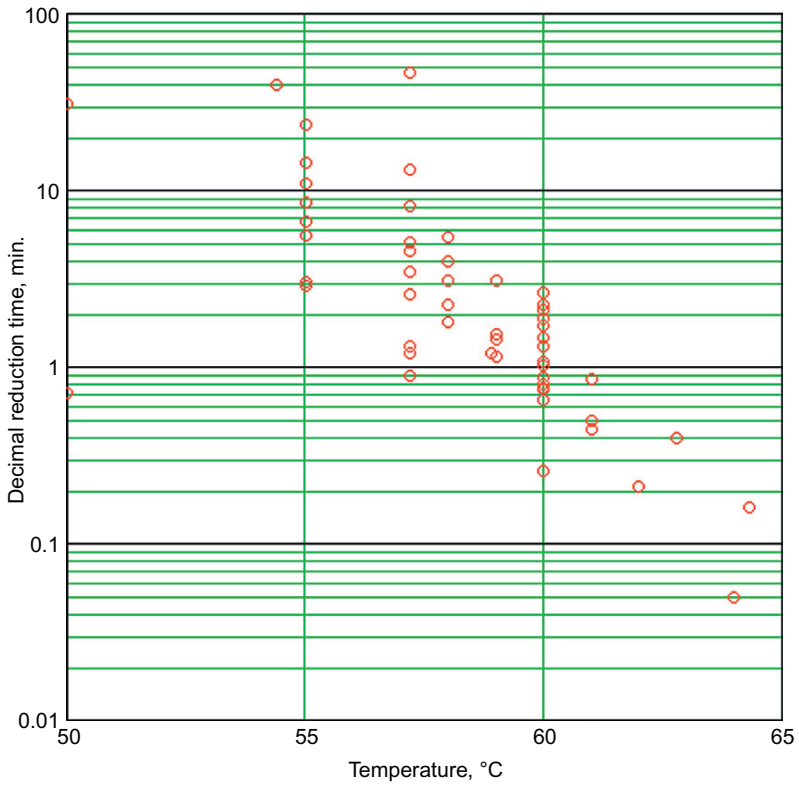

FIGURE 33.7

Decimal reduction time vs. temperature for pathogenic *Escherichia coli.*

of a single ideal value for the rate constants is inadequate due to biological variability and other factors such as the procedures used to isolate and enumerate the microorganisms (data taken from ICMSF [10]).

When the practical interest centers on the most risky conditions that may be realistically found, statistical tools can be used to place prediction bounds around our estimate to assure that the probability of failure of the corresponding estimates is understood. Placing a 95% prediction interval around the decimal reduction time vs. temperature numbers (Figures 33.8 and 33.9) demonstrates that for a given temperature, the upper prediction interval will provide us with numbers that will represent 95% of the data. The confidence interval may be adjusted as needed. Some of the heuristic "rules of thumb" currently in use should be reviewed in light of this. The use of a tolerance interval is advantageous in presenting the variability found in the cold-spot temperature because it allows for adjustment due to the precision of the data and for the sample size (Figure 33.10). In this case, Student's *t*-distribution was used and the corresponding tolerance intervals would be adjusted to account for small sample sizes. Temperature at the cold spot may then be used to calculate

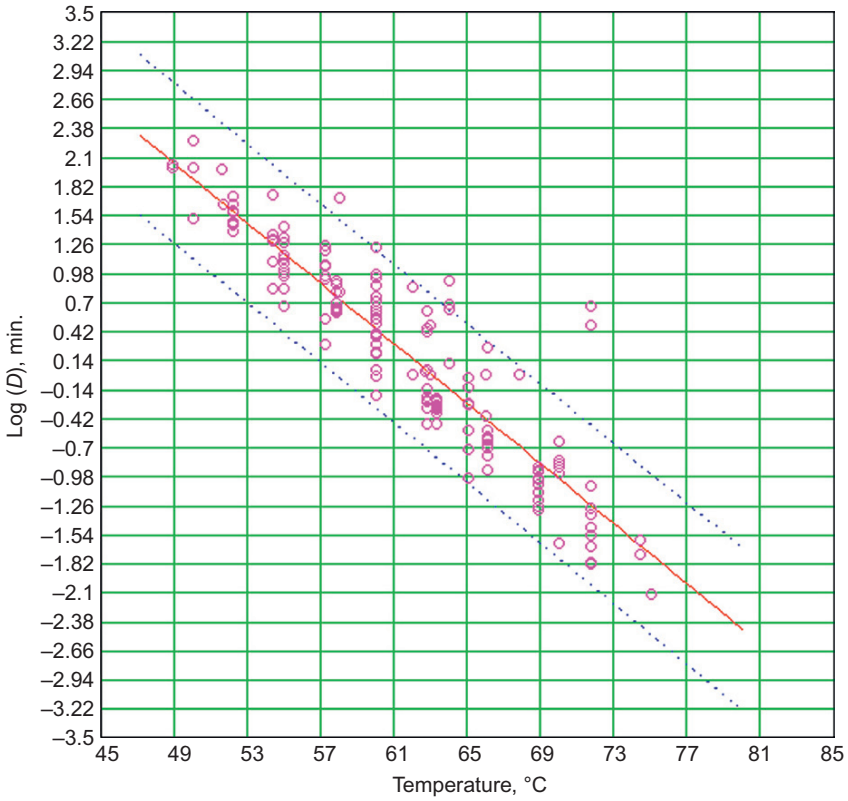

FIGURE 33.8

Listeria monocytogenes D values vs. temperature with corresponding upper and lower 95% prediction intervals.

corresponding values for Fo that take into consideration important sources of risk. The linkages between temperature, Fo, population density, and, finally, the probability of failure (with a corresponding confidence level) may be clearly established.

Cleaning

Detailed information is available from other sources on the engineering aspects of cleaning and disinfecting [3] and on processes for cleaning and disinfecting equipment and materials [11]. Cleaning and contamination receive significant attention by the research community, and interesting results are abundant. However, validated models of practical use are mostly still in a developmental stage. Thus, procedures are often developed empirically and validated by experience. Validation of cleaning and disinfection processes is a strong area for companies that produce reusable medical

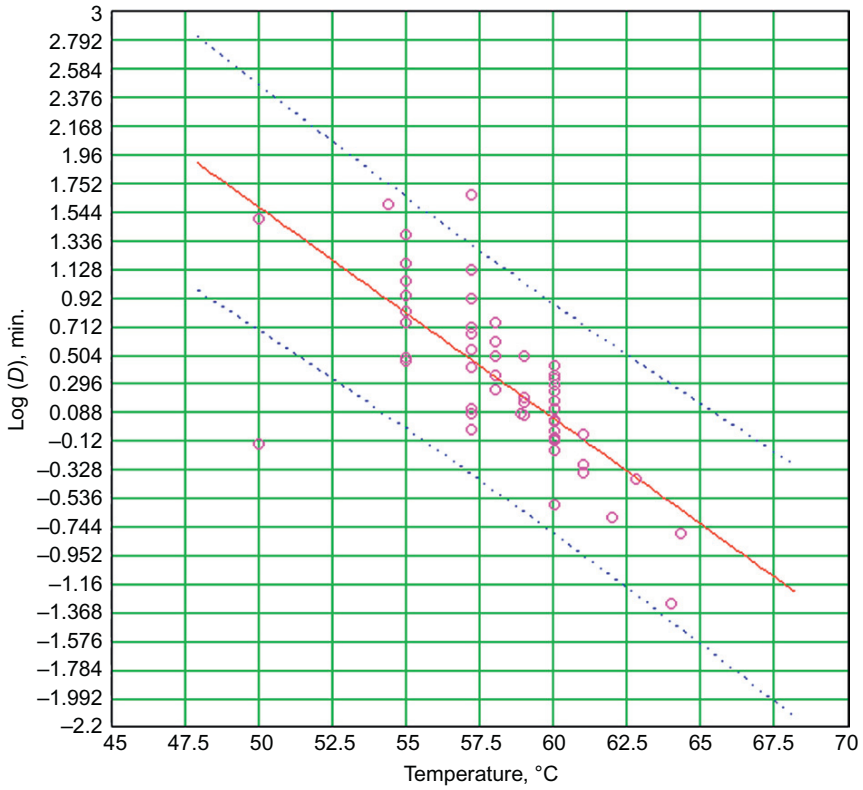

FIGURE 33.9

Pathogenic *Escherichia coli D* values vs. temperature with corresponding upper and lower 95% prediction intervals.

devices, and procedures in their guidance documents (e.g., AAMI TIR30 [2]) may be used by the food industry to develop equivalent technology without the need to repeat the intense and extensive developmental work already performed.

The kinetics of cleaning has some similarities to the kinetics of inactivation. In many instances, the logarithmic nature of the processes is evident. Often, cleaning will occur at a rate that diminishes with time and, theoretically, may never reach a point at which the contaminant(s) have been completely removed. However, the behavior of microorganisms concerning adhesion and motion, among other factors, is more complicated than that of inert materials [12]. Microorganisms often have organelles such as flagella or cilia that potentially enable motility beyond that expected from inert particles. In addition, microorganisms may produce substances that enhance their adhesiveness to some surfaces, and, finally, populations of microorganisms may develop into biofilms that enhance their resistance and adhesiveness to equipment or ingredients, or both [13].

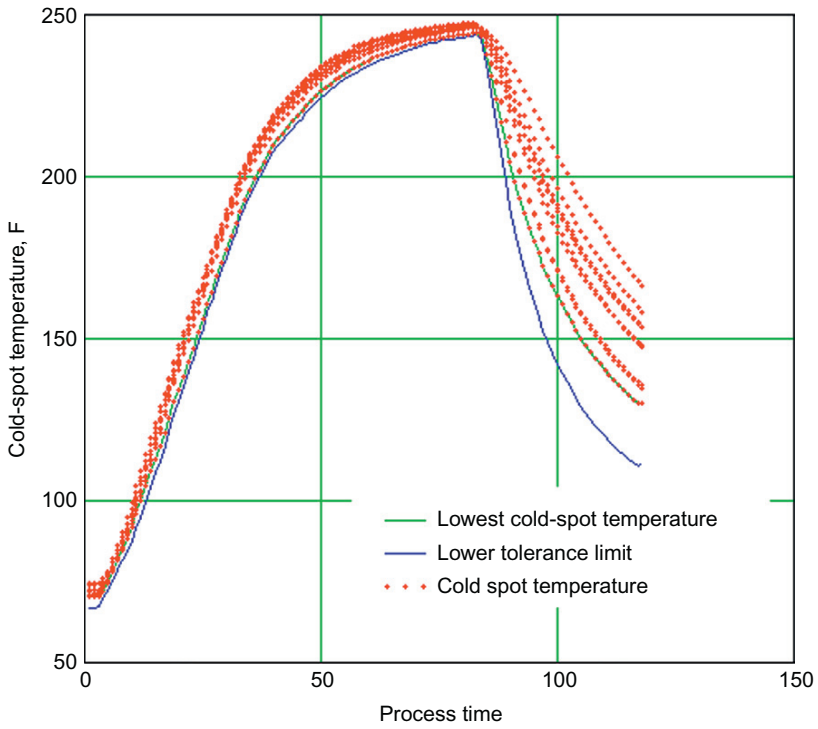

FIGURE 33.10

Tolerance limits applied to cold-spot temperature data.

Post-process growth

In principle, each survivor or residual CFU is capable of duplicating itself when appropriate conditions surround it. Thus, after an adaptation time the number of CFUs may be expected to double in a periodic fashion until the conditions change, e.g., when the nutrients are exhausted. The time required for duplication is a function of multiple factors, and when these factors are defined a prediction of the population dynamics is technically feasible. Predictive microbiology allows for the description of the expected behavior of microorganisms of interest as functions of initial contamination, pH, temperature, and other factors.

The University of California at Davis has a web page that captures the main pathogen modeling programs: http:ucfoodsafety.ucdavis.edu/Food_Safety_Links/Pathogen_Modeling_Programs/. An excellent example is the USDA Pathogen Modeling Program, which is maintained and supported by FSIS/USDA (http://ars.usda.gov/Services/docs.htm?docid=11550. A series of considerations warning us about the limitations of the current knowledge is presented in the FSIS Notice 25-05: "It is not possible or

appropriate to rely solely upon a predictive modeling program to determine the safety of foods." However, the increasing power of modern predictive microbiology is an excellent resource to support practical related decisions.

Conclusion

This chapter presents the concepts, transformations, and systems that define the effect of process on the microbiological safety of a food product, including the application of material from the medical devices industry related to bioburden and cleaning. This information is presented with the idea that our food industry does not need to repeat the related development efforts and may benefit from these available concepts and tools once they are adapted to our particular needs. The conceptual aspects discussed here should prove valuable in understanding and evaluating the effect of process on food safety. An important concept is that knowledge of the values of the microbial population density (and their variability) enables us to determine the corresponding probability of failure of a process aimed to assure food product safety from its initial production steps till product reaches consumer.

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CHAPTER

34

Food Safety Post-processing: Transportation, Supermarkets, and Restaurants

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Introduction

As we learned in previous chapters of this book, foodborne illness is a significant cause of personal distress, disease, death, and economic burden. Foodborne diseases affect tens of millions of people and kill thousands in the USA each year. They also cause billions of dollars in health care-related costs, industry costs, and personal costs annually. As a result, the public has become increasingly aware of, and concerned about, the safety of the food they eat. There are multiple sources of contamination and means of prevention during the flow of food from the farm to the consumer's table. Each step in the flow, from farm to food manufacturing facility to retail and food service operations to the consumer's table, has some degree of risk and different methods of prevention. For the purpose of this chapter, three important components in the post-processing flow of food have been identified for potential food safety risks and means of prevention. They include transportation, retail food establishments, and food service operations.

Like growers and processors, transporters, retail food stores, and food service establishments have a responsibility to use food handling practices that reduce the risk of foodborne illness within their operation. Since the retail and food service components are often the last steps in the flow of food prior to consumption, proper food handling practices at these steps are especially critical for assuring that the food is safe prior to being consumed. The retail and food service sectors also are very complex. The restaurant industry in the USA employs over 12.8 million Americans in 960,000 locations, and sales in 2010 exceeded $604 billion [1]. The US supermarket industry houses over 36,000 stores and employs over 3.4 million people, and 2010 sales exceeded $363 billion [2].

Foodborne Infections and Intoxications.

There are over 3000 state, local, and tribal agencies that serve as the primary regulators of retail food stores and food service establishments in the USA. They are responsible for the inspection and oversight of over 1 million retail food establishments including restaurants, retail food stores, vending operations, and food service operations in institutions such as schools, hospitals, nursing homes, and child care centers [3]. While state and local agencies have the authority to enforce food safety regulations, remove unsafe products from the marketplace, and bring action against violators within their state/local jurisdiction, one of the challenges is that food safety regulations for each state/local jurisdiction can vary greatly. The FDA keeps an active website (http://www.fda.gov/Food/FoodSafety/RetailFoodProtection/FederalStateCooperativePrograms/ucm122814.htm) that provides further information about retail- and food service-related laws and regulations for different states/jurisdictions [4].

The FDA publishes the *FDA Food Code* to bring uniformity to food safety regulation and to ensure that food at retail is safe and properly protected and presented [5]. Local, state, tribal, and federal regulators use the *FDA Food Code* as a model to develop or update their own food safety rules and to be consistent with national food regulatory policy. The FDA also provides the guidance and training these agencies need to protect food in their jurisdictions.

Transportation of food

There are several examples of food becoming contaminated during transportation or which demonstrate the potential for food to become contaminated during transport. In 1989, multiple outbreaks of staphylococcal food poisoning were linked to canned, imported mushrooms that had been contaminated by post-harvest and pre-processing collection and transportation practices used in China [6]. In 1994, a salmonellosis outbreak affecting over 200,000 people was correlated with cross-contamination of pasteurized ice cream transported in tanker trailers that had previously been used to transport non-pasteurized liquid eggs. In 1999, unpasteurized orange juice was contaminated with *Salmonella* Muenchen, leading to over 400 illnesses and one death. The juice company and regulatory officials that investigated the outbreak traced the source of the outbreak to a tanker truck from Mexico that was chilling the unpasteurized orange juice with contaminated ice during transit. The overall public health and economic impacts of foodborne contamination attributable to transportation is unknown and is likely to be grossly under-reported and underestimated. In 1993, conservative estimates placed the financial impact at $2 billion annually [6].

In 2007, the Michigan Department of Agriculture published information from the Interstate Food Transportation Assessment Project that was conducted in the states of Michigan, Illinois, Indiana, and Ohio. The study's focus was to examine food safety and food defense risks for foods in transit via interstate commerce. The study revealed several areas of concern in food transport that could increase the likelihood of food contamination, such as improper refrigeration, transport of raw meat and poultry simultaneously or sequentially in trucks also used to carry fruit and

vegetables, food products lacking label or source information, improper packaging, infestation with insects, insanitary storage (e.g., roof leaks and moldy walls, animal blood and food on bed floors), lack of security seals or locks, low driver awareness of safe food temperatures, and inadequate food safety training of drivers [7].

The US Congress passed the Sanitary Food Transportation Act (SFTA) in 1990 [8]. In 2005, the SFTA was revised to shift the responsibility for safe transportation of food from the US Department of Transportation to the FDA. Among other things, the SFTA amended the Food, Drug, and Cosmetic Act to include a section that requires the FDA to develop regulations addressing sanitation, packaging, limits on transport vehicles, information exchange among carriers, manufacturers, and other persons involved in transportation of food, and transportation-related record-keeping. The 2005 SFTA provides the foundation for the FDA to promulgate regulations setting forth sanitary transportation practices to be followed by shippers, carriers by motor vehicle or rail vehicle, receivers, and others engaged in food transport and to ensure that food is not transported under conditions that may render the food adulterated.

Food safety risks and preventive measures during transportation

The FDA has addressed the transportation of food with several different regulations (Table 34.1a), standards, and guidance documents (Table 34.1b) [9]. The challenge with food transportation is that each of these regulations, standards, and guidance documents is limited in scope to a particular circumstance or to a particular segment of the food supply. To assist the food transport industry in preventing food safety problems during transport, the FDA has identified problem areas (food handling risks) where food may be at risk for physical, chemical, or biological contamination during food transport (including temperature abuse, cross-contamination, improper loading and unloading practices, and inadequate employee hygiene and training) and recommended preventive controls that could be used by the transportation industry [9], such as:

- appropriate temperature control during transport;
- appropriate sanitation;
- appropriate packaging/packing of food products and transportation units;
- good communication between shipper, transporter, and receiver; and
- employee awareness and training.

Retail and food service

Food safety risk factors for food service, restaurant, and retail food establishments

In 1998, the FDA National Retail Food Team initiated a three-phase, 10-year study, to evaluate food handling practices and behaviors commonly identified by the Centers for Disease Control and Prevention (CDC) as the most important contributing factors in foodborne illness outbreaks. Data for this study were collected in

Table 34.1a Regulations Addressing the Transportation of Food

Title (CFR) Reference	Description
Current good manufacturing practice for medicated feeds; equipment cleanout procedures (21 CFR 225.65)	Requires adequate cleanout procedures for all equipment used in the manufacture or distribution of medicated feeds that are essential to avoiding unsafe contamination of feeds with drugs
Current good manufacturing practice in manufacturing, packing, or holding human food; warehousing and distribution (21 CFR 110.93)	Requires that storage and transportation of finished food be under conditions that will protect food against physical, chemical, and microbial contamination as well as against deterioration of the food and the container
Listing of specific substances prohibited from use in animal food or feed; requirements for renderers; requirements for protein blenders, feed manufacturers, and distributors; and requirements for persons that intend to separate mammalian and non-mammalian materials (21 CFR 589.2000 (c)–(e))	Requires distributors of mammalian and non-mammalian materials for animal food to provide for measures to avoid commingling or cross-contamination of the materials
Hazard analysis and critical control point (HACCP) systems; process controls (21 CFR 120.24 (c))	Requires that juice processors complete a 5-log pathogen reduction treatment and final product packaging within a single processing facility operating under CGMPs
Establishment, maintenance, and availability of records: what information must transporters establish and maintain?; what are the record retention requirements?; what are the record availability requirements?; what records are excluded from this subpart?; what are the consequences of failing to establish or maintain records or make them available to FDA? (21 CFR 1.352, 1.360–1.363)	Requires persons who transport food for humans and animals to establish and maintain records identifying the immediate previous source of all food received, and the immediate subsequent recipient of all food released, as well as certain other information related to the transported food; sets forth the record retention and record availability requirements for transporters
Cattle materials prohibited in animal food or feed to prevent the transmission of bovine spongiform encephalopathy (21 CFR 589.2001(c))	Requires the use of dedicated equipment for handling and transporting cattle materials prohibited in animal feed
Production, storage, and transportation of shell eggs (21 CFR 118.1(b) and 118.4(e))	Establishes requirements for refrigeration of shell eggs during storage and transportation

Adapted from [9].

Table 34.1b Guidance Documents and Standards Addressing the Transportation of Food

Title (Year)	Description
Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables [22]	Includes recommendations regarding microbial food safety hazards and good agricultural and management practices common to the growing, packing, and transporting of most fresh fruits and vegetables
Guidance on Bulk Transport of Juice Concentrates and Certain Shelf Stable Juices [23]	Provides industry with recommendations for appropriate control measures to use in the bulk transport of covered juice products to ensure that the products do not become contaminated or recontaminated with microbial pathogens during bulk transport, and states the FDA's intent to consider the exercise of enforcement discretion with respect to the single facility requirement in 21 CFR 120.24(c) provided that certain conditions are met
Dairy Farms, Bulk Milk Transporters, Bulk Milk Transfer Stations and Fluid Milk Processors: Food Security Preventive Measures Guidance [24]	Identifies the kinds of preventive measures operators of bulk milk transportation operations may take to minimize the risk that fluid milk under their control will be subject to tampering or other malicious, criminal, or terrorist actions
Food Producers, Processors, and Transporters: Food Security Preventive Measures Guidance [25]	Identifies the kinds of preventive measures operators of human or animal food establishments (including firms that distribute or transport food or food ingredients) may take to minimize the risk that food under their control will be subject to tampering or other malicious, criminal, or terrorist actions
Guidance for Industry #122: Manufacture and Labeling of Raw Meat Foods for Companion and Captive Non-companion Carnivores and Omnivores [26]	Provides guidance on transport of foods that contain raw meat, or other raw animal tissues, for consumption by dogs, cats, other companion or pet animals, and captive non-companion animal carnivores and omnivores
A Notice from FDA to Growers, Food Manufacturers, Food Warehouse Managers, and Transporters of Food Products on Decontamination of Transport Vehicles [27]	Provides information and references that can be used for the decontamination of food transport vehicles that have been flooded or otherwise impacted by hurricanes, before being placed back in service to transport or store food
Grade A Pasteurized Milk Ordinance, Appendix B—Milk Sampling, Hauling and Transportation [28]	Sets forth training requirements, evaluation criteria, and standards to be met by bulk milk haulers and milk transporters
Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables [29]	Recommends practices for transporting fresh-cut produce under conditions that will protect the food against physical, chemical, and microbiological contamination

Adapted from [9].

1998, 2003, and 2008 [10]. During the study, FDA personnel visited approximately 850 food service operations and retail food establishments to observe and document practices and behaviors related to operational risk factors commonly associated with foodborne illness outbreaks. The study covered nine facility types in three categories, including: 1) institutional food service (hospitals, nursing homes, elementary schools [K-5]); 2) restaurants (fast food restaurants, full service restaurants); and 3) retail food stores (deli departments/stores, meat and poultry markets/departments, seafood markets/departments, produce markets/departments). When selecting establishments to participate in the study, an effort was made to include a sampling of establishments throughout all regions of the USA. Operations that handled only prepackaged food items or conducted low-risk food preparation activities were excluded from the study.

The FDA study tracked the retail food and food service industry's efforts to reduce the occurrence of employee behaviors and practices related to five key CDC foodborne illness risk factors: obtaining food from unsafe sources, poor personal hygiene, inadequate cooking, improper holding of food (time and temperature control), and contaminated food surfaces and equipment. For each of the nine different facility types, FDA specialists determined whether the observations made of the employee food safety practices or behaviors related to the 42 individual data items within the risk factors were IN compliance, Out of Compliance, Not Observed, or Not Applicable. When analyzing the data, the FDA used three levels of analysis: 1) Out of Compliance percentage for each individual data item; 2) Out of Compliance percentage for each foodborne illness risk factor; and 3) Overall IN compliance percentage for all 42 data items.

Overall compliance improved over the three data collection periods (1998, 2003, and 2008) in all nine of the establishment types studied [10]. Improvements in compliance were statistically significant in elementary schools, fast food restaurants, full service restaurants, meat and poultry markets and departments, and produce markets and departments. Improvements in compliance, although not statistically significant, were also seen in hospitals, nursing homes, deli departments/stores, and seafood departments/stores. A key finding of this study highlighted the need for continued improvements with regard to three of the five CDC risk factors: poor personal hygiene, improper holding of food, and contaminated food surfaces and equipment.

For the "poor personal hygiene" risk factor, a statistically significant improvement was observed in seven of the nine facility types [10]. Despite that improvement, the "IN compliance" percentages for this risk factor remained low in 2008 in some facility types, including hospitals (83%), nursing homes (84%), elementary schools (85%), fast food restaurants (76%), full service restaurants (59%), delis (80%), meat and poultry markets/departments (93%), seafood markets/departments (91%), and produce markets/departments (85%). In facility types that had relatively low "IN compliance" percentages for the poor personal hygiene risk factor, the specific data item most frequently low was proper and adequate hand washing. The

"IN compliance" percentages for proper and adequate hand washing by facility type were hospitals (64%), nursing homes (66%), elementary schools (72%), fast food restaurants (61%), full service restaurants (24%), delis (48%), meat and poultry markets/departments (82%), seafood markets/departments (78%), and produce markets/departments (75%).

Relative to the "improper holding/time and temperature" risk factor, a statistically significant improvement in compliance was observed in five of the nine facility types [10]. However, the "IN compliance" percentages for this risk factor remained low in 2008 for some facility types, including hospitals (64%), nursing homes (71%), elementary schools (73%), fast food restaurants (62%), full service restaurants (45%), delis (49%), meat and poultry markets/departments (80%), seafood markets/departments (68%), and produce markets/departments (65%).

For the "contaminated equipment/protection from food contamination" risk factor, a statistically significant improvement in compliance was observed in one of the nine facility types: full service restaurants [10]. The "IN compliance" percentages for this risk factor were as follows: hospitals (82%), nursing homes (83%), elementary school (85%), fast food restaurants (83%), full service restaurants (65%), delis (81%), meat and poultry markets/departments (83%), seafood markets/departments (86%), and produce markets/departments (84%).

This FDA study also highlighted the importance of food safety knowledge by validating that the presence of a certified food protection manager (CFPM) to oversee effective food safety practices had a positive impact on food safety behavior and risk factor compliance. A correlation between improved control of certain risk factors and the presence of a CFPM was found in every facility type, except for nursing homes [10]. The *FDA Food Code* defines a CFPM as one who "demonstrates knowledge of foodborne disease prevention, application of the Hazard Analysis and Critical Control Point (HACCP) principles, and is familiar with the requirements of the *FDA Food Code*" [5]. A CFPM demonstrates knowledge by passing an approved exam that is accredited by the American National Standards Institute (ANSI). Current approved testing programs include: The National Restaurant Association Educational Foundation's ServSafe program (http://www.servsafe.com/), the National Registry of Food Safety Professionals (http://www.nrfsp.com/), and Prometric (http://www.prometric.com/foodsafety/default.htm). Many states and localities now require a CFPM and many sectors of the retail and food service industries employ them voluntarily as a matter of good practice.

Along with the 10-year study, the FDA also has initiated the Retail Food Safety Initiative. The new initiative is part of the agency's overall prevention-based, farm-to-table food safety strategy to reduce foodborne illness. The new initiative contains four action areas that are to: 1) make the presence of certified food protection managers common practice; 2) strengthen active managerial control at retail and ensure better compliance; 3) encourage widespread, uniform, and complete adoption of the *FDA Food Code*; and 4) create an enhanced local regulatory environment for retail food operations [10,11].

The Conference for Food Protection

The Conference for Food Protection (CFP) is a non-profit organization that is structured to provide thoughtful and broadly representative guidance and recommendations to food safety regulatory programs [12]. The CFP brings together representatives from the food industry, government, academia, and consumer organizations to identify and address emerging problems of food safety and to formulate recommendations for their elimination and control. The organization seeks to balance the interests of regulatory and industry stakeholders while providing an open forum for the consideration of ideas from any source. The group meets at least biennially to provide this forum. Though the CFP has no formal regulatory authority, it is a powerful public voice that profoundly influences model laws and regulations among many food-related government agencies and minimizes disparate interpretations and implementation. One of the key outcomes of the CFP process is input into the development, modification, and updating of the FDA's published *FDA Food Code.*

The CFP promotes food safety and consumer protection by the following [12]:

- Identifying and addressing problems in the production, processing, packaging, distribution, sale, and service of foods, with primary focus on the food protection programs governing the food service, retail food store, and food vending segments of the food industry;
- Adopting sound, uniform procedures which will be accepted by food regulatory agencies and industry;
- Promoting mutual respect and trust by establishing a working liaison among governmental agencies, industry, academic institutions, professional associations, and consumer groups concerned with food safety;
- Promoting uniformity among states, US territories, and the District of Columbia; and
- Utilizing the following as the primary channels for dissemination of information:
  - The US Department of Agriculture/Food Safety and Inspection Service in matters under its purview, such as food production, meat and poultry processing, and consumer information; and,
  - The US Public Health Service/Food and Drug Administration in matters under its purview, such as food processing and assistance to food regulatory agencies based on the model food codes and related documents.

The *FDA Food Code*

The FDA publishes the *FDA Food Code*, a model code that assists food control jurisdictions at all levels of government by providing them with a scientifically sound technical and legal basis for regulating the retail, food service, and vending segments of the food industry. The *FDA Food Code,* which establishes practical, science-based guidance and enforceable provisions for mitigating risk factors known to cause foodborne illness, is a model code and reference document for state, city, country, and tribal agencies that regulate restaurants, retail food stores, vending operations, and food service operations in institutions such as schools, hospitals, nursing homes, and child

care centers [5]. Local, state, tribal, and federal regulators use the *FDA Food Code* as a model to develop or update their own food safety rules and to be consistent with national food safety regulatory policy.

Between 1993 and 2001, the *FDA Food Code* was issued in its current format, every 2 years. With the support of the CFP, the FDA decided to move to a 4-year interval between complete *FDA Food Code* revisions. The 2009 *Food Code* is the first full edition to be published since the 2005 edition. At the midpoint between new editions, an *FDA Food Code* Supplement that updates, modifies, or clarifies certain provisions in the *Code* is made available. *FDA Food Code* adoption and implementation is important for achieving uniform national food safety standards and for enhancing the efficiency and effectiveness of our nation's food safety system. As of January 2009, 49 of 50 states and 3 of 6 territories of the United States report having retail codes patterned after prior editions of the *FDA Food Code* [5]. Many federal agencies and tribal governments have also adopted the *FDA Food Code.*

The *FDA Food Code* supports many key national food safety efforts. It supports the Food Safety Working Group created by the Obama administration to modernize statutes that require effective sanitation and preventive controls in food establishments. Implementation of the *FDA Food Code* also supports many of the food safety objectives of *Healthy People 2020*, the comprehensive, nationwide set of health promotion and disease prevention objectives designed to serve as a 10-year strategy for improving health in the United States. *Healthy People 2020* objectives include reducing infections caused by foodborne pathogens, reducing outbreaks of foodborne illness, and improving food employee behaviors and food preparation practices that directly relate to foodborne illness in retail food establishments.

Food safety management programs for transportation, retail, and food service

In a HACCP food safety system, the focus on foods during transportation and at retail and food service is on their safety and how they are handled during transportation, storage, preparation, display, and service [13,14]. A sanitary environment is important for safe food production, however food can still be contaminated by employees if proper food handling techniques are not used, good personal hygiene is not practiced, cross-contamination controls are not in place, and/or food temperatures are not controlled. Whether during transportation or in restaurants, food establishments, institutions, health care facilities, or other food service operations, the primary goal is always the same—production of safe food.

When correctly designed and implemented, the HACCP system helps food transporters and food managers to identify and control potential problems before they happen. HACCP is the preferred approach to food safety because it provides the most effective and efficient way to ensure food products are safe. When a potential problem is identified, the food transport professional or food establishment manager can initiate procedures to reduce or eliminate the risk of foodborne illness and monitor actions to make sure the procedures are being followed.

For retail and food service, HACCP systems control time and temperature and other specific risk factors that are known to contribute to foodborne disease outbreaks. Retail and food service HACCP plans focus on foods (or ingredients) that are called "Potentially Hazardous Food—Time/Temperature Control for Safety Food," or PHF (TCS) foods. PHF (TCS) foods are defined in the *FDA Food Code* as "a food that requires time/temperature control for safety to limit pathogenic microorganism growth or toxin formation" [5].

The approach to HACCP is a little different for this segment of the industry compared with food manufacturing. In food manufacturing, development of HACCP plans is "process specific" *and* "product specific." In this way, HACCP plans can be developed for each product that is made. However, because of the complexity and number of products prepared and sold in many retail establishments and food service operations, HACCP plans are often more generic, focusing on similar processing steps from receiving to service/display in general (Figure 34.1).

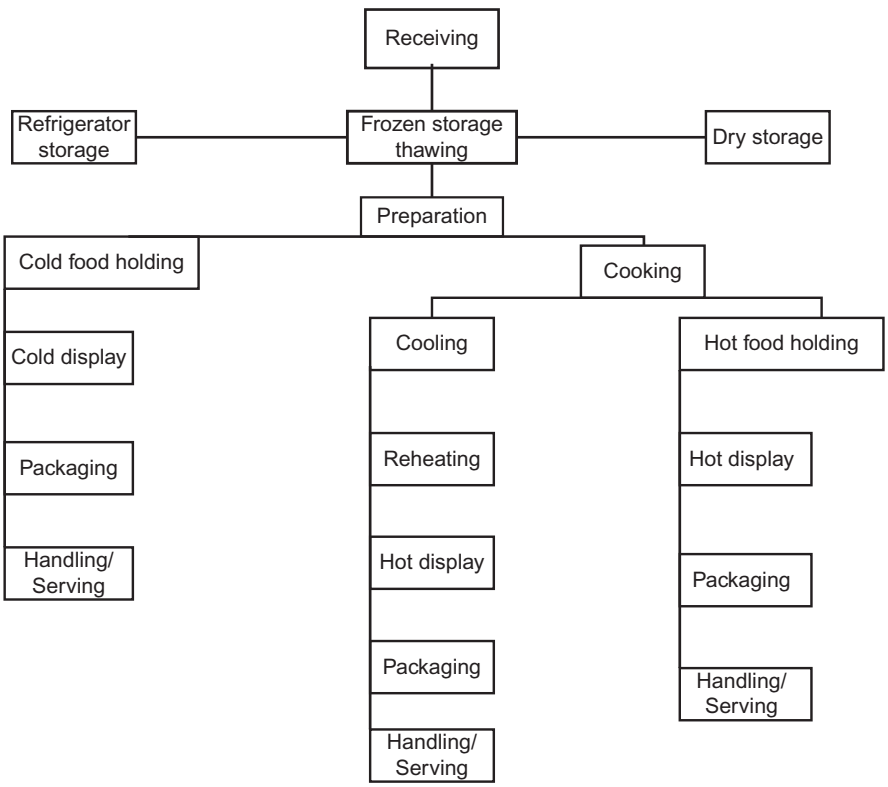

FIGURE 34.1

Common steps in the flow of food for retail and food service operations (used with permission of the Food Marketing Institute [14]).

HACCP plans for retail and food service that are developed for PHF (TCS) foods often are "process specific" for nearly all steps in the flow of food, with the exception of cooking [14]. The cooking step is "product specific" and different time/temperature parameters are required to make different products safe. Recommendations for time and temperature control of PHF (TCS) foods have been provided in the *FDA Food Code* in an effort to reduce the risk of foodborne illness within these establishments (Table 34.2) [10].

Good manufacturing practices (GMPs)

HACCP is the most recognized food safety management system in the world, but it is not a standalone system. HACCP requires that other effective food safety and food quality "prerequisite" programs be in place to complement the HACCP system. In food manufacturing and transportation industries, good manufacturing practices (GMPs) focus on minimum sanitary and processing requirements necessary to assure the production of safe and wholesome food products. GMPs include provisions for food handling related to personnel, building and facilities, equipment and utensils, and production and process controls. GMPs help to control contamination by implementing good personal hygiene and effective cleaning and sanitizing procedures [15].

Good retail practices (GRPs)

While HACCP programs for retail and food service effectively control two of the identified CDC risk factors that involve time and temperature control (inadequate cooking and improper holding of food), they do not address the other risk factors (food from unsafe sources, poor personal hygiene, and contaminated food surfaces and equipment). These risk factors are best managed in retail and food service operations by using good retail practices (GRPs). GRPs are the key prerequisites to instituting a HACCP plan or risk control plan for retail food establishments. GRPs are preventive measures that include practices and procedures which effectively control the introduction of pathogens, chemicals, and physical objects into food. GRPs may include programs such as:

- Educational and training food safety programs;
- Facility design criteria;
- Vendor certification programs and buyer specifications for approved suppliers;
- First In, First Out (FIFO) rotation procedures for ingredients and prepared foods;
- Personal hygiene and hand washing procedures;
- Allergen management programs;
- Cleaning and sanitation programs;
- Proper equipment and maintenance programs; and
- A commitment from management to facilitate the HACCP and total food safety management system.

Table 34.2 Time and Temperature Control for PHF (TCS) Foods for Food service Operations and Retail Food Establishments [10]

Step in the Flow of Food	Temperature/Time Control
Receiving frozen foods	Temperature where food is solidly frozen (recommended below 0 °F [−18 °C])
Freezing frozen fish—parasite destruction	−4 °F (−20 °C) or below for at least 7 days, or −31 °F (−35 °C) until solid and stored at −31 °F (−35 °C) or below for a minimum of 15 hours, *or* −31 °F (−35 °C) until solid and stored at −4 °F (−20 °C) or below for a minimum of 24 hours
Receiving refrigerated foods Receiving whole shell eggs	At 41 °F (5 °C) or below At 45 °F (7 °C) or below
Thawing Under cold storage In cool water	At 41 °F (5 °C) or below Water temperature not to exceed 70 °F (21 °C) Product temperature not to exceed 41 °F (5 °C) for raw foods for less than 4 hours Product temperature not to exceed 41 °F (5 °C) for ready-to-eat foods
Cooking Meat roast (rare)	At 130 °F (54.4 °C) or above for 112 minutes At 131 °F (55.0 °C) or above for 89 minutes At 133 °F (56.1 °C) or above for 56 minutes At 135 °F (57.2 °C) or above for 36 minutes At 136 °F (57.9 °C) or above for 28 minutes At 138 °F (58.9 °C) or above for 18 minutes At 140 °F (60.0 °C) or above for 12 minutes At 142 °F (61.1 °C) or above for 8 minutes At 144 °F (62.2 °C) or above for 5 minutes At 145 °F (62.8 °C) or above for 4 minutes At 147 °F (63.9 °C) or above for 134 seconds At 149 °F (65.0 °C) or above for 85 seconds At 151 °F (66.1 °C) or above for 54 seconds At 153 °F (67.2 °C) or above for 34 seconds At 155 °F (68.3 °C) or above for 22 seconds At 157 °F (69.4 °C) or above for 14 seconds At 158 °F (70.0 °C) or above for 0 seconds
Raw eggs	At 145 °F (63 °C) or above for 3 minutes At 150 °F (66 °C) or above for 1 minute At 158 °F (70 °C) or above for 1 second
Meat and pork (other than roast), fish	At 145 °F (63 °C) or above for 15 seconds
Ground meat, mechanically tenderized meat, ground pork, ground game animals	At 155 °F (68 °C) or above for 15 seconds
Meat roast (medium), pork roast, ham	At 145 °F (63 °C) or above for 4 minutes At 165 °F (74 °C) or above for 1 second
Poultry, ground poultry, stuffed meats, and stuffed food products Microwave cooking for raw animal foods	At 165 °F (74 °C) or above
Hot food holding	Held at 135 °F (57 °C) or above
Cooling	Cooled from 135 °F (57.2 °C) to 70 °F (21 °C) or above within 2 hours, *and* cooled from 135 °F (57.2 °C) to 41 °F (5 °C) or above within 6 hours
Cold food holding	Held at 41 °F (5 °C) or below
Reheating	Heated to 165 °F (74 °C) or above within 2 hours; heated to 135 °F (57.2 °C) or above within 2 hours for ready-to-eat packaged foods

An example of a GRP for receiving raw sushi-grade fish as developed by the Food Marketing Institute is shown in Figure 34.2.

Risk-based inspections

Both the regulatory community and the food industry advocate risk-based inspections of retail and food service establishments whereby the frequency of inspections is based

GRP Example: Receiving and Storage of Raw Sushi-grade Fish

1. Receiving Raw Sushi-grade Fish

All ingredients should come from approved sources. Seafood products identified as a parasite hazard in the *Seafood Hazard Guide* should be obtained from a supplier who has frozen the product throughout using one of the methods recognized in the *FDA Food Code* and identified below. If the species is identified as a histamine producer, documentation from the supplier should show temperature control from harvest through delivery was sufficient to prevent any state of decomposition.

General Receiving Instructions:

- Check the incoming product for date code, physical condition of packaging, physical defects, insect infestation, and food quality.
- Check the temperature to assure the product is frozen solid or has remained refrigerated at 41 °F (5 °C) or below.
- Place ready-to-eat foods in the cooler immediately.
- Place frozen product delivery into the freezer immediately.
- Rotate delivery of the product to facilitate the first-in, first-out (FIFO) method of stock rotation.
- Obtain a letter of guarantee from the supplier stating all sushi-grade fish has been frozen at −4 °F (−20 °C) for at least 24 hours before it is released for distribution.
- Obtain a letter from the supplier verifying that, based on microbiological testing, the fish is high sushi-grade.
- Reject products that do not meet the above criteria.

2. Storage Guidelines for Raw Sushi-grade Fish

To maintain product quality once the retailer has accepted shipment:

- Place product in the appropriate storage unit.
- Equip refrigeration unit with a calibrated thermometer easily accessible for viewing.
- Use recording devices and alarm systems.
- Avoid cross contamination.
- Store product in a specific, predesignated area in the refrigeration unit, away from other raw and cooked foods.
- Keep product containers 6 inches above the floor, away from walls and ceilings, and preferably on non-wooden supports, shelving or surfaces.
- Use an inventory system to make sure the first product in is the first product out (FIFO)
- Check the temperature of the refrigerator and freezer units at least twice daily to assure they are operating at 41 °F (5 °C) and 0 °F (−18 °C) or below respectively.
- Notify store manager or person in charge immediately if improper temperatures are detected at times other than the unit's defrost times; remove food from defective unit; and place it in one that works properly.
- Store raw foods below or away from ready-to-eat foods.

FIGURE 34.2

Good retail practice (GRP) example: receiving and storage of raw sushi-grade fish (used with permission of the Food Marketing Institute [24]).

on such factors as the sanitation history of the establishment, the number and type of meals prepared and sold, the amount of food preparation and handling conducted on-site, and the type of consumers served. Whether inspections are conducted by regulatory officials or internally by representatives of the food industry, they should be proactive and use interventions outlined in the *FDA Food Code*. Five important interventions have been described in the *FDA Food Code* that encompass a wide range of control measures specifically designed to protect consumer health. These include demonstration of food safety and food handling knowledge, implementation of employee health policies, hands as a vehicle of contamination, time/temperature relationships, and consumer advisory. These five interventions are helpful when assessing strengths and weaknesses of the food safety management system that is in place [16].

Global food safety initiative

The global food safety initiative (GFSI) is a global food network that is coordinated by The Consumer Goods Forum to foster continuous improvement in food safety management systems that will ensure confidence in the delivery of safe food to consumers worldwide [17]. The goals of the GFSI are convergence between food safety standards through maintaining a benchmarking process for food safety management schemes; improvements in cost-efficiency throughout the food supply chain through the common acceptance of GFSI recognized standards by retailers around the world; and provision of a unique international stakeholder platform for networking, knowledge exchange, and sharing of best food safety practices and information [18].

The tool used to do the benchmarking is called the GFSI Guidance Document. The Guidance Document sets out the key elements for production of food as requirements for food safety management schemes and provides a framework in which food safety management schemes can be benchmarked. It also sets out the requirements for the delivery of conforming schemes and contains guidance on the operation of certification processes. The GFSI Guidance Document is not itself a food safety standard and the GFSI is not involved in certification or accreditation activities. Rather, the Guidance Document is a tool which can be used to determine equivalency between food safety management systems. The most recent version of the Guidance Document can be found at: http://www.mygfsi.com/gfsifiles/Guidance_Document_Sixth_Edition_Version_6.1.pdf.

The GFSI program also provides the framework for a system of independent certification to validate that a supplier's food safety and quality management system complies with international and domestic food safety regulations. Unlike other audit programs, Accredited Third Party Certification utilizes a system of checks and balances that strengthen the audit system and provide confidence that products produced by certified manufacturers are performing at the highest possible levels. GFSI began with the goal, "once certified, accepted everywhere." This group of over 650 global retailers and manufacturers benchmark potential certification schemes to ensure compliance with globally recognized best practices and harmonization under the GFSI banner. This enables suppliers to assure their customers that food has been produced, processed, prepared, and handled according to the

highest possible standards, at all levels of the supply chain, including transportation, retail, and food service.

Among others, the following food safety certifications are currently recognized by the GFSI:

1) The Safe Quality Food Institute (a division of the Food Marketing Institute);
2) The British Retail Consortium (the UK's leading retail trade association); and
3) The International Food Standards (an accredited Certification Body for IFS).

These are the primary standards benchmarked, and several more are currently being reviewed and are under consideration for approval by the GFSI.

FDA Food Modernization Act

In 2011, a new federal statute called the FDA Food Safety Modernization Act (FSMA) was promulgated [19]. The FSMA is divided into four areas: prevention of food safety hazards, detection of and response to food safety problems, improving the safety of imported foods, and miscellaneous provisions. The new law enables the FDA to focus more on preventing food safety problems rather than reacting to problems after they occur. The FSMA provides greater authorization for the FDA and the Secretary of Health and Human Services (HHS) to increase inspections of many domestic food facilities, enhance detection of foodborne illness outbreaks, and order recalls of contaminated food products. The new law requires most food companies to write and implement new safety protocols to control or eliminate potential foodborne hazards. More regulatory oversight of imported food products is also included in the law and more provisions are in place to deny entry of food products into the USA when there is reason to believe they are adulterated or misbranded. The FDA Food Safety Modernization Act is the most significant update of US food safety laws since adoption of the US Federal Food, Drug, and Cosmetic Act in 1938, and it establishes several new regulatory requirements that have a direct impact on transportation and the retail and food service industries, including registration, HACCP, and record-keeping requirements [19].

Education, training, and food safety culture

There are a wide variety of other educational resources that are helpful for retail and food service operators and food employees (Table 34.3). Education and training activities are core to protection of the food supply for the entire food system—from farm to fork. Since food employees in retail and food service operations are often the last ones to touch and handle food, proper education is critical. Most education and training programs have focused on teaching important food safety information that correlates with the Certified Food Protection Manager credential. The National Registry for Food Safety Professionals [20] has identified core areas of knowledge that are important to reduce risk of foodborne illness in retail and food service operations,

Table 34.3 Educational and Training Food Safety Resources for Retail and Food service

Resource	Description
SafeMark Guide to Food Safety (http://www.fmi.org/foodsafety/)	Textbook, with training curriculum, used to prepare candidates for the Certified Food Protection Manager's Exam—Retail Food Store focused
SafeMark Essentials of Food Safety and Sanitation—Food Safety Fundamentals (http://www.fmi.org/foodsafety/)	Textbook,with training curriculum, used to prepare candidates for the Certified Food Protection Manager's Exam—Restaurant focused
ServSafe Coursebook (http://www.servsafe.com/FoodSafety/)	Textbook, with training curriculum, used to prepare candidates for the Certified Food Protection Manager's Exam—Restaurant focused
Food Safety Culture: Creating a Behavior-Based Food Safety Management System (http://www.springer.com/food+science/book/978-0-387-72866-7)	Book that describes importance of behavior-based food management programs
FDA Food Protection website (http://www.fda.gov/Food/FoodSafety/RetailFoodProtection/default.htm)	Website that provides regulatory-based information about retail food safety
Retail Food Safety Consortium website (www.retailfoodsafety.org)	Website that provides national information about retail food safety
Food Safety Icons (http://www.foodprotection.org/resources/food-safety-icons/icons.php)	Food safety messages provided by the International Association of Food Protection
Partnership for Food Safety Education (http://www.fightbac.org/)	Website that provides educational materials for consumer-based food safety

improve the "food safety culture," and focus on human behavior. Achieving food safety success requires going beyond traditional training methods, testing, and inspectional approaches to managing food safety risks. It requires a better understanding of organizational culture and the human dimensions of food safety. To improve the food safety performance of retail and food service operations, an organization must work to change behavior and change the way people do things [21].

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CHAPTER

HACCP and Other Regulatory Approaches to Prevention of Foodborne Diseases

35

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Introduction

Food safety law in the United States is an evolutionary product. Each time a food safety concern captured congressional attention, Congress enacted a new requirement. Rather than being integrated, typically each new requirement was simply pasted onto the existing statute. As a consequence, food safety law is not a single standard but a conglomeration of various requirements [1].

For most of the twentieth century, the law largely reactive. If a poisonous or deleterious substance was found at a level that was or might be injurious to health, the food could be deemed adulterated and condemned. This regulatory scheme was preventive only to the extent that the risk of government action created a prophylactic incentive to avoid adulteration.

The law was (and remains) preventive also to the extent that it is precautionary regarding insanitary conditions where a food *might* become contaminated with pathogens. Section 402 of the Food, Drug, and Cosmetic Act (FDC Act) states in part:

> *A food shall be deemed to be adulterated . . . 4) if it has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health; or (5) if it is, in whole or in part, the product of a diseased animal or of an animal which has died otherwise than by slaughter."*

Note that these adulteration provisions only indirectly relate to the safety of the food. For a violation to be established, the Food and Drug Administration (FDA) need not prove that the food in fact will cause foodborne illness.

A science-based, systems approach to preventive regulation, however, was slow in coming [2]. From development of HACCP—Hazard Analysis and Critical Control Point—in the 1950s, more than half a century elapsed before comprehensive,

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science-based, preventive regulation applied across the food supply. This chapter summarizes implementation of science-based risk control systems into food regulation in the United States, beginning with HACCP and finishing with enactment of the Food Safety Modernization Act in 2011.

Hazard Analysis and Critical Control Point (HACCP)

Hazard Analysis and Critical Control Point (HACCP) is a systematic approach to identifying, evaluating, and controlling food safety hazards. HACCP was developed in the late 1950s and early 1960s by the Pillsbury Company with the National Aeronautics and Space Administration (NASA), the Natick Laboratories of the US Army, and the US Air Force Space Laboratory Project Group. NASA wanted food for space flights that was 100 percent assured of safety from pathogens, toxins, and other hazards because the consequences of a foodborne illness in space would be catastrophic.

HACCP grew from recognition that end product testing alone is incapable of assuring food safety because it is reactive rather than preventive. In HACCP, the inherent risks in ingredients, process, and the final food are analyzed. Steps necessary to control the identified risks are established. Those control steps are then monitored and verified.

The system has undergone considerable study, refinement, and testing over the years since introduction. HACCP was first described in detail to a larger audience at the Conference for Food Protection in 1971. In 1989, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF)[1] issued their definition of the seven HACCP principles to be considered when developing an HACCP plan. The NACMCF system was revised in 1992 to add a new hazard analysis procedure. Codex Alimentarius adopted an HACCP system similar to NACMCF but somewhat simplified and with slightly different terms. NACMCF adopted a revised HACCP system in 1997, which was simplified like the Codex version, and it addresses prerequisite programs. HACCP is now widely accepted in the United States and internationally as an effective system of food safety control (Table 35.1).

Pre-HACCP implementation of systems control

Even before we had adoption of anything called HACCP, there was the application of systematic, risk-based controls applied in certain food processing segments.

[1]The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) is an advisory committee chartered under the US Department of Agriculture and comprised of participants from the USDA Food Safety and Inspection Service, the Food and Drug Administration, the Centers for Disease Control and Prevention, the National Marine Fisheries Service, the Office of the Army Surgeon General, academia, and industry and state employees. NACMCF provides guidance and recommendations to the Secretary of Agriculture and the Secretary of Health and Human Services regarding the microbiological safety of foods.

Table 35.1 The Seven HACCP Principles

1. Hazard and risk assessment.
2. Determine the critical control points (CCPs) to control the identified hazards.
3. Establish critical limits for the preventive measures.
4. Establish procedures to monitor the critical control points (CCPs).
5. Establish corrective actions to be taken when monitoring shows that a critical limit has been exceeded (or other deviation occurs in CCP monitoring).
6. Establish effective record-keeping systems that document the HACCP system is working correctly.
7. Establish procedures for verification that the HACCP system is working correctly.

FDA [3].

Acidified and low-acid canning process control provides a good example because the severity of the risk of botulism led to the development of critical control points, critical limits, and systemic risk control. After a number of botulism food poisonings, the US Food and Drug Administration (FDA) proposed the federal low-acid canning regulations in 1974. Although not called HACCP, the FDA referred to the HACCP principles in the rule promulgations, and the rule essentially mandated HACCP for acidified and low-acid canning. The rules nearly eliminated the incidence of botulism associated with canned food.

HACCP implementation

HACCP implementation for seafood

In 1995, FDA established HACCP requirements for the seafood industry that require processors of fish and fishery products to develop and implement HACCP systems for their operations.

In addition to the desire to create a more effective and efficient system for ensuring the safety of seafood, there were a number of other factors why seafood was selected by the agency for the application of HACCP preventive controls. The seafood industry had already undertaken many measures, and leaders in the industry requested mandatory, HACCP-type inspection system for their products.

The agency also recognized that seafood safety presents special challenges because there are hundreds of edible species around the world. The resulting range of hazards is wide and includes bacteria and viruses, toxic chemicals, natural toxins, and parasites. In its preamble to the final rule, FDA noted:

> *Because of seafood's unique characteristics (e.g., the fact that it is predominantly wild caught and presents a wide range of possible hazards), the FDA began to question whether the current Federal regulatory system, which was developed for the general food supply, is best suited for the seafood industry. Seafood processors are subject to periodic, unannounced, mandatory inspection*

by the FDA. These inspections provide the agency with a "snapshot" of conditions at a facility at the moment of inspection, but assumptions must be made about conditions before and after that inspection. Concern about the reliability of these assumptions over the intervals between inspections creates questions about the adequacy of the system.

Inspections today verify the industry's knowledge of hazards and controls largely by inference. Whether a company produces products that are adulterated, or whether conditions in its plant are consistent with current good manufacturing practice (CGMP) are measures of how well the company understands what is necessary to produce a safe and wholesome product. This system places a burden on the government to find a problem and to prove that it exists, rather than on the firm to establish for itself, for the regulator, and for consumers that it has adequate controls in place to ensure safety.

Given the nature and frequency of the current inspection system for seafood, it has failed to produce a situation in which the public has full confidence in the safety and wholesomeness of these products. There has been a similar failure with respect to imports.

A significant aspect of the FDA's rollout of seafood HACCP was the agency's recognition of the importance of education and training. Among the agency's efforts was the publication of *Fish and Fishery Products Hazards and Controls Guide*, which listed the likely food safety hazards associated with many species of seafood and many processing methods. The guide also made recommendations on ways to control those hazards. The FDA also invested in a nationwide train-the-trainer curriculum.

HACCP implementation for raw juice

After a number of outbreaks of various illnesses associated with juice products, including some directly affecting children, in 1996 there was a high-profile outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized apple juice which caused at least 66 cases of illness, with 14 cases of HUS, and the death of one child. This event prompted the FDA's examination of the regulation of juice. Mandatory pasteurization was considered, but the FDA decided that would eliminate the incentive to develop innovative methods to accomplish the same purpose. In addition, pasteurization only controls microbial hazards. HACCP systems can control all food hazards that are reasonably likely to occur.

In 1998, the FDA proposed HACCP rules for the processing and importing of juice. The final rule had a phased-in effective date through January 20, 2004, for very small businesses.

Around this time, the FDA considered developing regulations that would establish HACCP as the food safety standard in other areas of the food industry, including both domestic and imported food products. However, no other specific proposals were offered.

HACCP implementation for meat and poultry

In 1989, the US Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) announced plans to implement HACCP for the country's meat and poultry inspection program [4]. In 1993, the Jack in the Box restaurant *E. coli* O157:H7 outbreak, which sickened over 700 people and killed others, added impetus to the FSIS development of HACCP requirements for meat and poultry. In 1996, The USDA FSIS issued proosed rules to establish HACCP for meat and poultry processing plants (codified at 9 C.F.R. § 417). Most of these establishments were required to start using HACCP by January 1999. Implementation was complete on January 25, 2000, when very small plants had to comply.

FSIS HACCP involved significant adjustment and change in the organization of the FSIS, changing roles for inspectors, and changing responsibilities for the regulated industry. Unlike other adoptions of HACCP up until this time, the FSIS's proposal was more conceptual than product-specific. HACCP systems had to be developed by each processor. Written HACCP plans had to be developed for every product and process at each plant. Operators had to tailor the plans to their individual products, processing, and distribution conditions.

The FSIS HACCP regulations provided flexibility for the industry to develop and implement innovative measures for producing safe food, but the rules also imposed clear responsibilities on the manufacturers for ensuring safe food. Prior the FSIS inspections were based on a quality assurance approach. Inspectors found deficiencies and the firms corrected them. This traditional approach was largely reactive rather than preventive.

The role of the FSIS inspectors and compliance officers began to change from examination of products to verifying that a company's written plan and control systems were appropriate and effective at preventing adulteration. Under HACCP, the plants are responsible for finding and documenting deficiencies, and for using this information to strengthen their preventive process controls. Rather than focusing on detecting and documenting deficiencies for enforcement as in the past, the FSIS inspects for verification that the HACCP systems are functioning properly. So long as the firm detects, documents, and corrects deficiencies, there is no need for the FSIS to take enforcement action.

As in all HACCP systems, accurate record-keeping is essential for both the manufacturer and the inspector. Manufacturers need records to verify that their control measures have worked and that their products are safe. Inspectors rely on record reviews to assess whether the food safety systems are functioning properly. This added a new responsibility to facility managers and inspectors of giving priority to ensuring that records are adequate and truthful. The FSIS made enforcement a priority for cases involving incomplete, false, or deceptive records.

Pathogen reduction performance requirements

The USDA's implementation of HACCP included performance standards for finished products that called for reducing levels of specified pathogens. Under the

FSIS regulations, in addition to developing and implementing a written HACCP plan that systematically addresses all significant hazards associated with its products, all slaughter plants were required to regularly test for generic *E. coli* to verify their procedures for preventing and reducing fecal contamination. Raw products from slaughter plants and plants that grind meat and poultry are subject to *Salmonella* testing by the FSIS [5].

An important challenge to these performance standards came from Supreme Beef Processors. In *Supreme Beef Processors*, *Inc. v. USDA*, the Fifth Circuit Court of Appeals held that the FSIS could not enforce the agency's *Salmonella* performance standards because these regulations exceeded the agency's authority under the Federal Meat Inspection Act (FMIA). The FMIA authorizes the USDA to establish sanitation rules for meat and poultry establishments. However, the 1996 FSIS rules required plants to reduce bacteria on raw meat and poultry products and defined an establishment's failure to meet the performance standard three consecutive times as a "failure to maintain sanitary conditions," (FSIS, [5], § 310.25(b)).

The court reached this conclusion, in part, because the FSIS performance standard did not measure food safety or adulteration. The FSIS was trying to use the performance standards as a proxy for pathogen controls in an establishment, but the FSIS conceded that its performance standard regulated more than the presence of pathogen controls. Supreme Beef maintained that the salmonella had entered its facility in beef that it had purchased, and the court ruled that the FMIA did not grant the USDA the authority to regulate an establishment's meat procurement.

HACCP for retail food establishments

Although the FDA has authority over most retail food establishments, largely due to limits on resources, it leaves the regulation of retail establishments to the state and local governments. To foster uniformity and to further its mission, the FDA publishes *The Food Code* [6], a model document and reference for state and local regulatory agencies. Although it is neither federal law nor federal regulation, nearly all localities adopt some version of the model *Food Code* [7].

The Food Code includes a number of provisions for mandatory and voluntary use of HACCP. For instance, juice packaged in retail establishments must be treated under an HACCP plan (*Food Code*, 2009, § 3−404.11). A retail establishment that packages potentially hazardous food using a reduced oxygen packaging must operate under an HACCP plan (*Food Code*, 2009, § 3−502.12). A molluscan shellfish life support system used to store or display shellfish that are offered for human consumption must be operated and maintained in accordance with an HACCP plan (*Food Code*, 2009, § 4−204.110). A variance from the chart of required cooking temperatures can be granted by the regulatory authority based on an approved HACCP plan (*Food Code*, 2009, § 3−401.11). The required contents of an HACCP plan are specified in *Food Code* section 8−201.14.

FSMA and new science-based, preventive controls

Prevention of foodborne illness, not reaction to problems, is now the guiding principle of our food safety law—with the primary responsibility for prevention resting squarely on the shoulders of food producers and processors.

Michael Taylor, FDA's Deputy Commissioner for Foods [8]

The FDA Food Safety Modernization Act (FSMA) for the first time in US history shifts the focus of the US food law from reacting to food safety problems to prevention. Previous HACCP regulations were based on the FDA's interpretation and application of general food safety requirements in the Food, Drug, and Cosmetic Act (FDC Act) [9]. In the FSMA, Congress gave the FDA a legislative mandate to require comprehensive, science-based, preventive controls across the food supply. The FSMA requires that all the FDA-regulated food companies implement hazard analysis and preventive controls unless specifically exempt.

Mandatory risk-based preventive controls

The FSMA amended the FDC Act to add section 418, which requires that food facilities implement a written hazard analysis and risk-based preventive control plan (FSMA § 103 amending the FDC Act by adding § 418 [21 USC 350g]). Although not called HACCP, this is essentially an HACCP system. The single difference is that the FSMA hazard analysis and preventive control plan (HAPCP) is slightly broader because it requires identification and control of hazards generally, not just critical control points (CCPs) (Table 35.2).

The FSMA requires a food establishment to re-analyze the preventive controls whenever a change at the facility creates a reasonable potential for a new hazard or significantly increases the potential of a previously identified hazard. If there are no changes, re-analysis must be conducted not less than every 3 years.

Table 35.2 The Seven HAPCP Requirements (Section 418 Preventive Control Plans)

Under the FSMA the "operator, or agent in charge of a facility" must:

(1) Identify and evaluate known or reasonably foreseeable hazards,
(2) Develop a written analysis of the hazards,
(3) Implement preventive controls for significantly minimizing or preventing the identified hazards (including hazards intentionally introduced, such as terrorism),
(4) Monitor these controls to ensure they are working,
(5) Establish procedures for corrective actions,
(6) Verify the plan is carried out and effective, and
(7) Maintain records for 2 years.

FDC Act § 418.

Critical Control Points (CCPs)

Congress did not explain why it avoided the HACCP terminology in writing the FSMA, but clearly there was a decision to eliminate emphasis on the establishment of CCPs. CCPs identify those points in an HACCP process that must be controlled to ensure the safety of the food. As part of establishing CCPs, critical limits are established in order to document the parameters that must be met at each CCP.

CCPs make sense in a process like low-acid canning of food where there are clear critical limits in cooking temperature and time. On the other hand, critical limits are not so clear in many food establishments. For instance, pathogen reduction in raw meat can increase consumer safety, but many would say that pathogen reduction is not a CCP unless there is a critical limit, and the CCP for raw meat is the final cooking step. Critical limits are also not always clear in retail establishments. For example, proper employee hand washing before handling ready-to-eat food is critical to food safety but establishing a critical control point is difficult because critical limits cannot be nailed down to a clear-cut measurement like time or temperature.

Situations like those described above make for debate among food safety experts as to whether HACCP can be applied to those situations without clear critical limits. When the experts debate, one can imagine the confusion of those trying to implement HACCP in such situations. The concept and application of critical control points can be one of the most difficult to convey and most confusing in employee training for food establishments.

With the FSMA, there was a decision to broaden the application of systematic control of risks. In particular, the FSMA requires establishment of science-based mitigation strategies to prepare and protect the food supply chain against intentional contamination at vulnerable points (FDC Act § 420). This type of risk control plan would not fall within the traditional definition of HACCP.

For all these reasons, the new terminology used by the FSMA is more flexible and better suited for diverse types of establishments.

Regulations and guidance

The FSMA directs the FDA to promulgate rules establishing "science-based minimum standards" for HAPCP compliance. The FDA also must issue a guidance document explaining the hazard analysis and preventive control principles and requirements. The details in these rules and guidance document will be key to understanding the agency's thinking and expectation on what will be needed in a HAPCP system to satisfy the FDA inspectors.

Establishments would be prudent to treat guidance and even draft guidance as being nearly the same as a regulation. Although guidance has no force of law itself, it provides a look at the agency's opinion on what is necessary to comply with the law. That is, an establishment that fails to follow a guidance document cannot be charged with a violation of the guidance—however, they may be charged with a violation of the law based on general language in the statute.

Small food producer exemption

Strong lobbying by small farm holders resulted in exemptions being carved out for small "qualified" food producers (FDC Act § 418(l) [21 USC § 350g(l)]). A "qualified" facility is one that has a limited monetary value of food sales *and* sells its food directly to "qualified end-users." The monetary value of sales must have an average yearly value of less than $500,000. "Qualified end-users" are consumers or restaurants and retail food establishments that sell directly to consumers that are located in the same state as the qualified facility or are located no farther than 275 miles from the qualified facility.

Qualified food producers do not have to meet the hazard analysis and preventive control plan hazard analysis requirements of the FSMA. However, these facilities must "demonstrate that the owner, operator, or agent in charge of the facility has identified potential hazards associated with the food being produced, is implementing preventive controls to address the hazards, and is monitoring the preventive control to ensure that such controls are effective," or, as specified by the FDA, the facility must present documentation showing compliance with state, local, or county food safety laws. If a foodborne illness outbreak is directly linked with a qualified facility, then the FDA may withdraw this exemption.

The section 418 HAPCP requirements only apply to those facilities that must be registered (FDC Act § 415 [21 USC § 350d]). Thus, also excluded from HAPCP are facilities solely engaged in the production of animal feed; sites for the storage of raw agricultural commodities (other than fruits and vegetables) intended for further distribution or processing; and sites for storage of packaged goods not exposed to the environment.

As mentioned above, seafood, juice, and low-acid canning facilities are already subject to HACCP. To avoid duplication, the section 418 HAPCP requirements do not apply to low-acid canned food, juice, and seafood subject to HACCP. In addition, facilities subject to the special produce safety requirements of FDC Act section 419 are also exempt from section 418's HAPCP requirements.

Mandatory produce safety standards

The FDA historically has had little involvement in raw produce safety. Changing that course, the FSMA directs the FDA to work with the USDA to propose "science-based minimum standards for the safe production and harvesting" of fruits and vegetables that are raw agricultural commodities for which the FDA has determined such standards will minimize the risk of "serious adverse health consequences" (FSMA § 105 amending FDC Act § 419 [21 USC § 350h]). The rules must consider naturally occurring hazards, as well as those that may be introduced either unintentionally or intentionally, and must address soil amendments (such as compost), hygiene, packaging, temperature controls, animals in the growing area, and water (*ibid*).

Due to inherent difficulties in regulating raw agricultural commodities, the law requires that any proposed rule "provide sufficient flexibility to be applicable to various types of entities engaged in the production and harvesting of fruits and vegetables" (FDC Act § 419(a)(3)(A) [21 USC § 350h(a)(3)(A)]). In addition, section 419 requires the FDA prioritization of the implementation of regulations based on the known risks accompanying raw agricultural commodities (FDC Act § 419(a)(4) [21 USC § 350h(a)(4)]). Past foodborne illness outbreaks are likely to form the basis of the FDA's prioritization.

The effective date of the final rule is to be delayed for "small businesses" and "very small businesses" (terms to be defined by the FDA by regulation) by 1 year and 2 years, respectively. A state or foreign country from which food is imported may request a variance from the FDA from any of the requirements in a regulation under this section (FDC Act § 419(b)(3)(B) [21 USC § 350h(b)(3)(B)]). The request for a variance must present information demonstrating that the variance does not increase the likelihood that the food for which the variance is requested will be adulterated under section 402 and whether the variance provides the same level of public health protection as the requirements of the regulations adopted under Section 419.

Again, food establishment operations that fall under the FDA guidance documents are well advised to treat guidance and draft guidance for operational purposes as being the same as a law. Although guidance has no force of law itself, an establishment that fails to follow a guidance document can be charged with a violation of the law based on general language in the statute.

The Jensen Farms 2011 *Listeria monocytogenes* outbreak, which infected at least 146 persons in 28 states, with 30 deaths and one miscarriage, provides a dramatic case in point [10]. Auditors ignored violations of the FDA guidance documents because they were not law. However, in response to the outbreak, the FDA inspected the company and issued a warning letter for packing and holding cantaloupe under insanitary conditions in violation of Section 402(a)(4) of the FDC Act [11].

Contaminant-specific, science-based performance standards

The FSMA contemplates an evolving set of science-based performance standards to protect against significant foodborne contaminants. The FSMA section 104 requires the FDA to review and evaluate relevant health data, including toxicological and epidemiologic studies and recommendations of the FDA's Food Advisory Committee, to determine the most significant foodborne contaminants (21 USC § 2201). Based on this review and when appropriate to reduce the risk of serious illness or death, the FDA is required to issue, by regulation, action level, or guidance document, contaminant-specific, science-based performance standards. The FDA is required to coordinate with USDA to avoid duplicative performance standards for the same contaminants, and the review is to be completed at least every 2 years.

Reportable food registry

One regulatory tool for the FDA to track patterns and apply limited resources in a scientific manner is through the Reportable Food Registry (RFR). The RFR is

an electronic portal for food establishments to report to the FDA any occurrences where there is a reasonable probability that an article of food will cause serious adverse health consequences. The RFR was created by the Food and Drug Administration Amendments Act of 2007 (Pub. L. 110-085 effective September 27, 2007), which created a new section 417 in the FDC Act.

In the first year of the RFR, the FDA received 2600 submissions. Of those, the FDA deemed 360 to be non-reportable, 229 were primary industry or regulatory reports, 139 were amended reports, and 1872 were secondary upstream or downstream reports [12]. These data reveal how a small number of recalls can have a far-reaching impact. A single contaminated ingredient can spread through the food supply chain and result in a cascade of recalls. For example, an initial report of salmonella in hydrolyzed vegetable protein led to 1001 reports across at least 11 commodity categories by upstream and downstream facilities. In the end, 177 products were removed from commerce and no illnesses were reported [12].

Michael Taylor, Deputy Commissioner for Foods, FDA, noted:

> *The data in this report represent an important tool for targeting our inspection resources, bringing high risk commodities into focus, and driving positive change in industry practices—all of which will better protect the public health. Several key US industries are already re-evaluating their hazards and preventive controls [12].*

Inspection and compliance

Preventive control standards improve food safety only to the extent that producers and processors comply with them. Therefore, the FSMA increases the FDA oversight for compliance with these requirements. One of the foremost of these compliance tools is expanded records access. The FDA will have authority to access the required written food safety plans and the records firms are required to keep documenting implementation of their plans (FDC Act § 418[g]-[h]). These records are to be kept for at least 2 years, and the records must be made available "promptly" to a duly authorized agent of the FDA upon request (FDC Act § 418[g]-[h]). The FDA also has expanded authority to access records for foods where there is a reasonable belief that the food is adulterated and may cause serious adverse health consequences (FSMA § 101 amending FDC Act § 414[a]). An importer must keep records of importer verification for at least 2 years.

Some points about compliance

Food law in the United States puts the responsibility for food safety clearly on the shoulders of the manufacturer and seller of that food. Ultimately, that is the best reason for implementing a systematic risk control plan. Complying mechanically with government regulations will not bring about the degree of confidence or safety that comes from management's sincere commitment to systematically implementing the highest degree of food safety.

Prerequisites neglect

When first learning HACCP, a common mistake is to overemphasize the critical control points (CCPs) to the detriment of standard operating procedures (SOPs) and other prerequisite programs. HACCP, of course, does not include SOPs and other prerequisites, and including them in the HACCP plan can dilute attention to the critical limits. One of the great strengths of HACCP is that recognition of the CCPs in an operation allows focus on preventive control measures on specific areas rather than doing everything one can think to do to improve sanitation and other conditions with the hope that something will work to prevent problems.

Nonetheless, prerequisites are essential to implement HACCP effectively. Prerequisites include education and training of appropriate personnel, basic sanitation, customer complaint handling, product recall procedures, and preventive maintenance. The FDA and USDA HACCP regulations in 1995 and 1996 added a new element to the traditional HACCP: SSOPs—sanitation standard operating procedures. The existing regulations already required sanitation, but adding the requirement for food establishments to write SSOPs with their HACCP plan by design emphasized how basic sanitation specific to each operation needed to be addressed by the operator and the SSOPs verified as effective in order for the success of the HACCP plan. For example, failure to properly clean could create a bacterial load that contaminates and overwhelms existing controls. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) revised their HACCP system in 1997 to recognize the importance of prerequisite programs.

Moreover, food establishments must heed not only critical items but also other requirements of the applicable statutes and regulations. A customer who breaks a tooth on a foreign object in your product will not be mollified by the knowledge that the foreign material was not a critical control point. Similarly, the inspectors will be unimpressed that no critical limited has been exceeded if your processing plant has gross violations of basic good manufacturing practices regulations.

Preparing a risk control plan

There are many approaches to hazard analysis and risk assessment even within a particular food industry. This is an area where one must have expert knowledge of the hazards, their severity, and their probability in the foods being handled. For example, with raw ingredients, an understanding of the microbiology of the foods being prepared is essential. To assess the severity of a risk may require extensive knowledge of epidemiology. The understanding of hazards associated with the food must then be mated with understanding of the food production, food processing, and preparation methods.

For the full benefits of prevention and control to be realized, HACCP/HAPCP-focused training of those involved in an establishment's food safety, production, processing, and preparation is essential. In fact, training could be considered the missing eighth step of HACCP.

Verification, validation, and monitoring

No matter who develops a risk control plan, it is wise to have an outside party validate that the known hazards have been identified, the correct controls have been implemented, the monitoring and record-keeping are effective, and the plan is effective in reducing health risks to the specified levels. Firm management must be committed and involved in verification (or internal auditing) that the plan is implemented properly and that it is effective. Validation is an initial review of the risk control system to determine effectiveness of design and proper implementation.

One area that benefits from a do-it-yourself attitude is the monitoring. Those involved in the day-to-day operation must monitor the control points. Management must also be actively involved in monitoring to ensure that it is done properly. Supervisors must review logs to see that they have been filled out properly. They must also ensure that monitoring has in fact been done and done properly, and that corrective actions are taken when needed and are effective.

Supervision must also ensure that records are properly maintained. "If it isn't documented, then it didn't happen" is good refrain to remember. Documentation has never been more important for demonstrating compliance to government regulators. This documentation can also be essential in any litigation involving injury from a food safety problem. Some worry that records will simply provide the rope needed to hang them, but if a risk control system is properly implemented, the documentation will more often exonerate. Furthermore, one of the best ways to prevent foodborne illness liability is to prevent incidence of illness, which a properly designed and implemented HACCP plan can do.

Conclusion

Systematic preventive regulation was slow coming to food law, taking more than half a century for Congress to give the FDA a mandate to apply comprehensive, science-based, preventive controls. This new statutory direction will require the FDA to prepare and issue regulations and guidance documents as well as develop a host of reports, plans, and notices. Therefore, full implementation will likely take several years.

Moreover, adequate funding will be necessary to implement the new law. A provision authorizing the FDA to collect fees for facility registration was removed at the last hour to facilitate passage. A number of smaller fee provisions remain, but these involve only small portions of law, such as fees to be paid by third-party auditors to fund the accreditation program and fees for participants in the Voluntary Qualified Importer Program. Without a fee structure to pay for the cost of implementation, the FDA must rely on congressional appropriation, which has generally underfunded or left unfunded new mandates to the agency.

Nonetheless, the trend is clear. Risk-based, preventive control systems are here to stay. Prudence advises keeping abreast of the proposed rules and draft guidance documents as well as final rules and final guidance documents. However, food establishments should also not wait for details from the FDA before implementing risk-based, preventive control systems in their operations. The benefits of a risk

control system like HACCP go far beyond regulatory compliance and reduced risk of regulatory action. HACCP provides a higher degree of assurance of food safety than seen with traditional approaches. Effective implementation can reduce risk of emergencies, consumer complaints, costly litigation, and high rejection costs.

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CHAPTER

The Legal Basis for Food Safety Regulation in the USA and EU

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That, in view of the extensive and increasing adulteration, misbranding, and debasing of food, liquors, and drugs, and in view of the fact that such practices can not be entirely and effectually regulated by State laws, owing to the numerous complications arising from interstate commerce, it becomes, therefore, necessary, that State laws should be supplemented by national law on this subject.
Ref. [1]

Introduction

Food safety laws were adopted very early in our nation's history as a way to protect trade with Europe and promote interstate commerce. There were also elements of consumer protection in the earliest laws, such as proper weights and measures, purity of ingredients, and fair pricing. By the early 1900s, states had adopted a patchwork of laws and there was a determined movement for national legislation. Comprehensive legislation was added in 1906, when Congress adopted two laws that covered meat products and non-meat products separately. That framework remains in place today, but it is being modernized to bring greater alignment to the different programs. Recent changes to the laws focus on adopting legal approaches that can more readily apply modern scientific approaches to food safety.

This chapter will review food law from its earliest adoption in colonial America, and move forward to the Food Safety Modernization Act, which was signed into law in 2011. It also discusses the evolution of laws in Europe, ending with the modern era.

Early food laws in the United States

Massachusetts had the first food safety laws in the American colonies, and the evolution of its laws shows the clear emphasis on both trade and domestic consumption.

Foodborne Infections and Intoxications.

The Massachusetts Meat and Fish Inspection Law of 1641 dealt with meat destined for export to ensure that the colony produced and exported only high-quality food products. The Massachusetts Bread Law of 1646 regulated the quality and price of bread, and required bakers to mark each loaf of bread so that its origin would be traceable. To root out economic deception, the law gave inspectors the authority to enter bakeries and weigh the loaves.

Massachusetts also passed the first comprehensive food adulteration law in 1785, titled "An Act Against Selling Unwholesome Provisions." Violators could be sent to the pillory for selling "any such diseased, corrupted, contagious, or unwholesome provisions, whether for meat or drink." Many other states and territories adopted food safety laws and regulations in the 1800s, including Iowa, Oregon, California, New York, and Virginia [2].

The advent of federal regulation

When most food production occurred locally in the United States, state and local governments were responsible for food safety regulation. But by the late 1800s, the population had increased and shifted to a more urban nature, food was shipped across state lines, and the food supply became more national in scope and distribution. In many areas, people no longer had a local connection with the producers of their food; much of it came from outside the community.

Problems rapidly emerged in a number of sectors. For example, drinking milk in New York City and many other jurisdictions in the mid-1800s could be a risky proposition. When the mortality rate in New York City for children under 5 tripled from 1843 to 1856, bad milk was suspected as a major contributing cause. While it was commonly suspected that water was added to milk sold in the city, the swill milk scandals brought to light a more serious problem. Cows were housed in crowded stables next to distilleries and were fed nothing but the hot alcoholic mash left over from the whiskey-making process. When cows became too sick to stand up, they were hoisted up on slings to be milked. To thicken and cover up the bluish color of this milk, swill herd farmers added flour, starch, chalk, plaster of Paris, and other ingredients. In 1864, New York outlawed swill herds. Massachusetts, Pennsylvania, Illinois, Kentucky, and Indiana also banned this practice [3].

Other scandals captured national attention. During the Spanish–American war in the late 1800s, more US soldiers died from "embalmed beef" than from battle wounds. The first veterinarians to the US army were appointed after this scandal to inspect the meat served to the troops [4]. It was not long before such protection was considered essential for all US consumers.

The United States Department of Agriculture (USDA) was founded in 1862 by President Lincoln, and it was the first home for food safety in the US government. USDA chemists first suggested a general food law for the United States in 1879. From 1887 to 1901, they also compiled and published reports documenting mass adulteration in all sectors of the food supply, including *Bulletin 13, Foods and Food Adulterants* and a less technical version entitled *A Popular Treatise on the Extent*

and Character of Food Adulterations, intended for consumers. This treatise documented adulteration or mislabeling issues in many common foods, and elevated the issue in the public conscience. Notwithstanding, there were many opponents to a federal law who argued that the federal government had no business policing what people ate, drank, or used for medicine.

The distinction between meat and non-meat foods emerged early. There were distinct proposals for meat, which required on-sight government inspection, and non-meat foods, where limited inspection was the norm. This distinction was crystalized when legislation was finally passed in 1906.

Dr. Harvey W. Wiley, who became the chief chemist of the USDA Division of Chemistry (the forerunner of the US Food and Drug Administration) in 1883, is considered to be the father of the Food and Drugs Act of 1906, which focused food regulation on prohibiting the sale of products that were "adulterated or misbranded." Wiley worked on a number of fronts to help gain passage of the 1906 Act, igniting public interest through his many speeches and writings in popular newspapers and magazines. He made consumers aware of the risks posed by adulterated foods and drugs [5].

Another important factor that triggered Congressional action was publication of *The Jungle* by Upton Sinclair [6]. This largely fictionalized investigative report described the filthy conditions and adulteration that was common in Chicago stockyards and meat packing plants. Although Sinclair meant to highlight the poor treatment of workers, the public was more shocked and appalled about the horrifying nature of the meat they were eating. Within weeks of the publication of *The Jungle,* domestic meat sales declined by half. Congress quickly passed the Pure Food and Drugs Act [7] and the Federal Meat Inspection Act [8], both of which President Theodore Roosevelt signed into law on June 30, 1906. These were the first national laws designed to protect American consumers from unsafe foods, rather than to protect American trade.

Meat, poultry, and eggs

The federal meat inspection acts

By the 1880s, most European countries had banned the import of American meat products due to their reputation as unfit to eat [9]. To address this trade issue, the Meat Inspection Act was passed in 1890 with the support of the meat industry to ensure that salted pork and bacon intended for export were safe. The Act was amended in 1891 to cover inspection and certification of all live cattle for export. In addition, the Act required the inspection of all cattle, sheep, and hogs if they were to be sold in interstate commerce. Post-mortem inspections of meat could also be made if deemed necessary. This was the first time Congress authorized the use of the "Inspected and Passed" labeling of meat. However, Congress did not allocate money for the cost of the federal inspection program, weakening its effect [4].

When Congress passed the Federal Meat Inspection Act of 1906, the focus of meat inspection changed from protecting American meat exports to protecting

the American public from meat products that were unfit for human consumption. The Act required mandatory inspection of all cattle, sheep, pigs, goats, and horses before slaughter and mandatory post-mortem inspection of every carcass. It required the continuous presence of USDA inspectors in slaughter and processing operations and established sanitary standards for the industry. Originally, the Act placed the burden of cost for inspection on the packers by mandating an inspection fee for every animal [8]. The meatpacking industry lobbied hard against this, and in the end, Congress appropriated federal funds for meat inspection. By 1907, over 2200 USDA inspectors were deployed to approximately 700 establishments, all at government expense [10]. The same law, with minor changes, is in effect today.

Additional legislation for meat, poultry, and eggs

The Federal Meat Inspection Act did not cover poultry because at that time consumers commonly bought chickens directly from a farmer or a local butcher. During and after World War II, the demand for poultry products greatly increased and the poultry industry became more centralized. The US military purchased poultry only from establishments that met the military's sanitation standards; the USDA inspected these establishments to ensure compliance. Shortly thereafter, the USDA required that poultry processing plants only purchase poultry from establishments that met USDA sanitation requirements. This process laid the groundwork for the passage of the Poultry Products Inspection Act in 1957 [10]. The Act required any poultry products that moved in interstate commerce to be continuously inspected: prior to slaughter, after slaughter, before processing, and, if the poultry was imported, at the point of entry into the United States. The Act imposed sanitation standards similar to the 1906 Federal Meat Inspection Act. The Act was revised in 1962 to include poultry products in intrastate commerce if the state did not have its own inspection program [11].

Congressional investigation of meat inspection programs in the early 1960s revealed that 15% of all commercially slaughtered animals and 25% of all commercially prepared meat products were not subject to inspection because they were intended only for intrastate commerce, and the Meat Inspection Act only covered meat intended for interstate commerce. Furthermore, only 29 states imposed mandatory inspection during slaughter of animals intended for sale as food. In response, Congress enacted the Wholesome Meat Act of 1967 [31] and the Wholesome Poultry Products Act of 1968 [32]. These Acts applied federal standards to state inspection programs to regulate products sold in local commerce, requiring state inspection programs to be "at least equal in rigor to" their federal counterparts.

The Egg Products Inspection Act of 1970 [33] established federal regulatory authority over the inspection of eggs and egg products, mandating continuous egg inspection in plants that produced liquid, frozen, and dried egg products. Egg hatcheries and packers were also under inspection but the inspection was not continuous.

Dairy products and seafood, though considered animal products, are regulated by the FDA with assistance by the states [12].

Non-animal products, dairy, and seafood

In the early 1900s, Wiley sought and received Congressional approval to form a "Poison Squad," a cadre of chemists whose mission was to study additives and preservatives used in food products. They accomplished this by consuming food made with the various chemicals under study, and then noting the effects on their health. These studies included boric acid, sulfurous acid, sulfites, benzoic acid, copper sulfate, saltpeter, and formaldehyde. While none of the Poison Squad succumbed to their job, they did find that many of these chemicals were harmful to human health. This was the start of the federal government's role in approving chemicals used in food production. The work of the Poison Squad captured the public's attention, further in flaming the desire for improved federal oversight of the food industry [13].

The Pure Food and Drugs Act

The Food and Drugs Act of 1906 (also called the Pure Food and Drugs Act) [7] covered food, drink, confectionery, and condiments used by man or other animals, "whether simple, mixed, or compound." The law prohibited the addition of any ingredients that would substitute for the food, conceal damage, pose a health hazard, or constitute a filthy or decomposed substance. It defined for the first time in a federal statute the terms "misbranding" and "adulteration," and gave the federal government the authority to seize adulterated or misbranded foods and punish violators with fines of up to $500 and/or imprisonment of up to 1 year for the first offense. The FDA could sample imported foods for adulteration, misbranding, or health hazards. The Act transformed the Division of Chemistry from a scientific bureau to a regulatory agency.

Although it was a good start and accomplished much, from the beginning the Food and Drugs Act had some very large flaws that made it almost impossible to prove adulteration of a food. For example, without knowing how much strawberry was supposed to be in strawberry jam, the FDA could not prove that a product containing almost no strawberries was not strawberry jam. Furthermore, the Act required the government to prove that alleged offenders of the law *intended* to deceive or poison consumers with their product. When brought to court, defendants pleaded ignorance of the results of their actions. While the law prohibited false labeling, producers did not have to list ingredients in their products [4]. Nevertheless, the Act endured as the major law regulating the food supply until the early 1930s when the movement for reform started anew.

The Food, Drug, and Cosmetic Act (FDCA)

Evidence grew that the 1906 law was outdated; new kinds of products, new methods of manufacturing, and new scientific discoveries all demanded a more modern method of control. Public opinion in the 1930s played a strong role in educating Congress on the need for reform. In 1930, the USDA's Division of Chemistry was renamed the Food and Drug Administration (FDA), later to be transferred out of the

USDA altogether. In 1933, the FDA introduced a bill into the US Senate to replace the 1906 Act. As it did in 1906, the FDA went directly to the public to discuss the need for reform, speaking to women's clubs and civic organizations, and on the radio.

At one point, while preparing for Senate hearings on the bill, the FDA collected hundreds of products (both food and drugs) that had injured or cheated consumers, to emphasize that the 1906 Act did not regulate these products enough to prevent such occurrences. The exhibit was christened the "Chamber of Horrors," leading to the 1936 FDA publication *The American Chamber of Horrors* [14]. This publication recounted some of the little-known behind-the-scenes details of the food industry. For example, when discussing a new method that an FDA scientist invented for analyzing butter for contamination, the agency recounted:

> *Examination of only a few samples by this new method was enough to fill regulatory officials with dismay and incredulity. Butter that looked perfectly clean and wholesome to the naked eye disclosed a history of filth leading all the way back to the farm. Hay; fragments of chicken feathers; maggots; clumps of mold—blue, green, white and black; grasshoppers; straw chaff; beetles; cow, dog, cat and rodent hairs; moths; grass and other vegetable matter; cockroaches; dust; ants; fly legs; broken fly wings; metallic filings; remains of rats, mice and other animals were revealed to the astonished eye—all impregnated with yellow dye from the butter.*
>
> **Ref. [14]**

In 1938, Congress passed the Federal Food, Drug, and Cosmetic Act (FDCA) [15]. It continued the overall intent of the 1906 Act, but broadened the scope of federal regulation and plugged many of the loopholes. The new Act covered all kinds of foods sold across state lines and all substances found in foods, including those added intentionally or unintentionally. It also covered imported and exported foods. The FDCA for the first time:

- Defined adulteration to include: bacteria or chemicals that are potentially harmful; insect parts, rodent hairs, and other unintentional substances; and deceptive degrading of the quality of a product.
- Allowed the FDA to inspect food manufacturing and processing facilities.
- Required ingredients of non-standard foods to be listed on labels.
- Prohibited the sale of food prepared under unsanitary conditions.
- Gave the FDA the authority to monitor animal drugs, feeds, and veterinary devices.
- Authorized mandatory food standards for identity, quality, and fill of container.

This law was considered a model law, providing consumer protections from unsafe food and unfair marketing, for decades.

Dairy

In 1924, the United States Public Health Service (US PHS) developed the Standard Milk Ordinance, now known as the Pasteurized Milk Ordinance (PMO), to assist

states with voluntary milk pasteurization programs. The US PHS and the FDA administer the PMO, which defines practices relating to milk parlor and processing plant design, milking practices, milk handling, sanitation, and standards for the pasteurization of Grade A milk products. Each state regulates milk processing within their own state, but dairy products must meet the regulations stated in the PMO for products that will enter interstate commerce.

Although the FDCA covered dairy products, the FDA deputized a coalition of state and industry officials to recommend standards for the regulation of milk. This coalition is called the National Conference on Interstate Milk Shipments. The Conference meets periodically to set policy for the safety and quality of milk, subject to the FDA's approval. States enforce the standards that are established through this process. While the programs are considered "voluntary" for the industry, companies cannot ship milk across state lines unless they participate in the program, ensuring a high degree of participation. The names of participating companies are placed on the Interstate Milk Shippers List, which certifies that they have an approved program subject to regular inspection and laboratory sampling. Cheese, ice cream, and other non-fluid milk dairy products are regulated by the FDA.

Seafood

The Seafood Inspection Act of 1934 [34] regulated seafood via a voluntary program for seafood inspection. In the early 1930s, the FDA seized increasingly large amounts of decomposed product due to poor fishing practices and poorly supervised packing operations. Under the Seafood Inspection Act, packers of any seafood product could request that an inspector examine the premises, equipment, methods, containers, and materials they used. If the inspection was favorable, they could use that information on their label. The new seafood inspection program almost immediately corrected conditions in the canned seafood industry. Product quality improved and the industry was able to regain consumer confidence in seafood products. In 1943, seafood was added to the FDCA.

Shellfish products are regulated under the Interstate Shellfish Sanitation Conference, a joint federal–state conference similar to the one used for milk. The program is enforced by the states and limits interstate sale of shellfish to the shippers listed by the states.

Pesticides and food additives

In the 1950s, several amendments to the Food, Drug, and Cosmetic Act further increased the FDA's regulatory responsibility. The Food Additives Amendment of 1958 [35] and the Color Additive Amendments of 1960 [36] required manufacturers of new food and color additives to establish the safety of additives before they could be used in food. This requirement for "prior approval" meant that, for the first time, it was illegal for companies to introduce any substance into the food supply

without being assured of its safety, rather than withdrawing a product after reports of injury. The law stipulated that additives in use prior to January 1958 with a substantial history of safe use are Generally Recognized as Safe (GRAS) and exempt from the provisions in the additives and color amendments. The 1958 Delaney Clause of the Additives and Color Amendments prohibited the approval of any food additive shown to induce cancer in humans or animals.

The Pesticide Residue Amendment of 1954 [37] articulated procedures for setting safety limits for pesticide residues on raw agricultural products. The original 1938 Food, Drug, and Cosmetic Act defined as adulterated any food to which a poisonous substance was added. However, Congress determined that some pesticides cannot be avoided in the production of food and are not dangerous at low levels. The 1954 amendment allowed a food to be exempted from being considered adulterated if the pesticide was within tolerance levels set by the FDA. This authority was transferred to the Environmental Protection Agency (EPA) in the 1970s.

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)

The Federal Insecticide, Fungicide, and Rodenticide Act of 1947 (FIFRA) [38] regulated pesticide registration, protection of the environment, use of pesticides, and the safety of those using them. Pesticides are required to be registered and any claims made in respect to the efficacy of the product to be substantiated. FIFRA required stricter labeling, including ingredients, directions for use, and a warning statement. Originally the USDA administered FIFRA, but in 1970 the EPA took responsibility for FIFRA. Approximately 25,000 pesticide products are registered under FIFRA. Amendments to FIFRA in 1988 authorized the EPA to conduct the pesticide re-registration program—a comprehensive review of the human health and ecological effects of pesticides first registered before November 1984 to ensure that they met current scientific and regulatory standards. In 2008, the EPA completed its review of the approximately 1150 pesticide active ingredients that fell into this category.

The Food Quality Protection Act (FQPA)

In August 1996, President Clinton signed into law the Food Quality Protection Act (FQPA) [16]. The new law amended the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Food, Drug, and Cosmetic Act (FDCA), fundamentally changing the way the EPA regulates pesticides. Highlights of the FQPA are:

- *Special Provisions for Infants and Children*. Allows the EPA to make an explicit determination that tolerances are safe for children. This includes an additional 10-fold safety factor to take into account potential pre- and post-natal toxicity, unless on the basis of reliable data a different factor will be safe. In addition, the EPA must take into consideration which foods children consume at high frequencies, children's special sensitivity, and exposure to pesticide chemicals when setting tolerance levels.

- *Tolerance Re-evaluation*. Requires the EPA to review all existing tolerances within 10 years to make sure they meet the requirements of the new health-based safety standard.
- *Enforcement*. Gives the FDA the authority to impose civil penalties for pesticide residue tolerance violations.
- *Pesticide Registration Renewal*. Requires the EPA to periodically review pesticide registrations, with a goal of establishing a 15-year cycle to ensure that all pesticides meet updated safety standards.
- *Endocrine Disruptors*. Incorporates provisions for endocrine testing, and also provides new authority to require chemical manufacturers to provide data on their products, including data on potential endocrine effects.
- *Health-Based Safety Standard for Pesticide Residues in Food*. Establishes a health-based safety standard for pesticide residues in all foods. It requires the EPA to consider "a reasonable certainty that no harm" will result from all non-occupational sources of exposure, including drinking water, as well as exposure to other pesticides with a common mechanism of toxicity when setting tolerances.

This last facet of the FQPA allowed for the repeal of the Delaney Clause of the FDCA, which prohibited the addition of any cancer-causing substance, no matter how small the amount, to be added to foods [39]. Although the Delaney Clause appeared to be a reasonable law when it was proposed, advances in science and technology made it obsolete.

To better understand the neurotoxicity effects of pesticides on children, the EPA began developing risk assessment models to look at factors unique to infants and children, including different dietary patterns and behaviors, such as playing on the floor, or on a lawn where pesticides are commonly applied, or putting objects in their mouths. In 1999, the EPA announced cancellation of major "kid's food" uses of the organophosphate (OP) pesticide methyl parathion and significant restrictions on the use of another OP, azinphos methyl.

By the 10-year anniversary of passage of the FQPA, the EPA had re-evaluated 99% of existing tolerances and tolerance exemptions, leading to confirmation of the safety of 5237 tolerances and to the recommendation to revoke 3200 tolerances and modify 1200 tolerances. The remaining tolerance reassessments were completed the following year. Following FQPA passage, the EPA developed the Endocrine Disrupter Screening Program to screen pesticide chemicals for their potential to produce effects similar to those produced by human hormones and for other endocrine effects [17].

In assessing the aggregate risk from a pesticide, the EPA developed risk assessments that looked at multiple routes of exposure, including food, drinking water, and other non-occupational sources such as lawn and garden care, household insect control, exposures to humans due to pet treatments, and wearing clothes impregnated with insect repellents. Cumulative risk assessments take into account aggregate risks and risks from pesticides that share common mechanisms of toxicity. By

2011, the EPA completed cumulative risk assessments for organophosphates (OP), N-methyl carbamate, triazine, chloroacetanilide, and pyrethrins/pyrethroid groups of pesticides.

A modern food safety system

In the early 1990s, a major outbreak erupted on the West Coast of the USA, which helped trigger the regulatory adoption of the industry-developed system known as HACCP (hazard analysis and critical control points). The system was already in use in many parts of the processed foods industry. Both the USDA and FDA developed proposals to apply the system to raw protein products, including all meat, poultry, and seafood.

Hazard Analysis and Critical Control Points (HACCP)

Traditional inspection, which relies on inspectors applying sensory tools to examine meat, was not effective against foodborne pathogens. But this was the only regulatory system in use in 1993 when an outbreak of *E. coli* O157:H7 in hamburgers in the northwest USA sickened over 700 consumers and killed four children. This outbreak supplied a major impetus to change the meat inspection system. In 1996, the USDA issued its Pathogen Reduction: Hazard Analysis and Critical Control Points (HACCP) System rule [18]. This rule required all meat and poultry processing plants selling meat in the USA to operate under an HACCP system.

The HACCP system is a written plan that identifies likely hazards and the critical points during processing where food contamination is most likely to be controlled. This allows food industry personnel to focus on these critical areas and utilize controls and monitoring systems to prevent contamination. HACCP places primary responsibility for the safety of food on the food industry. The government's role is to verify that industry has properly identified the hazards and is carrying out its responsibility. The government also initiates appropriate regulatory action.

Consumer organizations effectively advocated for the adoption of a *Salmonella* performance standard and testing protocols to be included in the USDA HACCP program, and those additions provided valuable verification information for the agency. In addition, the government testing data allow the USDA to identify trends over time in the different meat and poultry sectors, initiate improvements based on findings, and validate the overall effectiveness of the government's control programs.

In 1995, the FDA developed HACCP regulations for the seafood industry, "Procedures for the Safe and Sanitary Processing and Importing of Fish and Fishery Products," and mandated that seafood processing facilities have in place an HACCP plan by 1997 [19]. The agency published a *Hazards and Controls Guide* [40] to help the seafood industry with implementation, but failed to establish any performance standards or verification testing, so there is little evidence documenting the program's effectiveness in controlling illnesses or outbreaks linked to seafood. In 2001,

the FDA also adopted "HACCP Procedures for the Safe and Sanitary Processing and Importing of Juice" for fruit and vegetable juices after several high-profile foodborne illness outbreaks from juice [41].

The 2011 FDA Food Safety Modernization Act

The FDCA served as the principal food safety law for FDA-regulated foods in the USA through 2011, but unlike the Meat Inspection Act, the law's reactive approach provided inadequate protection to both consumers and the food industry. The introduction of process control food safety systems like HACCP in the late 1990s, combined with international shifts toward preventive food safety systems as a prerequisite to trade, led to efforts to reform the law.

The effort was jump-started in 2002 with Congress's adoption of the Bioterrorism Act. Food was recognized as a potential target, and Congress adopted several provisions ensuring that food facilities, both domestic and foreign, were registered and that importers provided the FDA with notice of food shipments.

The 2000s were also marked by a period of major outbreaks linked to FDA-regulated foods, including such diverse products as spinach, peanut butter, and eggs. Like the 1890s *Popular Treatise on the Extent and Character of Food Adulterations* [1] and the 1930s *American Chamber of Horrors* [14], these outbreaks provided clear evidence that the FDA's program was failing. They also triggered a public outcry for Congress to take action to improve the law. Consumer organizations worked alongside the processed food industry and actual victims to educate members of Congress on the need for comprehensive legislation encompassing the HACCP model for the processed food industry, production guidelines for farmers, and more stringent control of imported foods.

In 2010, Congress adopted the FDA Food Safety Modernization Act (FSMA) [20] which refocused the law to preventing outbreaks rather than responding to problems in the food supply. The FSMA introduced many new concepts into the US food law, several of which had already been adopted in other countries and regions such as the European Union.

The new law implements significant changes to inspections of domestic food firms and applies new approaches to imports, including requiring more inspections of foreign firms that import into the USA. Unlike the older law where inspections were discretionary and hampered by restrictions on the inspector's authority to review records, the new law mandates inspection schedules and makes access to a firm's food safety plans, associated records, and testing results part of the inspection routine.

The law implements process control systems throughout the food industry and allows the agency to set performance standards for the most significant foodborne contaminants, to provide a benchmark for measuring firms for compliance. Regulatory testing is done through accredited laboratories, and certain test results are reported directly to the FDA.

Imported products are subject to a number of new mandatory and voluntary requirements. Importers must operate through the Foreign Supplier Verification

Program which requires an import agent to verify that firms are in compliance with the process control requirements of the Act. A variety of third parties, including foreign national governments, foreign cooperatives, and other recognized third-party auditors can certify that food is in compliance with the law. Private auditors must report public health risks they discover in the course of certifying the safety of a foreign firm. Certification can be either mandatory for high-risk products, or voluntary under the Voluntary Qualified Importer Program.

The FSMA added important enforcement authorities as well, providing the agency with the power to suspend registrations, administratively detain food where it presents a threat to public health, and levy civil penalties when firms refuse recall orders.

These new powers are viewed as transformative of the FDA's authority. Whereas the FDA's enforcement historically has been limited to conditions inspectors observed during infrequent visits to food processing plants or in post-outbreak investigations, the new authority provides knowledge about operations over time, and focuses on prevention.

Development of food safety law in the European Union

European food law from the Middle Ages to today

The roots of food safety law in the USA lie in the European nations that colonized the new world. The Massachusetts Bread Law of 1646 descends from the Assizes of Bread, Beer and Lucrum Pistoris that regulated weight, price, and quality of bread and beer in thirteenth-century England. Purity laws, echoed in post-colonial acts governing unwholesome foods, emerged in Europe toward the end of the Middle Ages; and trade with Europe dominated development of food laws such as the US Meat Inspection Act in more recent times. This trans-Atlantic influence is reflected in Wiley's 1901 bulletin on *Pure-Food Laws of European Countries* [21]. Noting suspicion that European countries used food laws to discriminate against American products, it nonetheless argued for adherence to stronger standards applied both domestically and to exports.

Developments in European food safety law continue to influence US law today. In some regards, this is a process of cross-fertilization. HACCP, developed for the US space program, was mandated for US meat and seafood plants in the late 1990s. However, Europe in 2004 was the first to apply mandatory HACCP to all food production. The USA would not follow suit until 2011 with adoption of the FDA Food Safety Modernization Act. However, Europe's economic consolidation into first the European Economic Community (EEC) and later the European Union (EU) allowed for more rapid regional adoption of its food law, due to the EU's exercise of control over its member states in the area of food regulation. The six countries that signed the Treaty of Rome in 1957 to form the EEC sought to create an internal market that would allow goods to circulate freely between member states [22]. Under this focus on market issues, the EEC developed food safety regulations in a piecemeal fashion [23]. The first hygiene rules governed fresh meat and were

adopted in 1964 [24]. Further rules developed over time into a comprehensive set of directives on food hygiene for specific food groups by the mid-1990s.

Food safety crises 1990–2002 and modern developments

The Treaty of Maastricht established the EU in 1992 but did not provide an explicit basis for food safety laws. Instead, food safety legislation within the EU is based on combinations of provisions for agricultural trade and consumer protection [22].

As occurred in the USA, a series of food safety crises highlighted inadequacies in the EU's food safety system. In the 1980s, foodborne disease outbreaks from *Salmonella* linked to eggs in the United Kingdom, botulism in canned mushrooms and soup from Italy, and shrimps from France and the emergence of *E. coli* O157:H7 in meat raised public health concerns within the EU [24]. Discovery of bovine spongiform encephalopathy (BSE) in British cattle and its linkage to fatal variant Creutzfeldt-Jakob disease in people proved a turning point.

In 1997, the European Commission (the executive body of the EU) released its green paper *The General Principles of Food Law in the European Union* [25]. This paper identified the need for a whole food chain approach to food safety—usually simplified as providing "farm to fork" protection. Discussions started by the green paper developed into the *White Paper on Food Safety* [26] issued in January 2000. It proposed a radical new approach for food safety within the EU and led to development of the General Food Law. Prior to its publication, the EU's food laws had focused on developing a common market; afterward they were to focus on assuring high levels of food safety [22].

The basic principle developed in the *White Paper on Food Safety* was the need for a comprehensive, integrated approach with clearly defined roles for each stakeholder in the food chain [26]. Feed manufacturers, farmers, and food operators had primary responsibility for food safety; the government in each country would monitor and enforce this responsibility. The European Commission would evaluate the national systems to ensure conformance. Finally, consumers would be responsible for proper storage, handling, and cooking of food. To assure confidence, an independent European Food Safety Authority (EFSA) was given responsibility for scientific risk assessments, while risk management was left to the Commission.

The European Commission adopted the General Food Law in 2002. This was followed with adoption of the Food Hygiene I and II laws in 2004. These are collectively identified as EU food law.

Elements of European food law

The General Food Law primarily defines stakeholder responsibilities. It holds food businesses liable for food safety failures, requires them to maintain traceability records, and makes them responsible for recalls. It also establishes the EFSA and the rapid alert system, and sets out general principles of the food safety system [27]. Hygiene I requires food businesses to have HACCP systems and applies to all stages of food production [28]. Hygiene II sets out additional requirements for foods of

animal origin. It requires food business operators to have authorization before placing foods of animal origin on the market and restricts their importation [29].

The EU law regulates food in three broad classes:

Food of animal origin is anything made from animal products and so includes fish, cheese, milk, etc., in addition to meat. Food of animal origin is subject to safety requirements that are stricter than those applied to other foods. Businesses that handle food of animal origin must be inspected and authorized before they can operate. Importers can only import food of animal origin from listed establishments within eligible countries. Additionally, imported food of animal origin must be presented for inspection at the border before entry. The importer is responsible for ensuring imported food has been inspected and meets the EU standards.

Food of non-animal origin covers all other foods. It must be produced under an HACCP system. (An exception to this requirement is that farms need only follow good practices unless they are selling to a commercial processor, in which case the farm must have an HACCP plan.) A food facility handling food of non-animal origin only has to provide notice before operating and the food safety authority may or may not inspect prior to start-up. The importer is responsible for ensuring imported food meets the EU standards.

Composite food refers to foods that combine products of animal and non-animal origin (such as a processed kabob that has meat and vegetables on a skewer). If the composite food contains meat, then it is handled as a food of animal origin. If it contains other forms of food of animal origin, as long as the cheese, butter, etc. does not make up more than 50% of the composite food, it is treated as a food of non-animal origin.

Each member state is required to design the mechanisms for enforcing the food law. Each member state has its own food safety agency (or agencies) that draw up control plans for inspection of food. The food law sets minimum requirements and member states can adopt requirements that are more restrictive. For example, Sweden has a zero-tolerance standard for *Salmonella* that is not an EU-wide requirement [30].

The EU's role is to maintain trust between the member states, assist in implementing the food law, and carry out crisis management functions. Because the law relies on state governments for implementation and enforcement, trust that food safety measures in Greece result in the same high standard as those in France is essential to maintaining confidence. This trust applies to imported food as well. Once it has entered the EU, imported food moves through the common market the same as food grown and processed in any EU country would. To assure that trust, the Food and Veterinary Office enforces consistent application of the food law. If necessary, the EU will bring a case against a member state in the Court of Justice. When food safety failures occur, the EU utilizes a rapid alert system to share information about the risk to all member states.

Challenges for European food law

Since the Treaty of Rome, the EU has grown to 27 member states, speaking 21 languages. It is home to more than 450 million people with diverse food cultures and

traditions. This presents an enormous challenge for managing a comprehensive system of food safety regulation. To arrive at an integrated system, differing national attitudes about food and food safety among the various member states had to be accommodated. Prior to adoption of the General Food Law, there were wide variations in how each country implemented and enforced food safety legislation. That meant consumers could not be confident of receiving the same level of protection across the EU [26]. When the BSE crisis highlighted the flaws in this decentralized system, the European Commission responded with a radical approach that kept elements of member state regulation under an EU-wide umbrella food safety system. The result was a food safety system that established key concepts of industry responsibility for the safety of its products, traceability, and independent, science-based risk assessment as part of a food safety system.

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