Natural Food Toxins of Bacterial Origin

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Abstract

One in six people in the USA acquire a foodborne illness each year. *Food poisoning* is a general term used to describe the physiological effects caused by ingestion of contaminated food or water. The effects of ingesting contaminated food range from short-lived symptoms such as vomiting and diarrhea to paralysis and sometimes death. Some foodborne bacterial toxins have enzymatic properties that allow incredibly small quantities of toxin to exert potent physiological effects. Part of the challenge of sustaining a safe food supply is the ability to rapidly detect low levels of these toxins and in particular within a

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biological matrix. Development of useful assays suitable for analysis of complex food matrices is more challenging than traditional laboratory studies of toxin–substrate interactions in a buffer. This chapter surveys the current methods used to detect a variety of bacterial toxins and explores recent research findings that show promise for adaptation to industry. Highlighted here specifically are three genera of gram-positive pathogens that cause foodborne illness through the action of toxins produced while growing in food, namely, *Bacillus, Staphylococcus*, and *Clostridium*. Also discussed is what is currently understood about their pathogenesis, the potent toxins they produce, and the efforts to accurately detect these preformed toxins in a biological matrix, specifically food. A special emphasis is placed on botulinum neurotoxins as they are the most lethal foodborne toxins to humans. Since the area of pathogen and neurotoxins detection is a rapidly evolving one, there is also a discussion of a number of important factors one should consider when developing new diagnostics.

Introduction

Food poisoning is a generic term that encompasses two types of disease – one being foodborne infections (where pathogens enter a host and cause a disease state through contaminated food or water) and the second being foodborne intoxications (where preformed toxin molecules enter a host through food and/or water and cause disease by altering the normal functioning of host cells). Pathogens that can cause foodborne illness are bacteria, viruses, fungi, protozoa, and helminths. The degree and severity of pathogenesis caused by foodborne illnesses range from self-limiting diarrhea and vomiting to life-threatening medical emergencies. Although the groups of foodborne pathogens listed above represent diverse biological taxa, they do share a common denominator with regard to their mode of transmission – through contaminated food and water. Food can serve as a favorable matrix for microbial growth, and depending on environmental conditions such as pH and temperature (refrigeration versus room temperature), pathogens can multiply to sufficient numbers to cause illness.

Bacterial pathogens are divided into gram-negative and gram-positive organisms. Gram negatives include *Escherichia coli, Salmonella* spp., *Shigella* spp., and other enterobacteriacea. These gram negatives can cause disease by infecting their hosts, colonizing and damaging the intestinal mucosa, and in some cases producing toxins that may cause acute diseases such as diarrhea and vomiting; however, several of these pathogens such as *E. coli* 0157:H7 can also produce hemorrhagic toxins that can cause life-threatening symptoms such as hemolysis and organ failure. This sequence of steps – pathogen enters a host, establishes and colonizes, and then produces toxins – is food infection. On the other hand, foodborne intoxication involves the growth of bacteria and subsequent toxin production in food prior to consumption. Examples here include the toxins produced by the gram-positive *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridium* spp. These three pathogens, and in particular their toxins, present the greatest risk of food poisoning in humans (Bennett et al. 2013).

Depending on the health status of the individual, food poisoning can progress from symptoms of self-limiting vomiting and diarrhea to becoming life threatening and in rare cases fatal disease. It has also been suggested that these toxins and their possible harm to humans presents an opportunity for their use in acts of bioterrorism. Techniques for sensitive, accurate, and rapid detection and inactivation of these toxins in food matrices (or any other biological matrix) will be critical to maintaining a safe food and water supply for humans and animals (Gould et al. 2013); some of these same technologies may be extended for use in clinical cases. In addition to these food safety concerns, the study of these bacterial toxins has also spurred interesting biological questions about their evolutionary significance and role in microbial biology and host–pathogen interactions.

A study published in 2013 by the Division of Foodborne, Waterborne, and Environmental Diseases and the Epidemic Intelligence Services of the Centers for Disease Control and Prevention focused on the distinguishing epidemiology and clinical characteristics of three known pathogens that cause foodborne illnesses (Bennett et al. 2013; Gould et al. 2013). Of the estimated 9.4 million foodborne illnesses in the USA each year, the majority of these cases are caused by known pathogens. Of these illnesses, 1.3 million (14 %) are caused by three gram-positive pathogens: *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridum perfringens*. These three pathogens synthesize toxins in food prior to its consumption (in the case of *B. cereus* and *S. aureus*) or in vivo after consumption of contaminated food (*C. perfringens*). Criteria for determining the etiology of an illness or outbreak include symptoms, incubation period, duration of the illness, and the suspected contaminated food. From 1998 to 2008, 1,229 foodborne outbreaks caused by *B. cereus, C. perfringens*, and *S. aureus* were reported in the United States. Thirty-nine percent of these outbreaks were reported with a confirmed etiology. Vomiting was the key symptom with both *B. cereus* and *S. aureus* illnesses. Meat and poultry were the implicated foods in *C. perfringens* and *S. aureus* cases, and rice dishes were most common in *B. cereus* cases. Problems with food preparation or processing were reported in 93 % of cases. Contamination by a food service worker was common in most *S. aureus* outbreaks (55 %). In the case of *C. botulinum*, in the United States alone, an average of 145 cases are reported each year. Of these, approximately 15 % are foodborne, 65 % are infant botulism, and 20 % are wound associated. Adult intestinal colonization and iatrogenic botulism also occur but rarely. Outbreaks of foodborne botulism involving two or more persons occur most years and are usually caused by toxins present in home-canned foods (Bennett et al. 2013; Gould et al. 2013). Table 1 summarizes important information regarding these organisms and the foodborne illnesses they cause.

For each of these pathogens, major virulence factors are toxin molecules produced by the bacteria while still in unconsumed food, and once ingested, can sicken people. Some of these molecules can be destroyed with heat, but some are heat stable. Since the primary cause of these foodborne illnesses are toxins rather than the presence of the pathogens themselves, their successful detection requires methods beyond standard DNA analysis such as polymerase chain reaction (PCR). Commercial kits are available for detection of many, but not all, of

Table 1 Summary of relevant information about foodborne illnesses caused by toxin-producing gram positive bacteria

Pathogen	Food sources	Incubation	Duration of illness
B. cereus	Variety, rice, leftovers	Diarrhea 6–15 h	24 h
		Emetic 30 min–6 h	
S. aureus	Food made with hand contact and no cooking	1–6 h	24–48 h
C. perfringens	Beef, poultry, gravies	6–24 h	24 h or less
C. botulinum	honey, home-canned low acid foods	Infants: 3–30 days	Variable
		Adults: 12–72 h	

these toxins. The efficacy of these kits varies, and the effects of complex matrices such as food complicate correct identification in many outbreaks. The lack of detection methods has diminished the ability to correctly identify the etiology of some outbreaks of foodborne illness (Khabbaz et al. 2014; Biggerstaff 2014).

The goal of this chapter is to provide a broad overview of foodborne illnesses caused by four toxin-producing pathogens: *Bacillus cereus, Staphylococcus aureus, Clostrodium perfringens*, and *Clostridium botulinum*. The first three organisms cause the most prevalent, toxin-associated foodborne illnesses in the US (Bennett et al. 2013; Gould et al. 2013; Khabbaz et al. 2014). *C. botulinum* is also included in this discussion since it produces the most potent foodborne toxin in humans and the vast number of studies on this toxin has provided an excellent model system for research and development of robust detection reagents against a deadly toxin. The biological properties of their toxins, their role in foodborne illness, and current methods to detect their presence in the environment and in food matrices will be explored. The reader is also encouraged to consult the US Food and Drug Administration's (FDA's) *Foodborne Pathogenic Microorganisms and Natural Toxins Handbook* for further information on these as well as other pathogens and toxins that cause foodborne illnesses.

Development of Detection Assays for Bacterial Toxins in Food: Important Factors to Consider

The development of a robust assay for the detection of any pathogen or biological product of a pathogen (such as toxins) requires consideration of several factors including sensitivity, specificity, matrix effects, and biological activity (Cheng et al. 2012). All of these factors will be discussed below in the context of what have been described using well-established model systems. A particular emphasis is placed on studies describing botulinum toxin detection assays. Many of these studies could be used as a basis for exploring assay development in other toxin systems.

Assay sensitivity is not a simple criterion to define in detecting any pathogen or toxin. Often, the level of sensitivity is determined in part by the method.

For example, the human lethal dose (LD) for oral intoxication with botulinum neurotoxin (BoNT) is estimated at 1 μg/kg or about 70 μg for a 70 kg adult (Cheng et al. 2012). Assays designed for evaluating food must detect at least this amount in a typical portion. Since portions vary widely between individual foods, assay sensitivity requirements may vary with specific matrices. Foods that typically have large portion sizes would require assays with lower detection limits. Furthermore, the dose to cause illness but not death might be lower. For instance, experience with BoNT exposure in rodents is that a level tenfold lower than the minimal lethal dose falls into this category. Projecting rodent data to humans is risky, but applying the same tenfold lower factor to humans suggests that an assay must have a sensitivity of at least 7 μg/serving, or 0.007 μg/mL (7 ng/mL) for a 100 mL portion. In contrast, tests designed for analysis of sera or other clinical matrices should be as sensitive as possible to account for low toxin levels. For example, in oral mouse toxicity studies, only a small portion of the ingested BoNT actually survives the harsh conditions in the gut to reach the bloodstream (Cheng et al. 2012; Dunning et al. 2012). Likewise, the lethal toxin intravenous dose varies between 20 and 200 ng in an adult human with approximately 5 L of blood (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012). Taking into account natural degradation and clearance of toxin in sera, the assay sensitivity for diagnostic evaluation must be in the low to sub-pg/mL range.

Many bacterial pathogens can produce multiple toxins depending on their environment, and different isolates within a single genus may produce different toxins. Some of these toxins share significant amino acid identity or similarity. For instance, there are currently seven known serotypes of BoNTs and 32 known subtypes (Kammerer and Benoit 2014). Undoubtedly, new subtypes will be identified in the future. Amino acid sequence differences can vary as much as 70 % among serotypes (Kammerer and Benoit 2014). This level of genetic diversity and variation can prove challenging for both molecular and antibody-based diagnostic methods. False-negative results could be obtained if a gene or protein structure of the toxin differs from established oligonucleotides/PCR primers or if critical antibody binding epitopes are modified. At the very least, assay performance needs to be established using as many toxin sero- and subtypes as practical. Ideally, assays should recognize all known subtypes of each serotype. Such an assay may require incorporating multiple primers for a PCR assay or antibodies for an immunoassay.

In almost all real-world scenarios, toxin samples to be tested are found in a matrix: a food or food product, a clinical sample (serum, sputum, feces, etc.), or an environmental sample (dust, soil, water, etc.) (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012). However, the majority of assay methods are designed, tested, and optimized in buffer conditions, and thus, assay performance, sensitivity, matrix effects, or influence of nearest neighbor compounds may be diminished or altered. For example, a food matrix may contain conditions such as high fat, high protein or salt content, or extreme pH; the presence of active proteases could also interfere with detection sensitivity, increase background signal, and be responsible for false-positive or negative results. Approaches to reduce

matrix interference range from simple sample dilution, pH rebalancing, addition of protease inhibitors, to specific affinity-binding steps prior to detection (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012). Ideally, examination of different matrices containing the target analyte as well as nearest neighbors is necessary to evaluate assay performance. These will help identify methods to circumvent matrix effects on assay performance.

The potent toxicity associated with bacterial toxins can be attributed to their enzymatic properties such as proteolytic (such as BoNT) or ADP ribosylating (such as some toxins produced by *B. cereus*) activity. The differentiation of active versus inactive forms of the toxin is needed for proper risk assessment and should be an important consideration in assay design. However, measuring only enzymatic activity is often insufficient to develop a real measure of activity. For example, measurement of the toxicity of BoNT must consider multiple factors in addition to just the presence of toxin. The toxin must be able to bind host cell receptors; it must translocate across cellular membranes and finally reach the host cell cytosol and cleave its target protein. Thus, the toxicity of BoNT is a multistep process culminating in a proteolytic event. Few assays if any, other than an animal-based bioassay, can measure all aspects of toxin function. Immunoassays (IA) generally do not differentiate between active and inactive toxin and may give false results even when no active toxin is present. An IA for BoNT that requires the presence of both toxin heavy and light peptide chains to obtain a positive signal can detect inactive toxin but a positive response is predictive of a structurally intact toxin (Cheng and Stanker 2013). For botulism, the presence of an intact toxin molecule is necessary for toxicity. Assays measuring endopeptidase activities of BoNTs are available but are not as sensitive and amenable to use in complex matrices. Genomic methods, while sensitive, detect the presence of toxin genes but not that of toxin. Depending on the diagnostic needs, a combination of methods may have to be used to get a more complete profile of the toxin activity.

For the widest application of an assay, it must be user friendly and allow for a timely diagnosis. Laboratory-based assays should use equipment or tools that are readily available and require minimal training to execute. Ideally, the assay should also be field deployable. In the discussion below, basic toxin biology of *Bacillus cereus, Staphylococcus aureus*, and *Clostridium* spp. are discussed along with what is known about current detection methods for these toxins. In addition to this discussion, the reader needs to know that the FDA sets scientific standards for testing foods for various contaminants. Laboratories, food companies, and government regulators worldwide use these standards to make sure food products are safe to eat and drink. The FDA's Bacteriological Analytical Manual (BAM) describes the agency's preferred laboratory procedures for the detection of pathogens (bacterial, viral, parasitic, as well as yeast and mold) and microbial toxins in food and cosmetics. The complete BAM is available online and is updated as needed (last update was April 2014). The reader is encouraged to consult this informative and excellent online text for detailed methods and criteria for presumptive or confirmative analyses.

Bacillus cereus and Its Foodborne Toxins

Bacillus cereus is a gram-positive, facultatively anaerobic spore-forming bacterium that belongs to the "*Bacillus cereus* group," also referred to as the *Bacillus cereus sensulato* group. This includes the five closely related species *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis*. These bacteria share a high degree of genetic similarity, and it has been difficult to separate them into different taxa. In fact, there is some debate as to whether they represent variants of a single species. Regardless of these issues associated with taxonomic classification, *B. cereus* is a major foodborne pathogen. Some strains are harmful to humans and produce toxins that cause either diarrhea or an emetic toxin that causes nausea and vomiting (Stenfors et al. 2008). *B. cereus*is is also considered an opportunistic pathogen causing bacteremia, meningitis, pneumonia, and gas gangrene–like cutaneous infections, primarily in immune-compromised patients. The United States Centers for Disease Control and Prevention estimates that there are 63,000 annual cases of foodborne illness attributed to *B. cereus* and 20 estimated hospitalizations associated with this pathogen (Bennett et al. 2013; Gould et al. 2013; Khabbaz et al. 2014). Sources of food poisoning by *B. cereus* include a variety of foods such as rice and leftovers as well as food stored at room temperature for prolonged periods of time. *B. cereus* produces one emetic "short incubation" toxin (EME) and three different enterotoxins: nonhemolytic Nhe, HBL (hemolysin B), and cytK (cytotoxin K) (Ramarao and Sanchis 2013; Ceuppens et al. 2011; Senesi and Ghelardi 2010). The large–molecular weight toxins cause diarrheal disease, while emetic disease is caused by a low–molecular weight toxin (1.2 kDa) often associated with rice prepared for a time and temperature insufficient to kill any spores present (Ramarao and Sanchis 2013; Ceuppens et al. 2011; Senesi and Ghelardi 2010). Studies defining the distinct role for each different enterotoxin have revealed that 90 % of the total toxicity can be attributed to the HBL and Nhe enterotoxins. Interestingly, a recent study revealed differential sensitivity to these toxins in different host cell types, suggesting that distinct receptors exist for each toxin (Ramarao and Sanchis 2013).

Biochemical details of HBL, Nhe, and CytK have been revealed through gene cloning and protein expression efforts as well as recent strides in genome sequencing. The emetic toxin, a small–molecular weight molecule (1.2 kDa) also called cereulide, is a heat-stable toxin that is synthesized by the bacteria while it contaminates food (Vangoitsenhoven et al. 2014; Rajkovic et al. 2014). Foodborne intoxication caused by cereulide occurs shortly after ingestion. Cereulide is a potassium ionophore–like compound that destroys the membrane potential of mitochondria and uncouples oxidative phosphorylation through a mechanism similar to the antibiotic valinomycin. Toxicity leads to emetic disease in primates. Chemically, its structure resembles valinomycin and is colorless, tasteless, and odorless. It is a cyclical tripeptide with three repeats of four α-amino and α-hydroxyacids [cyclo (l-O-Val-l-Val-d-O-Leu-d-Ala-)$_3$]. It is resistant to degradation by heat, acid, and alkali, and the minimal concentration of cereulide that causes disease in humans has yet to be determined accurately, although it has been purported that ingestion of 10 μg of cereulide per kg body weight may be sufficient to cause emesis.

The synthesis of cereulide occurs during vegetative growth in improperly refrigerated foods. The gene clusters encoding cereulide (called *ces*) are found on a 24 kb gene cluster contained within a megaplasmid called pBCE. Interestingly, the genes are flanked by 5′ and 3′ sequences that are homologous to the pXO1 toxin plasmid of *B. anthracis*. The toxin is synthesized using nonribosomal peptide synthetases and is difficult to control in food production given the ubiquitous nature of *B. cereus* and environmental resistance of spores. Mechanistically, cereulide acts similarly to valinomycin; it inhibits boar spermatozoa (as low as 1 nM) and causes swelling of their mitochondria, increases conductance across membranes when placed into KCl solutions, and forms adducts with K^+ ions (Rajkovic et al. 2007). These results were similar when tested on rat liver mitochondria. In HepG2 cells, transcription and cell proliferation were inhibited at 2 nM cereulide. Recently, using pancreatic beta cells, 24 h exposure to 5 ng/mL cereulide was sufficient to cause cell apoptosis, even though the concentration was lower than that associated with systemic disease (Vangoitsenhoven et al. 2014). Early studies utilized monkey-feeding experiments since other suitable models were unavailable (Rajkovic et al. 2014; Heilkenbrinker et al. 2013). Recently, cell culture assays using Vero cell and Hep-2 cells were developed (Rajkovic et al. 2014; Heilkenbrinker et al. 2013).

Past studies utilized the addition of antimicrobial compounds designed to inhibit the production of cereulide in food. The availability of compounds that block the synthesis of cereulide has been lacking. However, a recent study focused on polyphosphates and orthophosphates which are based on their successful use in the dairy and meat industries as emulsifying and stabilizing agents and flavor protectors in addition to their antimicrobial properties. Polyphosphates have been shown to inhibit nonribosomal peptide synthetases as well, including the enzyme that produces cereulide. Using an *in vitro B. cereus* growth system and HEp-2 cells, addition of polyphosphates did not block growth of *B. cereus* but did block cereulide gene transcription 70–100 % in reconstituted infant food and oat milk (Frenzel et al. 2011). Whether a similar observation is noted in other foods has yet to be described. Further, the large-scale use of polyphosphates specifically to inhibit synthesis of cereulide and similar toxins shows promise but has yet to be implemented in food-manufacturing settings.

The other toxins produced by *B. cereus* are associated with the diarrheal form of the disease and are referred to as enterotoxins; these are the hemolytic enterotoxin (HBL), the nonhemolytic endotoxin (Nhe), and cytotoxin K (CytK). Both HBL and Nhe are tripartite enterotoxins, while CytK is homologous to b-barrel pore-forming complexes (HBL B/L_1/L_2 = 37.8, 38.5, and 43.2 kDa respectively; and NheA/B/C = 41, 39, 40 kDa, respectively). Collectively, the pathogenesis is likely due to membrane damage of a number of different cells, including intestinal cells. The loss of fluid may also be attributed to activation of host cell adenylate cyclase. Of these three characterized toxins, HBL is the only one that has been shown to play a direct role in diarrhea (Ramarao and Sanchis 2013; Ceuppens et al. 2011; Senesi and Ghelardi 2010; Vangoitsenhoven et al. 2014; Rajkovic et al. 2014).

These toxins are heat labile and are formed 5–16 h post ingestion of contaminated food containing *B. cereus*. Table 2 summarizes information on both the

Table 2 Major emetic and enterotoxins produced by *Bacillus cereus*

Major toxin	Pathology	Molecular structure	Mechanism of action
Cereulide	Emetic food poisoning	Cyclical tripeptide	Ionophore/uncouples electron transport
Non-hemolytic Enterotoxin (Nhe)	Diarrheal food poisoning	Tripartite: NheA, B, C	Pore-forming complex
Hemolysin BL (Hbl)	Diarrheal food poisoning	Tripartite: Hbl B, L_1, L_2	Pore-forming complex
CytK	Diarrheal food poisoning	Single polypeptide	Pore-forming complex

emetic and diarrheal toxins. Other toxins may exist and may play a role in foodborne illness in addition to these noted major toxins.

To further dissect the mechanism of action of these toxins, *in vitro* cell culture models and in vivo animal studies have been used. *In vitro* pathology associated with HBL include hemolysis, vascular permeability, degradation of explanted retinal tissue, and necrosis in rabbit skin. In vivo models using rabbits have shown ocular necrosis and inflammation and rapid fluid accumulation in a rabbit ileal loop model (which is considered the gold standard test for determining diarrheal activity caused by an enterotoxin). In fact, the potency level of HBL is comparable to cholera toxin, highly suggestive of its role in pathogenesis on the surface of red blood cells, and then hemolytic activity arises through a colloid osmotic mechanism. The equilibration of ions through the pore gives rise to a net influx of ions, an accompanying movement of water, cell swelling, and then lysis. A proposed model for pore formation predicts oligomerization of the B subunit into a heptamer or octamer, and the L1 and L2 subunits help to induce conformational changes in the B subunit to facilitate oligomerization, or to stabilize the oligomer of B (Ramarao and Sanchis 2013; Ceuppens et al. 2011; Senesi and Ghelardi 2010; Vangoitsenhoven et al. 2014; Rajkovic et al. 2014).

The non hemolytic (Nhe) enterotoxin contains three components called NheA, NheB, and NheC. The Nhe complex is toxic to vero cells and supernatants from strains lacking HBL show no toxicity when monoclonal antibodies to NheB are added. The individual components of the complex are synthesized independently and assemble into a ratio of 10:10:1 (NheA:NheB:NheC). NheB is the binding component and the mechanism of action is through pore formation on the membrane and subsequent colloid osmotic lysis. Interestingly, hemolytic activity can be detected in Nhe, although much lower than observed in the HBL complex. Structural comparison shows similar features between NheB and NheC with HBL (Vangoitsenhoven et al. 2014; Rajkovic et al. 2014).

Cytotoxin K is a 34 kDa molecule with enterotoxigenic against intestinal epithelial cells and pore forming capability in planar lipid bilayers. Two forms of CytK have been identified, denoted as CytK1 and CytK2. The role of each form has not yet been deduced. Peptide sequence analyses of CytK reveal homology to *Staphylococcus aureus* leucocidins, γ-hemolysin, and α-hemolysin, *Clostridium perfringens* β-toxin, and *B. cereus* hemolysin II. All of these bacterial proteins

Table 3 A comparison of three currently available test kits for detection of *B. cereus* enterotoxins (Ceuppens et al. 2012)

	BDE VIA™	BCET-RPLA	Duopath™
Bacillus cereus enterotoxin	Nhe-AB	Hbl-L_2	Nhe-B and Hbl-L2
Minimal sample volume needed (μL)	200	50	150
Sample analyzed	Food	Food, isolate	Food, isolate
Detection limit (ng/mL)			
Manufacturer	1	2	ND
Published studies	2–5	≥0.6	6–20
Time to result (hours)	5	20	0.5

are predicted β-barrel channel forming toxins. Although the mechanism of CytK assembly and function has yet to be clearly described, if CytK follows other similarly beta-barrel toxins, then it would be predicted that they are secreted as water-soluble monomeric proteins and then assemble into beta-barrel-shaped pores/channels through membranes of target cells, causing cell death and lysis. A recent analysis of 616 isolates revealed expression of CytK in 85 % of *B. cereus* and *B. thuriengiensis* isolates (Vangoitsenhoven et al. 2014; Rajkovic et al. 2014).

Characterization of these toxins is critical to better understanding the distribution of isolates with potential of causing foodborne illness. For instance, epidemiological studies have shown that while the presence of *B. cereus* spores in the environment are ubiquitous, the prevalence of emetic-causing *B. cereus* isolates is rare (Stenfors et al. 2008). PCR methods are critical for conformation of cereulide-producing isolates. Stenfors and colleagues (Stenfors et al. 2008) tested a panel of 176 strains including *B. cereus* strains, *B. cereus* group strains, and other *Bacillus* spp. using PCR, immunoassays, and cytotoxicity tests and assessed the consistency of their results. A screening multiplex PCR for the detection of *hbl*, *nhe*, *ces*, and *cyt*K1 as well as two multiplex PCRs for the differentiation of Hbl genes (*hbl*C, *hbl*D, *hbl*A) and Nhe genes (*nhe*A, *nhe*B, *nhe*C) was applied. They observed a strong correlation for these three independent assays, and no false-negative PCR results were observed for any of the strains that tested positive by immunoassay and using cytotoxicity tests. The three multiplex PCRs proved to be a reliable method for the identification of enterotoxinogenic *B. cereus* isolates (Stenfors et al. 2008).

Given the importance of rapid identification of food contaminated with these toxins, it is necessary to discuss what tests are available to laboratory personnel. Currently, there are three commercial kits available for the detection of *B. cereus* diarrheal enterotoxins. These kits focus on the identification of Nhe and Hbl. A visual immunoassaykit (BDE VIA™ from 3 M Tecra) and two *B. cereus* enterotoxin reversed passive latex agglutination kits (BCET-RPLA from Oxoid) and Duopath™ Cereus Enterotoxins (Merck) (Ceuppens et al. 2012). *B. cereus* might also produce other toxins, but no kits for other toxins are currently available for any of these virulence factors. A comparison of these three kits and their use are shown in Table 3. No detection kit is yet available for cereulide or CytK (Ceuppens et al. 2012).

Genetic exchange among different *Bacillus* spp. complicates determination of the correct etiology in clinical cases. For instance, an isolate of *B. cereus* containing the genes encoding an anthrax-like toxin was probably acquired through lateral gene transfer. This molecule, called certhrax, genetically resembles anthrax and functions as an ADP-ribosylating toxin found similar to what is observed in *B. anthracis* (Visschedyk et al. 2012; Simon et al. 2013; Simon and Barbieri 2014). Cerethrax was first identified in *B. cereus* strain G9241. Certhrax shares 31 % sequence identity with anthrax lethal factor from *Bacillus anthracis* that may contribute to pathogenesis. Strain G9241 was isolated from a welder with cutaneous anthrax-like symptoms. Two plasmids encoding the toxin were isolated from G9241. Further investigation of the properties of this unusual toxin will indicate whether an anthrax-like disease, caused by cerethrax, could be acquired through a foodborne route. Whether this toxin, or others identified in the future, poses food safety problems has yet to be observed.

The genetic similarity and plasticity among different *Bacillus cereus* group members poses a taxonomic dilemma with many interesting biological questions to be asked. From a food safety standpoint, the high degree of similarity among members, such as *B. cereus* and *B. anthracis*, emphasizes the critical need to identify markers that can allow discrimination among pathogenic and nonpathogenic isolates. Perhaps the development of detection tools that focus on toxins discussed herein may help investigators rapidly identify isolates that pose threats to the food supply.

Staphylococcus aureus and Staphylococcal Food Poisoning (SFP)

Staphylococcus is a gram-positive, catalase-positive, spherical, non-spore forming, nonmotile bacterium that can exist in pairs, short chains, or grape-like clusters (Principato and Qian 2014; Hennekinne et al. 2012; Krakauer and Stiles 2013). They are facultative anaerobes and are ubiquitous in the environment. They can be found in the air, dust, sewage, water, on most surfaces, and on animals and humans. Some staphylococcal species are used in meat and dairy fermentation, and the potential for enterotoxin production by these "food grade" species is inconclusive. Some investigators have demonstrated toxin production, while others have not (Principato and Qian 2014; Hennekinne et al. 2012; Krakauer and Stiles 2013). Strain specificity appears to play a role in whether an isolate does or does not make a toxin that can lead to food poisoning.

Enterotoxigenic strains of *Staphylococcus aureus* are the primary cause of staphylococcal food poisoning (SFP). Twenty-two different enterotoxins have been described to date (as of September 2014) (Hennekinne et al. 2012; Krakauer and Stiles 2013; Omoe et al. 2013; Hait et al. 2014; Jenko et al. 2014). The toxins are single-chain globular proteins with molecular weights of approximately 22–29 kDa. Table 4 provides a summary of the staphylococcal enterotoxins (SE) identified to date. These toxins belong to a family of molecules known as pyrogenic toxin superantigens. Superantigens do not require intracellular processing by antigen-

Table 4 A brief summary of staphylococcal enterotoxins identified to date, their molecular weights, emetic potential, and diagnostic method of detection. *SE* staphylococcal enterotoxin, *SEl* staphylococcal enterotoxin-like (Krakauer and Stiles 2013; Jenko et al. 2014)

Toxin	Molecular weight (kDa)	Causes emesis	Diagnostic method
SEA	27.1	Yes	Commercial kits
SEB	28.4	Yes	Commercial kits
SEC	27.5–27.6	Yes	Commercial kits
SED	26.9	Yes	Commercial kits
SEE	26.4	No	Commercial kits
SEG	27.0	Yes	None
SEH	25.1	Yes	None
SEI	24.9	Weak	None
SE/J	28.5	Nd	None
SE/K	26.0	Yes	None
SE/L	26.0	No	None
SE/M	24.8	Nd	None
SE/N	26.1	Nd	None
SE/O	26.7	Nd	None
SE/P	27.0	Nd	None
SE/Q	25.0	No	None
SER	27.0	Yes	None
SES	26.2	Yes	None
SET	22.6	Weak	None
SE/U	27.1	Nd	None
SE/U2	Nd	Nd	None
(SEW)	Nd	Nd	None
SE/V	Nd	Nd	None

presenting cells before presentation to T cells and can activate T cells by cross-linking MHC class II molecules directly on antigen-presenting cells. This cross-linking can activate many T cells, leading to proliferation and subsequent release of chemokines. These chemokines can potentially cause toxic shock syndrome. SE toxins also can directly interact with epithelial cells and stimulate an inflammatory response. The most widely studied example is toxic shock syndrome (TSS) caused by toxins of *Staphylococcus* spp. (Principato and Qian 2014; Hennekinne et al. 2012; Krakauer and Stiles 2013). Staphylococcal toxins also cause food poisoning, and several SEs have been studied extensively, including SEB, which is a Category B Priority Pathogen as indicated by the National Institutes of Health, NIAID. These foodborne toxins utilize a primary mechanism of action (based on extensive studies using staphylococcal enterotoxin B, denoted as SEB) similar to TSS, an exacerbation of a cell-mediated immune response. In food poisoning, the preformed toxin enters the body, binds receptors, and gains access to immune cells. Specifically, SEB binds directly on major histocompatibility complex II on host cells without intracellular processing. This binding to MHCII then stimulates T-cell binding and activation. In fact, in the case with SEB, it has been reported than one in

five T cells may become activated as a result of SEB superantigen activity. This is in comparison to an estimated 1 in 10,000 T cells stimulated in a typical antigen presentation. Other T-cell populations then become activated in response and stimulate cytokine production and release. Crystal structures of several SEs have revealed similar three-dimensional structures despite differences in primary peptide sequence. The unique folding of these toxins might explain some of their resistant properties. For example, SEs are resistant to proteolytic enzymes, such as trypsin and pepsin, which allows them to remain intact during passage through the digestive tract. These toxins are also resistant to common environmental conditions that would inactivate other enterotoxins – such as freezing, drying, heat treatment, and low pH (Hennekinne et al. 2012; Krakauer and Stiles 2013).

Although a large number of putative enterotoxins have been identified using genomic techniques, the main criterion for categorizing an enterotoxin is whether or not it causes emesis in a primate animal model (Principato and Qian 2014; Hennekinne et al. 2012; Krakauer and Stiles 2013). As shown in the table above, whether all of these toxins causes emesis still remain to be determined, and most of the studies described herein utilized a single serotype. Whether multiple serotypes synergize pathogenesis associated with foodborne illness needs to be addressed. To date, analyses of toxic dosages in food have primarily focused on staphylococcal enterotoxin A (SEA). It has been reported that 0.5 μg of SEA can cause symptoms such as vomiting (Principato and Qian 2014). Others cited a 50 % emetic dose of ~0.2 μg SE per kg of human body weight (Jenko et al. 2014; Attien et al. 2014). Thus, an average adult human would require about 10–20 μg of SE to suffer symptoms. Other authors have argued that dosages as low as <1 μg could cause food poisoning symptoms. Still other studies have shown even lower dosages are sufficient to cause food poisoning (Jenko et al. 2014; Attien et al. 2014). Recovery of *S. aureus* from contaminated food or the detection of SE toxins in food is the primary means of diagnosing staphylococcal food poisoning. If doses lower than those reported can cause illness, detection methods will need to be more robust. *S. aureus* cells are heat sensitive but SE toxins are not, which can complicate diagnosis (Hennekinne et al. 2012).

Bioassays of food extracts in animals and/or superantigen activity in cell cultures can confirm the presence of active toxin. As described before, emetic activity using monkey-feeding or kitten intraperitoneal tests can confirm the presence of active toxin (Jenko et al. 2014; Attien et al. 2014). The house musk shrew is a more recently developed toxicity model (Attien et al. 2014). However, dosages above 2.3 μg are necessary to cause pathology. Since this amount of toxin is much higher than what is needed to cause disease in humans, many investigators do not consider the house musk shrew technique relevant and appropriate (Jenko et al. 2014).

Molecular diagnostic methods for staphylococcal food poisoning include PCR methods detecting enterotoxin genes in contaminated food (Simon and Barbieri 2014). Here, the presence of genes encoding SEs is detected by amplification. The limitation of this method is that it only demonstrates the presence of the SE genes but provides no information on the actual presence of toxin. Therefore, PCR methods measuring enterotoxin genes should not be the only means to determine

that *S. aureus* is the cause of a foodborne outbreak. Newer approaches include the extraction of RNA from contaminated food samples followed by reverse transcription and PCR analysis to detect SE toxin gene expression. Of course, the complex chemistry of food can interfere with the PCR (Jenko et al. 2014; Attien et al. 2014). Additional studies using a variety of food matrices are warranted.

Immunological methods for staphylococcal food poisoning are based on the use of antitoxin polyclonal or monoclonal antibodies (Jenko et al. 2014; Attien et al. 2014). Diagnostic tests are commercially available based on enzyme immunoassay (EIA) including enzyme-linked immunosorbent assay (ELISA) and enzyme-linked fluorescent assay (ELFA). The main challenge using these techniques is the difficulty of detecting the toxin in food matrices. An additional limitation is the lack of availability of antibodies to all the different SE toxins (antibodies only exist for SEA through SEE). Furthermore, some kits have low sensitivity, which can lead to false negatives. Since antisera are available only for SEA through SEE, commercial immunoassays for all SE toxins are not yet possible. Finally, immunoassays generally do not differentiate between active and inactive toxin.

Mass spectrometry (MS)–based methods have been successfully used to identify toxins and are considered the most sensitive of all methods for enterotoxins (Attien et al. 2014). However, sample preparation limits the widespread use of this method. In food matrices, there are many proteins, lipids, and other substances that can interfere with detection of the toxin. In a recent outbreak, MS-based methods were used successfully in the identification of toxins (Attien et al. 2014); however, the costs were less than optimal. The continued development of MS-based methods and production of ELISA-based methods for the other SEs should help investigators quickly identify toxins in food and other biological matrices. Other methods that have been used in the identification of the staphylococcal enterotoxins and may have application in foods are a T-cell proliferation assay and polyacrylamide gel electrophoresis (PAGE) combined with immunoblotting (Omoe et al. 2013; Hait et al. 2014; Jenko et al. 2014; Attien et al. 2014).

AOAC International has approved the microslide double diffusion method as their current standard for evaluating new methods to detect SE toxins (Jenko et al. 2014). Other methods used with food extracts should be at least as sensitive as the microslide method, which requires concentrating 600 mL extracts from a 100 g food sample to about 0.2 mL (Jenko et al. 2014). Less sensitive methods are deemed inadequate. However, radioimmunoassay (RIA), agglutination, and enzyme-linked immunosorbent assay (ELISA) require less or no concentration of food and therefore save time and are considered more sensitive. Latex agglutination is another serological tool for identifying staphylococcal enterotoxins. Several ELISA methods have been proposed for the identification of enterotoxins in foods, but except for a polyvalent ELISA and an enzyme-linked fluorescent immunoassay (ELFA), their specificity has not been studied extensively. Among ELISA methods, the "double antibody sandwich" ELISA is ideal, given that reagents are commercially available in polyvalent and monovalent formats for both toxin screening and serotype-specific identification. An automated enzyme-linked

fluorescent immunoassay has been developed and is commercially available. This method has undergone specificity and sensitivity evaluations and has proven to be an effective serological system for the identification of staphylococcal enterotoxin in a wide variety of foods.

Examining staphylococci isolated from foods for enterotoxin production helps to establish potential sources of enterotoxin in foods. Of the methods developed for laboratory testing for enterotoxin production, culturing cells in semisolid agar is approved by AOAC International (Hait et al 2014). It is simple to perform and requires minimal, routine laboratory equipment. Another simple approach is the use of pH 5.5 brain-heart infusion (BHI) broth. The major problem with identifying enterotoxins in foods is that minute concentrations are sufficient to cause food poisoning. Pasteurization and thermal processing may render most toxins serologically unreactive. Consequently, false negatives may result if detection methods lack sufficient sensitivity to detect active toxin (Hait et al. 2014; Jenko et al. 2014).

The FDA has now suggested that the VIDAS® SET2 be the preferential polyvalent test to ascertain the presence of staphylococcal enterotoxins type A and E (SEA-SEE) in foods. The second-generation antibody optimizes capture and detection by using a combination of SEA-SEE monoclonal anti staphylococcal enterotoxin antibodies. Removal of the Fc region of the antibodies allows for increased specificity by reducing nonspecific binding via the Fc portion of the antibody that may result in false-positive responses. The use of two different polyvalent kits to analyze one possible contaminated sample is also now recommended by the FDA.

The question of whether methicillin-resistant *Staphylococcus aureus* (MRSA) could present possible food safety issues has been discussed in recent years (Doyle et al. 2012; Wendlandt et al. 2013). Isolate CC398 is a lineage of MRSA that is most often associated with asymptomatic carriers in food animals. As of June 2009, only one case of food intoxication due to CC398 MRSA had been identified (Doyle et al. 2012; Wendlandt et al. 2013). Food may be contaminated with CC398, and handling contaminated food may facilitate transmission. Monitoring MRSA that carry toxin genes within the food supply should be a top priority. There is general agreement that more work needs to be done to pinpoint which toxins are directly related to vomiting and/or diarrhea.

Clostridium perfringens Produces a Foodborne Toxin

Clostridium perfringens is an anaerobic, spore-forming gram-positive bacterium that receives most of its attention by causing gas gangrene. However, *C. perfringens* is also the second most prevalent foodborne pathogen (Alves et al. 2014; Stiles et al. 2014; Li et al. 2013). The CDC has estimated that there are one million cases of foodborne *C. perfringens* infections each year (Bennett et al. 2013; Gould et al. 2013). *C. perfringens* is an intestinal bacterium of animals and humans and can be subsequently found in butchered raw meat and poultry (Alves et al. 2014; Stiles et al. 2014; Li et al. 2013). The ingestion and subsequent growth of vegetative cells can accompany toxin production in the intestines of humans. People who are

infected with *C. perfringens* develop abdominal cramping within 6–24 h (typically 8–12 h); vomiting and fever are not symptoms of infection or intoxication. The infective dose is estimated to be 10^6–10^7 cells (Stiles et al 2014). Most of the ~16 toxins produced by *C. perfringens* are encoded by large plasmids that range in size from ~45 kb to ~140 kb (Alves et al. 2014; Stiles et al. 2014; Li et al. 2013). These plasmid-encoded toxins are also closely associated with mobile genetic elements. *C. perfringens* strains have been observed to carry up to three different toxin plasmids, with a single plasmid encoding at least three distinct toxin genes (Alves et al. 2014). The five major types of toxins are labeled as (A, B, C, D, and E), theta toxin, and NetB.

Type A *C. perfringens* is found ubiquitously in the environment and can cause gas gangrene in animals and humans (Li et al. 2013; Stiles et al. 2013; Sully et al. 2014). In addition to producing the toxins listed above, it can produce a sporulation-linked enterotoxin (CPE) that causes a major form of food poisoning linked to meat. CPE is a 35 kDa protein, and the *cpe* gene is located on the chromosome or a plasmid (Li et al. 2013). CPE contains a nine-stranded β-sheet and binds claudin receptors located at the tight junctions of intestinal cells. The binding of CPE to these receptors leads to formation of a large complex that is internalized, compromising the function of tight junctions and giving rise to diarrhea. Furthermore, the internalization of the CPE:claudin complexes leads to apoptosis in some cells.

Methods used to determine the presence of *C. perfringens* in biological samples include animal models of infection and intoxication and diagnostic molecular methods (Stiles et al. 2013; Sully et al. 2014). Animal models include the rabbit ileal loop assay and the suckling mouse assay. The rabbit ileal loop assay has also helped to determine the synergistic roles some individual toxins play (Li et al. 2013; Stiles et al. 2013; Sully et al. 2014). Molecular detection methods include PCR, nested-PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP). The majority of isolates from the environment lack the CPE gene. However, *cpe*-positive isolates produce spores with higher levels of resistance and can therefore be selected for in food preparation environments.

During the process of food preparation, clostridial spores can withstand heating to 100 °C for at least 1 h, making it difficult to eliminate them completely. Foods like stews and other slow-cooked meat and poultry dishes are candidates for *C. perfringens* poisoning. Furthermore, improper refrigeration or allowing foods to stand at room temperature are also poor practices that contribute to perfringens food poisoning (Alves et al. 2014: Stiles et al. 2014). Therefore, most of the outbreaks are connected with poor temperature control (also termed *temperature abuse*). The general steps for confirming *C. perfringens* sporulation and enterotoxin production have been established by the FDA and utilize specialized culture media and conditions (to induce sporulation) and a commercially available reversed passive latex agglutination (RPLA) kit for confirming toxin production. Improving our understanding of the basic biology of *C. perfringens* toxins is needed given the large number of different toxins produced and the roles these molecules play in pathogenesis, including foodborne illness.

Clostridium botulinum: A Model System to Study Deadly Foodborne Toxins to Humans

*Clostridium botulinum*is a gram-positive, anaerobic spore-forming rod that produces botulinum neurotoxin. This is the most potent toxin against humans (Kammerer and Benoit 2014) and can paralyze animals by blocking acetylcholine release by neurons. The botulinum neurotoxin is classified into eight serotypes called A-H, of which A, B, E, and F are known to cause toxicity in humans; however, all serotypes are potentially toxigenic in humans. At least 32 additional subtypes have been described based on differences in both primary peptide sequence as well as structural distinctness. The parenteral lethal dosage for humans is 0.1–1 ng/kg, and the oral dose is 1 μg/kg. A single gram of BoNT released and subsequently inhaled can lead to the deaths of more than one million people (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012; Kammerer and Benoit 2014; Cheng and Stanker 2013).

Isolates categorized into Group I of *C. botulinum* are referred to as "proteolytic" and may produce toxin types A, B, or F. They are widely distributed in the environment and often found in a variety of raw foods. Botulinum toxin can cause symptoms at very low concentrations (possibly as low as 0.005 μg). The onset of symptoms typically takes 12–36 h but usually depends on the amount of toxin ingested and can take much longer. Symptoms include initial diarrhea and vomiting followed by neurological effects including blurred vision, weakness, and difficulty swallowing, talking, and breathing. If not diagnosed early, mortality rates can be as high as 40 %. Modern treatments have reduced this down to less than 10 %. Foods involved in outbreaks are usually incorrectly preserved meat or fish products, but a range of other foods may be implicated, including vegetables preserved in oil and cheese. The botulinum toxins are not heat stable and can be inactivated at cooking temperatures (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012; Kammerer and Benoit 2014; Cheng and Stanker 2013).

Strains in Group II are classified as "nonproteolytic." They produce toxin types B, E, or F. These isolates are able to grow at temperatures as low as 3 °C and are widespread in the environment. Type E strains are especially common in aquatic environments. There has been much concern that these isolates may produce toxin in refrigerated processed foods without any obvious signs of spoilage. The spores of this group of *C. botulinum* are much less heat resistant than those of Group I strains. Group II toxins are thought to be slightly less potent than Group I types, requiring at least 0.1 μg to cause symptoms. But in terms of other biological properties, they do not differ much. Foods that are involved in outbreaks of Group II botulism include cold-smoked fish and other preserved fish products (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012; Kammerer and Benoit 2014; Cheng and Stanker 2013).

BoNT serotypes can differ from one another by 34–64 % at the amino acid level (Kammerer and Benoit 2014). Genetic variation within each serotype is sometimes significant. Thirty-two toxin subtypes with amino acid sequence differences of 2.6–32 % have been identified thus far, with more likely to be identified in the

future. This extensive serotype and subtype diversity complicates direct antibody and molecular-based assay designs (Kammerer and Benoit 2014). Rarely does one reagent bind all possible serotypes and subtypes. In the bacterium, BoNT is synthesized as a holotoxin of ~150 kDa protein, and it is subsequently processed by a clostridial trypsin-like protease into two polypeptides that are connected by a single disulfide bond. The structure is similar to other known bacterial A-B dimeric toxins. The ~100 kDa fragment, known as the heavy chain (HC), facilitates toxin binding to specific host cell receptors and later in the translocation of the toxin from vesicles into the cell cytosol. The ~50 kDa fragment, known as the light chain (LC), contains the enzymatic domain and is often used for the development of activity-based laboratory assays. A number of different HC and LC specific antibodies have been developed for use in toxin neutralization and toxin detection immunoassays (Cheng et al. 2012; Dunning et al. 2012; Zhang et al. 2012; Kammerer and Benoit 2014; Cheng and Stanker 2013).

A rodent bioassay is considered the gold standard method for detecting BoNTs. Despite much effort to replace the use of animals, it is still the strongest assay to model all aspects of BoNT intoxication: binding, translocation and enzymatic activity, and pathology (Pellett 2013; Dorner et al. 2013; Singh et al. 2013). Studies to replace the mouse bioassay and improve assay time and sensitivity have led to the development of both in vitro and in vivo systems to detect BoNTs.

The animal assay measures BoNT in minimal lethal dose (MLD) units, which is the lowest dose at which all tested mice die. Mice are usually injected intraperitoneally with 0.5 mL of BoNT sample in a dilution series and then monitored over several days for signs of intoxication and death (Cheng et al. 2012; Pellett 2013; Dorner et al. 2013; Singh et al. 2013). When sufficient sample is available for testing, the identity of the unknown BoNT can be determined using neutralizing antibodies against each of the specific toxin serotypes (A-G). Hence, the serotype can be identified based on which antibody protects respective mice from death. While the mouse bioassay has high sensitivity, it also can detect different serotypes and subtypes as well as measure different aspects of active toxin. The mouse bioassay also is amenable to use in different matrices, one of the major factors discussed earlier. However, the mouse assay is not without drawbacks. Limitations include long assay times, requirement of specialized animal facilities, substantial costs, trained staff, or the use of animals (with death used as an end point). There is also substantial variation in results observed among different research laboratories.

Alternative refined animal assays that do not use death as an end point, such as the mouse phrenic nerve hemidiaphragm assay, have been evaluated (Cheng et al. 2012; Pellett 2013; Dorner et al. 2013). Although sensitive and faster than the use of whole animals, these assays require use of sophisticated equipment and training and are not amenable for use with large samples of complex matrices. A recently developed in vivo assay using the toe spread reflex model was tested for the detection of BoNT in buffer, serum, and milk samples (Pellett 2013; Dorner et al. 2013; Singh et al. 2013). This new assay can provide results more quickly than standard mouse bioassays. How well these results can be translated into a deployable kit that is user friendly has yet to be determined.

A number of different nucleic acid methods have been exhaustively developed. The use of the polymerase chain reaction (PCR) to identify the presence of *C. botulinum* DNA was originally used to detect the presence of bacterial spores. The method could detect the presence of as few as 100 spores per reaction mixture for serotypes A, E, and F and only 10 spores per reaction mixture for BoNT/B. Lindström and colleagues developed an enrichment method that could detect as few as 0.01 spores/g of sample for serotypes A, B, and F and 0.1 spores/g of sample for BoNT/E (Pellett 2013; Dorner et al. 2013; Singh et al. 2013). However, one critical drawback of this method is that enrichment often requires 5 days, which is probably too long for some clinical situations. Furthermore, the applicability of the assay for detection of food contamination became obsolete when beef was observed to interfere with the sensitivity of the assay. Also, if contamination were to occur with the actual toxin and not cells, this "traditional PCR" method would not be useful. Multiplex methods have also been tested as a way to analyze unknowns for multiple targets, such as different pathogens and/or associated gene products of those pathogens. This approach, known as multiplex technology, is straightforward for PCR-based approaches. Different combinations or sets of PCR primers, each one highly specific for a gene of interest, can be easily generated, allowing for the amplification of multiple targets in one PCR reaction tube. One such multiplex method was able to discriminate among BoNT serotypes A, B, E, and F, validating mouse bioassay results (Dorner et al. 2013; Singh et al. 2013). Furthermore, Peck and colleagues developed a culture enrichment method that when coupled with multiplex PCR could identify strains of *C. botulinum* that were nonproteolytic (BoNT serotypes B, E, and F) (Dorner et al. 2013; Singh et al. 2013). This method was robust and rapid enough for use with food samples contaminated with *C. botulinum*.

Real-time or quantitative PCR is useful in studies of gene expression: specifically differential expression of genes under different environmental conditions or for comparative studies among different organisms. For detection of clostridia, real-time PCR methods that examine expression of the nontoxic, nonhemagglutinin (NTNH) genes have been developed as well as methods to study toxin gene expression in *C. botulinum* serotypes A, B, E, and F (Pellett 2013; Dorner et al. 2013). In that study, 29 different strains of toxin-producing *C. botulinum* were screened and compared with expression profiles from non-toxin producing clostridia as controls. This assay has a sensitivity of 100-fg/1,000 f. total DNA in the PCR tube (equivalent to approximately 25–250 genomes). Converting this DNA concentration to its equivalent in cells/mL suggested a detection limit of approximately 10^3–10^4 cells/mL. Following a 48-h enrichment under anaerobic conditions, these investigators reported the detection of *C. botulinum* serotype A in a naturally contaminated sample of foie gras suspected in a botulism outbreak. Recently, pentaplex methods have been developed to simultaneously identify and discriminate among larger numbers of different serotypes using a wider array of different genes (Dorner et al. 2013; Singh et al. 2013). This technology should prove to be efficient and cost effective.

ELISA is a widely used detection assay format that uses anti-BoNT capture and detector antibodies, usually in a sandwich-type format. The read-out for the assay can be colorimetric, luminescence, or other formats. Most older-generation BoNT

immunoassays are about 10 times less sensitive than the mouse bioassay (Stanker et al. 2013; Stevens et al. 2013). Although not as sensitive, ELISA-based methods are relatively fast, inexpensive, and simple. They are also less subject to inhibitory matrix effects. Amplified enzyme-linked immunosorbent assay (ELISA) for detecting toxins in food matrices has been described. Here, toxins for serotypes A, B, E, and F could be detected in liquid, solid, and semisolid food. Assay performance in a range of foods was evaluated and included broccoli, orange juice, bottled water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meats, and dairy items (Wang et al. 2014). Assay sensitivity varied for each botulinum complex serotype, and sensitivities were reported as 60 pg/mL for BoNT/A, 176 pg/mL for BoNT/B, 163 pg/mL for BoNT/E, and 117 pg/mL for BoNT/F. The tests readily detected 2 ng/mL of serotypes A, B, E, and F in a variety of the foods tested. Recently, traditional-format sandwich ELISA assays using highly sensitive mAbs against BoNT/A and BoNT/B have detected as low as 5 pg/mL and 25 pg/mL BoNT/A, in buffer and in a milk matrix, respectively (Stanker et al 2013; Stevens et al. 2013; Singh et al. 2013); and 100 f. and 39 pg/mL of BoNT/B in buffer and milk matrix, respectively (Pellett 2013; Dorner et al. 2013; Singh et al. 2013; Stanker et al. 2013). These mAbs were used in an electrochemiluminescence ELISA assay using a Meso Scale Discovery (MSD) instrument. Detection sensitivities for BoNT/A using the MSD instrument were similar to traditional ELISAs in the buffer system but offered marked improvement in detection limits and reduction in backgrounds in liquid food matrices (Cheng and Stanker 2013). The higher sensitivity and reduced time required for these new ELISA methods make them possible alternatives or complements for the mouse bioassay.

Multiplex technology discussed before has been applied to the development of methods to analyze multiple epitopes on a single antigen or multiple targets in a single sample (Dorner et al. 2013; Wang et al. 2014; Dunning et al. 2014). This approach uses multiple mAbs as well as polyclonal antibodies to reduce false-positive and false- negative results. The LuminexxMAP technology utilizes microsphere beads conjugated with antibodies. The antibody–bead complexes detect multiple epitopes in a single sample; for instance, this technology was used to detect abrin, ricin, botulinum toxins, and staphylococcal enterotoxins in spiked food samples and used paramagnetic beads instead of nonmagnetic polystyrene beads to help in the analysis of food matrices (Dorner et al. 2013; Singh et al. 2013; Wang et al. 2014; Dunning et al. 2014).

Accurate and sensitive detection of contaminated food and other biological samples in the environment is critical. Brunt and colleagues have developed a number of rapid affinity immunochromatography column (AICC) assays for the detection of BoNT serotypes A, B, E, and F in food matrices. These investigators reported a detection limit for BoNT/A of 0.5 ng, twofold more sensitive than earlier reported lateral flow methods. For serotypes B, E, and F, the minimum detection limit ranged from 5 to 50 ng. Although not as sensitive as ELISA or mouse bioassays, immunochromatographic methods generally are rapid assays, requiring only 15–30 min to complete, do not require enrichment steps, and are amenable to use in the field (Wang et al. 2014; Dunning et al. 2014; Sachdeva et al. 2014).

The application of lateral flow methods for detecting toxins has also led to the development of a number of kits for sensitive and rapid testing. Here, capture antibodies are printed on nitrocellulose membranes. Detection antibodies are labeled with materials that can be visualized (e.g., colloidal gold or colored latex beads) The sample is added to a reagent pad containing labeled detection antibodies that bind toxin, wick across the membrane where toxin is retained, thus concentrating the labeled detection antibody. A positive reaction leads to a colorimetric change that is usually detected as a line. These assays are generally qualitative and determine the presence or absence of toxin. Sharma and coworkers tested different commercial lateral flow devices (such as the Bot-Tox-BTA kit) for their capacities to detect toxin in food samples (Dorner et al. 2013; Singh et al. 2013). They were able to detect concentrations of toxin as low as 10 ng/mL for BoNT serotypes A and B and 20 ng/mL for BoNT/E in a variety of liquids such as milk products, soft drinks, and fruit juices. Results by Stanker show sensitivity of 0.5 and 1 ng/mL for BoNT/A in buffer and milk, respectively, in lateral flow devices using the same mAbs described in the ELISA section above (Cheng et al. 2012; Ching et al. 2012). Although simple lateral flow tests have lower sensitivities compared to other methods, they produce rapid results, require no additional reagents or equipment, can be easily interpreted, and have many applications. They can be useful for the rapid screening of samples where the presence of BoNT may be more abundant.

An innovative approach for toxin detection combines antibodies with the amplification power of PCR in an assay called immuno-PCR (I-PCR). Here, instead of a secondary antibody conjugated to the detection enzyme, template DNA is conjugated to the antibody; upon binding of antigen by the antibody, an indirect test for the presence of the BoNT is carried out using PCR. Chao et al. described a sensitive I-PCR method (femtogram amounts, 10^{-15} g) for detection of BoNT/A. These investigators also compared standard ELISA as well as sandwich ELISA methods with the sensitivity of the I-PCR method. Both ELISA methods were sensitive for toxin detection down to 50 fg, and the I-PCR method was between 103 and 105 times more sensitive (Singh et al. 2013; Wang et al. 2014). The use of I-PCR for highly sensitive detection of BoNT in food matrices or other biological backgrounds has yet to be developed.

Rapidly distinguishing between the presence and absence of active versus inactive toxin is critical for intervention (Dorner et al. 2013; Singh et al. 2013). Since BoNTs are zinc metalloproteases, enzyme-substrate assays have been developed using knowledge of the human targets for these enzymes. Activity assays range from mixing toxin with recombinant versions of host targets (such as SNAP-25) and then using immunoblotting to detect cleavage of those substrates, to measuring fluorescence emitted from cleavage of fluorogenic peptide substrates. One such peptide, called SNAPtide, used in an assay with a reverse-phase HPLC with a fluorescence detector, can detect as low as 5 pg/mL of BoNT/A in skim milk (Dorner et al. 2013; Singh et al. 2013). Other peptide substrates, VAMPtide and SYNTAXtide, useful for their cognate BoNTs have been developed. The levels of substrate cleavage correlate well with toxin activity. Other investigators have looked for other indications of substrate cleavage by BoNTs. For instance, Nuss

and colleagues generated antibodies that specifically recognize the full-length version of human SNAP25 but not the cleaved form (Sachdeva et al. 2014; Bagramyan et al. 2013). Use of this antibody to confirm the absence of toxin activity by detecting only the intact, full-length substrate might be useful to confirm the absence of bioactive forms of the toxin.

Cell-based assays measure BoNT receptor binding, translocation, and enzymatic activity and can be viable alternatives to the mouse bioassay (Dorner et al. 2013; Hong et al. 2013; Basavanna et al. 2013). A number of different neuronal and non-neuronal derived cell lines have been generated for use in BoNT assays. These include rat spinal cord cells; chick embryo neuronal cells; neuroblastoma cells N2A, and BE(2)-M17 cells. The read-out for most of the cell-based assays for detection of BoNT/A is the cleavage of SNAP-25. Antibodies for SNAP-25 allow immunoblot detection of cleavage products, specifically detecting a decrease in size of endogenous SNAP-25 protein.

Investigators continue to examine different parameters in order to develop a more robust cell-based assay. The U.S. Food and Drug Administration approved a cell-based assay (developed by Allergan) for use as possible replacement of the mouse bioassay. Details of the assay have yet to be published. Cell-based assays may ultimately prove valuable for toxin detection in food. Detection methods can exploit the power of sensitive antibodies for enrichment or sample preparation as well as the signal amplification ability of enzymatic assays. For instance, the assay with a large immune-sorbent surface area (ALISSA) utilizes a two-step approach; first, an antibody-mediated step concentrates toxin onto a large bead surface. Captured toxin molecules are then used in a SNAPtide protease assay (Bragramyan et al. 2013). When compared to other established methods for toxin detection in food matrices, the ALISSA assay can detect toxin concentrations as low as 50 fg/mL, which is more sensitive than the mouse bioassay or either immunoassay or SNAPtide assay alone. The use of this method to evaluate a number of different food matrices suggests that it may be useful in food contamination studies.

The mass spectrometry-based method, ENDOPEP-MS, uses antibodies to concentrate and extract BoNT serotypes A, B, E, and F from test samples. The concentrated toxins are then subjected to an endopeptidase activity-based assay to generate target cleavage products. Finally, mass spectrometry is used to identify cleavage target products (Dorner et al. 2013; Singh et al. 2013). This approach has been successful in identifying BoNT serotypes A, B, E, and F in a variety of food and clinical sample matrices with sub-mouse bioassay sensitivities. To advance this technique even further, a single, high-affinity mAb (4E17.1) that can simultaneously identify BoNT serotypes A, B, E, and F has been developed (Dorner et al. 2013; Singh et al. 2013). The use of this mAb reduced assay time while maintaining assay sensitivity. The use of mass spectrometry can give fast and definitive results. With the future development of low-cost equipment, this method may be more readily available to investigators.

The analysis of all aspects of the botulinum toxin, from basic science to application, has provided a strong foundation ready to support similar work on other toxins, such as those isolated from the other pathogens discussed herein as well as those which might emerge in the future.

Conclusion and Future Directions

There are almost 10 million reported cases of foodborne illness each year in the USA (the number is actually much higher given the likely number of unreported cases). This large number encompasses all cases caused by bacteria, viruses, fungi, and parasites. Of these, strikingly, almost 14 % are caused by toxin-producing gram-positive bacteria. Furthermore, as pathogens acquire new toxin genes through lateral gene transfer, the emergence of new species, isolates, and serotypes expressing new and different toxins is eminent and unavoidable. Advances in genomic and proteomic analyses will also reveal new toxins in well-studied as well as understudied pathogens. It is an exciting time to be studying any aspect of bacterial toxins, from their basic biology and pathogenesis in foodborne illness to their manipulation as therapeutics for other diseases. Whatever the situation may be, both sensitive and specific methods to rapidly detect and identify these toxins are critical to maintaining a safe food supply and for identifying possible weak links in food preparation and more rapid and effective intervention.

Cross-References

- ▶ Applications of Nanotechnology in Developing Biosensors for Food Safety
- ▶ Model Fungal Systems for Investigating Food Plant Mycotoxins

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