Clostridium botulinum and the Most Poisonous Poison

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Abstract Botulinum neurotoxin (BoNT) is the most poisonous toxin known to humankind and is the cause of the neuroparalytic disease botulism in humans and animals. Due to the extraordinary toxicity of BoNT, control of botulism is a perennial concern of the food industry, medicine, and regulatory agencies. BoNT also poses a serious concern as a bioterrorism agent with the potential of causing mass casualties, and BoNT is designated as a Category A Select Agent, the most severe group comprising six agents, which also includes anthrax toxins and hemorrhagic viruses. There are seven serotypes of BoNT (designated A–G), which are defined by their neutralization against death in mice by homologous antibodies. BoNTs are produced by neurotoxigenic clostridia, particularly the diverse bacterial group *Clostridium botulinum*, and by sporadic strains of *Clostridium argentinense*, *Clostridium butyricum*, *Clostridium baratii*, and *Clostridium sporogenes*. These clostridia produce heat and chemical resistant endospores, which are widespread in soils, freshwater and marine sediments, and the gastrointestinal tract of certain animals (but not humans). Surveys have revealed that *C. botulinum* spores are present in the USA in about 35% of soil samples examined. BoNTs are ~150 kDa proteins comprised of a Heavy Chain (HC) and a Light Chain (LC). During the intoxication process, the HC binds selectively and with high affinity to peripheral nerve terminals, and the LC enters into the cytosol and inhibits the presynaptic release of acetylcholine, causing postsynaptic denervation of muscle and a characteristic flaccid paralysis that can last for several weeks to months in humans. BoNTs also cause disturbances in the sensory and autonomic nervous systems. The recognition of diversity of BoNTs has expanded in recent years mainly through DNA sequencing efforts, and within the seven serotypes (A-G) there are more than 40 sequence variants (subtypes) of BoNTs currently recognized (Hill et al. 2013. BoNTs are produced naturally in protein complexes, in which nontoxic associated proteins protect the heat-labile BoNT component in the digestive tract of humans and animals and likely in the environment. Six categories of botulism are recognized: (1) foodborne botulism whereby preformed toxin (and sometimes cells and spores) are ingested in foods, (2) infant botulism in which *C. botulinum* colonizes

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the infant gut and produces BoNTs, (3) adult intestinal botulism analogous to infant botulism, (4) wound botulism due to colonization and BoNT formation in surface wounds, (5) inhalational botulism which is extremely rare naturally but a concern of bioterrorism, and (6) iatrogenic botulism caused by poisoning from inappropriate injection of BoNT for pharmaceutical and aesthetic purposes. In all six categories, the symptoms and pathology are very similar. BoNT is absorbed into the lymphatic system and then into the blood stream, and is then transported to peripheral nerve terminals, primarily cranial and somatic nerves. BoNT is internalized into the nerve terminals at the neuromuscular junction and prevents the release of acetylcholine to muscles innervating the eyes, face, and mouth, and then the toxicity descends bilaterally to motor nerves of the torso causing a generalized paralysis. There is no antidote for botulism once it enters nerves, and currently the only means for treatment of the disease is by thorough nursing care, and in severe cases mechanical ventilation and parenteral feeding. Serum therapy by administration of human (BabyBIG®) in infants or heptavalent equine antibodies (currently HBAT) in adults into the circulation of poisoned toddlers and adults, can decrease the severity of the paralysis and duration of the illness while BoNT remains in the blood stream. This chapter focuses on aspects of neurotoxigenic clostridia and botulinum neurotoxins that have not been extensively reviewed recently, including the importance of BoNTs as a public health risk, properties of BoNT-producing strains and BoNTs, laboratory criteria needed for a definitive diagnosis of botulism, the pathology, recovery and treatment of botulism, and lastly a brief section on strategies to prevent foodborne and infant botulism.

Keywords *Clostridium botulinum* • Botulinum neurotoxin • Endospores • Flaccid paralysis • Select agent

Introduction

Lamanna proposed in 1959 that botulinum neurotoxin type A (BoNT/A) is the most poisonous substance known to humankind (Lamanna 1959); and his prediction has stood the test of time (Gill 1982; Morton 1961; Johnson and Montecucco 2008; Scott and Suzuki 1988). BoNTs have an estimated lethal dose in humans of 0.1–0.5 ng per kg by intravenous exposure, 0.10–1.5 μg per kg by the oral route, and 7–12 ng per kg by inhalation (BoNT/A). Botulism is a true intoxication caused solely by BoNT actions, while it is dependent on neurotoxigenic clostridia that produce the toxin. Botulism is a rare disease in humans, and is much more common in certain animals, particularly in fishes and waterfowl for which BoNT has caused massive outbreaks, involving thousands of animals every year (Anniballi et al. 2013; Eklund 1987; Smith and Sugiyama 1988).

From a global perspective, the worldwide impact of foodborne and waterborne diseases on human health and economics is vast (WHO 2015). Systematic evalua-

tions of the global impact of foodborne infections and intoxications on human morbidity, mortality, and economics have been evaluated for several industrialized countries by means of established infrastructure for estimates of epidemiology, disease diagnosis, treatment, and reporting (WHO 2015). In contrast, comprehensive information on the incidence and impact of foodborne disease is not yet available for many developing countries but the burden is undoubtedly also very large (Akhtar et al. 2014). Most foodborne infections and intoxications are caused by Gram-negative enteric bacteria with associated diarrheal syndromes, by certain vegetative Gram-positive bacteria including *Staphylococcus aureus* and *Listeria monocytogenes*, and enteric viruses, particularly norovirus (Doyle and Buchanan 2013). Natural toxins, including mycotoxins and algal toxins, are also an important cause of foodborne disease (Tu 1983–95).

Serious foodborne diseases of high health impact are also caused by spore-forming bacteria, most notably *Clostridium perfringens*, *Clostridium botulinum* and other neurotoxigenic clostridia (Doyle and Buchanan 2013; Hatheway and Johnson 1998; Johnson 2013). In particular, botulism is a paralytic illness that occurs worldwide and is caused by BoNT produced by neurotoxigenic clostridia. The primary categories are infant botulism, in which spores colonize the intestinal tract of infants and produce BoNT, foodborne botulism in which BoNT is ingested in foods, and wound botulism resulting from the colonization of *C. botulinum* and BoNT production in surface wounds (Hatheway 1995; Johnson and Montecucco 2008; Sam and Beynon 2010). Rare forms include adult intestinal botulism, inhalational botulism, and iatrogenic botulism due to injection of inappropriate formulations of pharmaceutical BoNT (Chertow et al. 2006; Ghasemi et al. 2012; Werner et al. 2000). BoNT-producing species include *C. botulinum*, and sporadic strains of *C. argentinense*, *C. butryicum*, *C. baratii*, and *C. sporogenes*. Owing to the high potency of BoNT and its stability in the gastric and respiratory tracts, BoNT is also considered to be a potential agent of oral and inhalational bioterrorism and a potential cause of mass casualties (Arnon et al. 2001). Currently there are no antidotes for botulism, and survival and recovery from botulism relies on intensive nursing care, and in severe cases mechanical ventilation, parenteral feeding, and rehabilitation (Brook 2006; Cherington 2004; Johnson and Monteuccco 2008). Serum therapy by administration of antitoxins has been used to alleviate the symptoms and shorten the duration of the disease. Remarkably, BoNTs have also been developed as highly effective pharmaceuticals (Schantz and Johnson 1992; Johnson 1999; Scott 1989) for the treatment of more than 100 neuronal disorders by direct injection of BoNT into the neuromuscular region of the disorders (Fig. 1) (Truong et al. 2013). Figure 1 also illustrates the diversity of neuromuscular symptoms that can occur in botulism. It is evident from phylogenomic studies that the genes encoding BoNTs have been horizontally transferred to other clostridia; furthermore many neurotoxigenic clostridia possess more than one BoNT gene cluster (Franciosa et al. 2003; Williamson et al. 2016). The interspecies transfer of genes encoding BoNT raises concerns of the generation of novel BoNT-producing organisms posing new health threats.

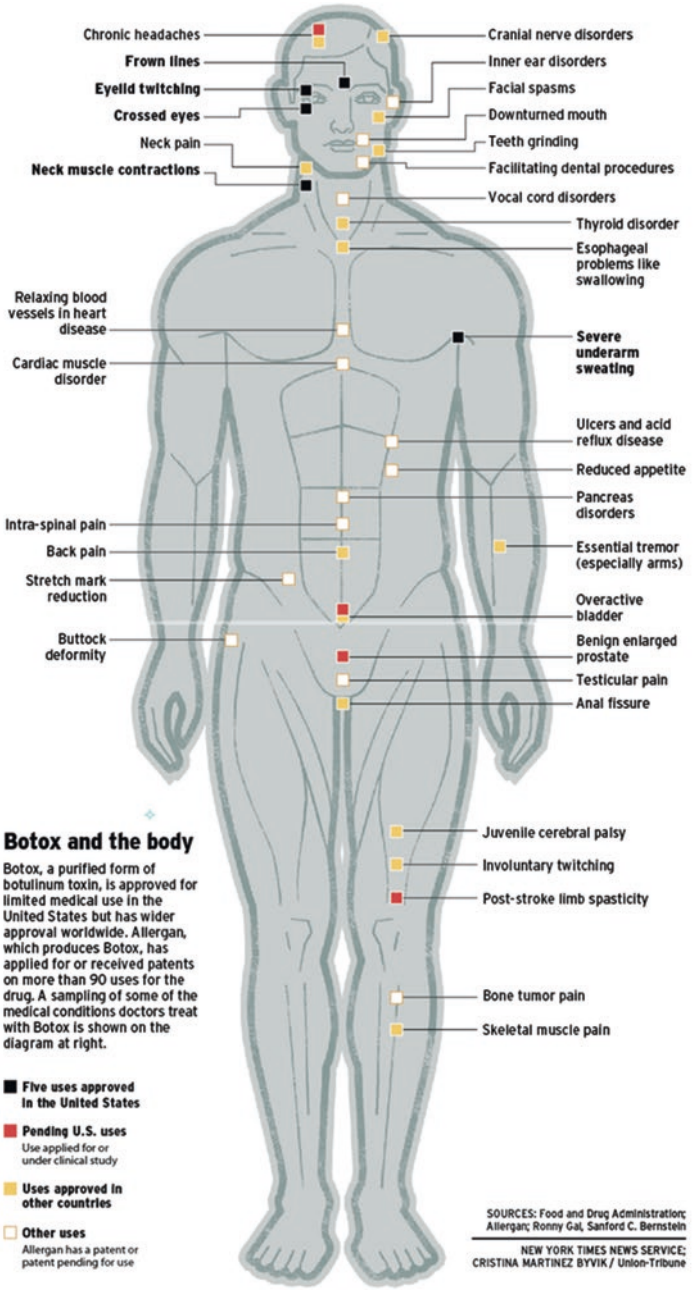

Fig. 1 Neuromuscular regions having shown successful treatment my pharmaceutical botulinum neurotoxin

Botulism as a Public Health Concern

Botulism is a serious public health concern to regulatory agencies, attending physicians and healthcare providers, and the food industry. Important considerations include:

1. Botulism is a reportable life-threatening disease (https://wwwn.cdc.gov/nndss/case-definitions.html; http://www.cdc.gov/nczved/divisions/dfbmd/diseases/botulism/#how_common). Botulism surveillance and emergency response measures have been described (Shapiro et al. 1997). Medical providers should be trained to recognize the signs and symptoms of botulism. Potential botulism cases with characteristic symptoms should be rapidly communicated to the appropriate state health department for possible occurrence and reporting of an incident, and management of the disease.
2. Botulism constitutes a public health emergency. The occurrence of a single case implies that other people may be at risk of contracting the disease.
3. In a probable botulism outbreak, the health care provider should contact their state epidemiology department for consultation. The CDC can also be contacted (www.cdc.gov; 770-488-7100) by the state epidemiologist for consultation and release of equine heptavalent antitoxin (HBAT). In the potential occurrence of infant botulism, the health care provider may contact the California Infant Botulism Treatment Program (www.infantbotulism.gov; 510-231-7600) who can provide valuable information and authorize the release of human-derived antitoxin (BabyBIG®). Both the CDC and Infant Botulism Treatment and Prevention Program provide 24-h, 7-day consultation (Maslanka et al. 2013).
4. The detection of early clinical symptoms should prompt appropriate medical observation and access to medical measures such as nursing care, neurological evaluation, mechanical ventilation, and administration of antitoxin as needed (Chalk et al. 2014; Maslanka et al. 2013; Shapiro et al. 1997).
5. A physical examination by a qualified physician for evaluation of typical symptoms of botulism should initially be performed. A confirmed case of botulism requires detection of BoNT in clinical samples and/or foods, generally by the mouse bioassay, and/or by culturing of neurotoxigenic clostridia from stool, wounds or foods (Hatheway 1988; Johnson 2013; Maslanka et al. 2013; http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070879.htm, accessed April 2016).

Current laboratory methods for the detection of botulinum toxin in clinical specimens and food samples generally require 2–4 days to perform (Hatheway 1998; Maslanka et al. 2013), and after consultation with the appropriate regulatory agency, samples should be refrigerated and sent as soon as possible to state health departments. Appropriate medical procedures and minimizing morbidity and mortality in a botulism outbreak depends on recognition of clinical signs in humans, an epidemiologic investigation, and rapid detection of botulinum neurotoxin and/or *Clostridium botulinum* in clinical specimens and foods (see Emergency Preparedness and Response procedures (http://emergency.cdc.gov/agent/botulism/, accessed April 2016; Arnon 2001).

Publicity Affecting the Public and Companies Involved in Putative Botulism Outbreaks

Botulism is a rare foodborne disease with unique symptoms compared to the more common diarrheal diseases. Botulism can be quite severe and may cause fatalities in humans and animals. Botulism often attracts much interest from the media and the public. The most common class of botulism in the USA and certain other countries is infant botulism, and illnesses in infants (as well as in adults) can be a particularly sensitive issue, and can lead to considerable publicity, often much of it presented in a sensational manner (Dickson 1920; Satin 2008). Important points should be kept in mind for industry and other responses to media releases and public perception:

1. Botulism is a foodborne illness that has been recognized for more than a century; it is not a new disease posing new threats to the human population.
2. Botulism is an extremely rare disease, and in the USA about 120 cases occur per year. Other countries for which epidemiologic data are available also have a very low incidence of botulism.
3. Botulism can be treated by appropriate nursing care and in severe cases by mechanical ventilation, parenteral feeding, and serum therapy.
4. Botulism is not a chronic disease and full recovery generally occurs unless there are underlying diseases. Botulinum toxin in foods is destroyed by heating the food for 10 min at 100 °C or for 30 min at 80 °C (Siegel 1993). However, spores are not destroyed by these heat treatments and the heated food should be discarded and not used in reformulated product.

Brief Historical Description of the Pathology and Microbiology of Botulism: The Foundation for Understanding the Disease and for Control of *C. botulinum* Intoxications

Although anecdotal evidence indicates that botulism was first recognized in ancient civilizations (Smith and Sugiyama 1988), botulism was first convincingly recognized as a distinct disease syndrome in Germany by Müller and Kerner in Germany in the 1700s and 1800s (Ergbuth 2007; Pellett 2012). The disease was noted to initially cause cranial neuronal disturbances of the eyes, mouth, and pharynx, followed by generalized muscle paralysis of the torso, and suffocation in severe cases. In 1820, Kerner published a seminal paper on the association of the disease with consumption of raw blood sausages by 230 people in Germany and termed the disease botulism, which derives from the Latin word “botulus” for sausage (reviewed in Pellett 2012). This important observation of the association of a toxic substance in food with a paralytic and often fatal human illness by Kerner was achieved several

decades before Pasteur, Koch and other founding microbiologists framed the germ theory of disease. Botulism was referred to as Kerner's disease for decades, and this discovery provided a background for studies of investigations of early outbreaks, primarily in the USA, Europe, Japan, and Russia (Dolman 1964; Meyer 1956). In these early outbreaks, the fatality rate was 40–60% (CDC 1998; Dembek et al. 2007; Meyer 1956). Presently, the fatality rate in developed countries has decreased to 5–10%, due to rapid diagnosis, supportive nursing care, parenteral feeding and assisted mechanical ventilation when needed, and serum therapy by administration of antitoxin. Serum therapy is derived by the methods of Von Behring and Kitasato for their work on antitoxins to diphtheria and tetanus (Grundbacher 1992). Von Behring was awarded the first Nobel prize in Physiology or Medicine in 1897 for serum therapy (Winau and Winau 2002). Early in the 20th century, antitoxin was tested against botulinum toxin extracts during the period 1897–1921 for neutralization of toxicity in animals using extracts from the van Ermengem strain (nonproteolytic type B) and *C. botulinum* type A strains (Dickson and Howitt 1920; Burke et al. 1921). They found that antitoxins against botulinum extracts were highly effective in neutralizing toxicity in animals. Serum therapy using antitoxins, although currently considered an archaic method for therapy of infectious diseases and intoxications (Manohar et al. 2015), is still the only method of preventive treatment for botulism today and new treatment modalities are urgently needed that can enter nerves and eliminate symptoms of botulism.

The fundamental information regarding the toxin-forming bacterium, toxicology of culture extracts, pathology, and inactivation and prevention of BoNT formation in foods was described in the late 1800s by van Ermengem (1897), and the principles he established are still valid today. In a remarkable investigation in 1894–96, the microbiologist Emile Pierre van Ermengem, who studied under Claude Bernard and Robert Koch and was eminently capable of recognizing new diseases, investigated a foodborne outbreak associated with 23 illnesses and 3 deaths that occurred from eating ham at a funeral ceremony in Ellezelles, Belgium (van Ermengem 1897). He discovered an anaerobic bacterium he termed "*Bacillus botulinus*" which produced a highly toxic substance. His primary findings of the properties of the bacterium and BoNT are integral for the current control of foodborne botulism: (i) foodborne botulism is an intoxication due to a heat-labile toxin and is not an infection; (ii) the toxin is produced by an anaerobic sporeforming bacillus; (iii) the toxin is active in the absence of the bacterium as revealed by filtration of culture extracts and microscopic examination (iv) the toxin is not inactivated by digestive enzymes in the gut of humans and animals; (v) the toxin is inactivated by moderate heat treatment such as boiling for a few minutes; (vi) the toxin is stable at low pH and in moderate NaCl concentrations; and (vii) BoNT is highly toxic to humans and to certain animals species via oral, intraperitoneal, and intravenous routes.

Following van Ermengem's pioneering study in 1894 and his discovery of BoNT type B (by today's *C. botulinum* taxonomy and BoNT criteria), five different serotypes (A-E) of BoNT were identified during the next 50 years (Johnson 2005). A serotype is defined by toxin neutralization and lethal protection in mice by antibodies raised against BoNTs of a homologous serotype, but not by heterologous

antibodies raised against different serotypes (Giménez and Giménez 1993; Hatheway 1988). Serotypes F and G were subsequently discovered, and currently there are seven known serotypes. Recently, a new serotype "H" was proposed (Barash and Arnon 2014; Dover et al. 2014), but this BoNT has been demonstrated to be a chimeric toxin comprised of BoNT/A and BoNT/F components, is neutralized by antibodies to type A, and thus by definition has been termed BoNT/FA and not BoNT/H (Maslanka et al. 2016; Pellett et al. 2016). Nonetheless, the discovery of a naturally occurring chimeric BoNT comprised of domains of BoNT/A and /F highlights the proclivity of BoNT gene transfer and recombination BoNT genes in the environment or animal hosts.

Researchers in the USA and in Europe mainly provided the foundations for the pathology of botulism in the early 20th century. The first detailed understanding of the pathology of botulism derived from studies of symptoms in animals and humans suffering from botulism (Bishop and Bronfenbrenner 1936; Dickson 1918; Dickson and Shevky 1923a, b), as well as by injection of BoNT into the intact gastrocnemius muscle of animals and careful measure of nerve and muscle activity, which is a technique that is widely used today in the study of BoNT (Guyton 1947; Payling Wright 1955). These studies revealed that BoNT acts on peripheral nerves that innervate striated muscle (Ambache 1949), and the toxin primarily acts at the neuromuscular junction (NMJ) of cholinergic neurons. In particular, BoNT acts on cranial and somatic motor nerves, and may affect the central nervous system (Bishop and Bronfenbrenner 1936; Dickson 1918; Dickson and Shevky 1923a, b). These early studies revealed that BoNT predominantly affects muscles controlling the eyes, mouth, pharynx, and gradually the muscles of the limbs and torso of the animal.

The molecular mechanisms of BoNT remained largely unknown until Burgen and colleagues (1949) used the isolated rat phrenic nerve-diaphragm preparation (Bülbring 1946) and later neuromuscular preparations to examine the effects of BoNTs A and B at the neuromuscular junction (NMJ) (Drachman 1964, 1971; Sellin 1981; Thesleff 1976). Administration of BoNT illicited paralysis of the phrenic diaphragm preparation in a dose-dependent manner with a substantial delay of paralysis compared to other NMJ toxins such as curare and saxitoxin. Kinetic studies and comparison with other toxins revealed that entry of the toxin into the nerves did not occur by penetration of the membrane, but involved a much slower process. This observation of a delay in action is a hallmark of BoNT intoxication, and was explained by the hypothesis that, unlike certain other neurotoxins such as saxitoxin which reach the nerve cytosol by rapid membrane penetration, BoNT requires several steps in the intoxication process, including entry into the lymphatic system and circulation, binding to receptors, endocytosis of BoNT, internalization into the nerve cytosol, and cleavage of SNARE substrates (Montecucco et al. 1994; Schiavo et al. 2000). These findings are highly relevant today since many substances can cause mouse lethality, but most of the nonspecific deaths by non-BoNT toxicants generally occur rapidly (within a few minutes to 1–2 h), whereas botulinum toxin action usually occurs after 4–24 h by oral gavage or intraperitoneal injection depending on the dose. An exception to the slow onset is intravenous injection of

high doses (e.g., 10,000 LD_{50s}), usually into the mouse tail vein (Boroff and Fleck 1966), and in this assay, paralytic symptoms and mouse death generally occur within 20–80 min depending on the dose. Burgen (1948) also provided evidence that acetylcholine was released from nonpoisoned neurons, but was blocked by BoNT in intoxicated neurons. Rats immunized with botulinum toxin were resistant to intoxication and of isolated neuromuscular preparations. Duchen (1971) and Duchen and Strich (1971, 1972) determined there were striking changes in the ultrastructure of motor endplates of NMJs following injection with BoNT. Taken together, these findings in intact animals and in diaphragm and NMJ preparations reveal the relatively slow onset of symptoms, including death, and provided the basis for our current understanding of the molecular mechanism of BoNT.

The fundamental aspects of the epidemiology and microbiology of *C. botulinum* and BoNTs were recognized during the period of 1920–1970 by prodigious microbiologists and physicians in the USA, Japan, Russia, and Europe (Hauschild 1989, 1993; Johnson 2005; Meyer 1956). In the USA, from 1913 – until his death in 1974, Karl Friedrich Meyer studied botulism and other diseases in California at the Hooper Foundation at the University of California San Francisco, which was the first (1914) medical research foundation incorporated into a university setting in the USA (history.library.ucsf.edu/hooper/html). It has been stated that *"Meyer influenced more microbiological and epidemiological domains than other scientists of his time; and he was driven by his deep-seated concern for the welfare of the people"* (Elberg et al. 1976). Meyer and colleagues (1956) discovered many cardinal properties of *C. botulinum*, its endospores, and BoNTs. They determined that the distribution of *C. botulinum* spores occurred worldwide and were present in approximately 35% of soil samples examined in the USA. Further, many regions of Europe had similar prevalences of *C. botulinum* spores. They also determined the high heat resistance of the spores and defined the thermal requirements for inactivation of *C. botulinum* spores (Esty and Meyer 1922), which led to the advent of the 12D "botulinum cook" treatment, which is still in use today and has been amazingly successful in preventing commercial outbreaks of botulism and spoilage in hermetically sealed (canned and pouched containers) (Lynt et al. 1975; Pflug 2010; Pflug et al. 1981). Meyer and Sommer also initiated studies on the isolation of botulinum neurotoxins, and determined that more than 90% of toxicity is precipitated from cultures by the addition of inorganic acids such as sulfuric acid to pH 3.5 (reviewed in Schantz and Johnson 1992). Acid precipitation is the first step in nearly all BoNT purifications of the seven serotypes currently performed. Meyer and colleagues also determined that growth of group I *C. botulinum* was inhibited at pH values $\leq$4.6 or $\geq$10% salt, and that growth of most strains did not occur at temperatures below 4–10 °C or above 50 °C, depending on the physiological group (I-IV) (Holdeman 1977) of *C. botulinum*. These parameters have served as the basis for formulation and extrinsic control of botulinogenic safe foods through the present.

During evaluation of early botulism cases, it was recognized that symptoms of botulism generally began 14–30 h after ingestion of the contamined food (Meyer 1956; Johnson and Montecucco 2008; Sobel 2005). Initial sporadic symptoms frequently are nausea, vomiting and diarrhea, but these gastrointestinal symptoms are

Table 1 Primary symptoms of botulism (Johnson and Montecucco 2008; Cherington 2004)

Symmetrical cranial neuropathies
Blurred near vision, blurred distant vision, dilated or nonreactive pupils, diplopia, drooping eyelids
Difficulty swallowing, dry mouth, difficulty speaking, facial ptosis
Descending bilateral flaccid paralysis, generalized muscle weakness progressing to neck, limbs and torso, paralysis of the respiratory diaphragm and death in the absence of mechanical ventilation

probably caused by the local action of the spoiled food in the gastrointestinal tract and not by BoNT itself. Nausea and vomiting are not usually seen in infant botulism, rather the first sign is constipation due to paralysis of intestinal musculature (Arnon 2006). In infants and adults, BoNT causes motor paralysis and the initial disturbances affecting cranial nerves, especially of the eyes, mouth and pharynx, including diplopia, mydriasis, nystagmus, strabismus, lack of response of the eye pupils, blepharoptosis, dysphagia, and aphonia (Table 1). The facial and mouth features include ptosis in the face, sluggish movements of the tongue, dry mouth, and accumulation of thick mucus in the pharynx (Cherington 1998, 2004; Johnson and Montecucco 2008; Shapiro et al. 1998; Sobel 2005). The intoxication then bilaterally descends to the torso and can paralyze all skeletal muscles. Other neurological signs are often seen in botulism depending on the BoNT serotype, including autonomic and parasympathetic symptoms. Retention of urine is commonly observed which is not due to kidney pathology but rather to weakened bladder contraction. Depending on the serotype, BoNT also causes disturbances in cholinergic-mediated secretions, including decreased sweating in the face, palms, and feet, lack of saliva formation, dysfunctional erection, constipation, and these symptoms are attributed to the effects of BoNT on the parasympathetic and autonomic nervous systems (Dressler and Benecke 2003; Merz et al. 2003; Tintner et al. 2005). BoNT does not cause fever (body temperature may be subnormal) or headaches in subjects, and thinking is clear except for the anxiety and depression due to the awareness of the disease (Cohen and Anderson 1986). Blood pressure and cardiac rate are generally unaffected. Analyses of recovered central and peripheral nerves have not shown permanent damage supporting that botulism is an acute and not a chronic disease syndrome.

In the 1940s and 1950s it was determined that the BoNT component existed in large protein complexes that formed paracrystals of high molecular weight (reviewed in Schantz and Johnson 1992), and, under certain conditions, the BoNT neurotoxic component could be separated from the complex (Sakaguchi 1983; Schantz 1964; Schantz and Johnson 1992). In the 1960s and 1970s, the neurotoxin component (BoNT) was isolated as a purified protein from the toxin complexes (DasGupta and Boroff 1967; Sugiyama 1980). BoNT was determined to consist of a heavy chain (ca. 100 kDa; HC) and a light chain (ca. 50 kDa; LC) linked by a disulfide bond (DasGupta and Sugiyama 1972). The availability of the purified BoNT component of the complex enabled definitive studies of biochemistry, structure and mechanisms of action. In the 1980s, the molecular mechanism of BoNT acting on the NMJ

was elucidated using purified BoNTs (reviewed in Schiavo et al. 2000; Rossetto et al. 2014). The three-dimensional structure of BoNT/A (Lacy et al. 1998, 1999) and later /B and /E (Swaminathan 2011) was determined along with variants (subtypes) within serotypes (Arndt et al. 2006), thereby providing critical insights into understanding the molecular mechanisms of BoNT, their immunological aspects, and the pathology of botulism.

The pathology of BoNTs has been extensively studied in mice (Simpson 2013), yet many aspects of the intoxication process are still unclear. BoNT intoxication involves a complex pathway, in which BoNT is absorbed into the lymphatic circulation and then into systemic circulation, from which it exits the main vasculature into capillaries and traffics to and binds at nerve terminals. The mechanisms of transport to the lymphatic system, into the circulation, and disposition from the circulation are not clear. After exiting the capillaries, the presynaptic mechanism of intoxication has been well studied (Montal 2010; Rossetto et al. 2014; Simpson 2000, 2013). The HC contains receptor-binding domains at its C-terminus, which have strong affinities to polygangliosides and, in some serotypes, for protein receptors including isoforms of SV2 and synaptotagmin within the neuronal membranes (Montal 2010; Rossetto et al. 2014). Following endocytosis and uptake in recycling synaptic vesicles, the HC N-terminal domain mediates translocation of the LC from the vesicle into the neuronal cytosol by formation of a channel within the H chain in the synaptic vesicle membrane (Montal 2010; Pirazzini et al. 2016; Rossetto et al. 2014). Once internalized in the cytosol, the LC acts as a protease with high specificity for SNARE (soluble N-ethylmalemide-sensitive fusion-associated) proteins (SNAP-25, VAMP, and syntaxin) which are responsible for trafficking and fusion of synaptic vesicles with the presynaptic membrane and release of acetylcholine (and other transmitters depending on the organ and innervation) at the neuromuscular junction (NMJ) (Schiavo et al. 2000; Rossetto et al. 2014). The deficiency in release of acetylcholine prevents muscle activation and contraction and results in the characteristic long-lasting flaccid paralysis, the hallmark property of botulism.

The inability of synaptic vesicles to fuse with neuronal membranes and to release neurotransmitter also prevents vesicle recycling and thus the nerve terminal presumably becomes resistant to further uptake of BoNTs by the major pathway of vesicular trafficking, and the toxin then affects other susceptible nerves (Dong et al. 2006), descending to large muscles with a comparatively low nerve-muscle ratio such as those in the limbs. The mechanism of vesicular trafficking within mammalian cells was elegantly revealed by in vitro reconstitution of the vesicle trafficking mechanisms by independent researchers. The elucidation of the mechanisms of SNARE proteins in trafficking of vesicles in cells resulted in the 2013 Nobel Prize in (Physiology or Medicine awarded to James Rothman, Randy Schekman, and Thomas Südhof http://www.nobelprize.org/nobel_prizes/medicine/laureates). The discovery of the function of SNARE proteins in vesicular trafficking and exocytosis, clarified the presynaptic mechanisms of BoNT since SNAP-25, syntaxin, VAMP I, II are the specific targets of BoNTs (Schiavo et al. 2000).

Clostridium botulinum and Other Neurotoxigenic Clostridial Species

Botulinum neurotoxins (BoNTs) are mainly formed by the species *Clostridium botulinum*, which is a diverse assemblage of clostridia comprising at least four independent clostridial lineages (Collins and East 1998; Collins et al. 1994; Hutson et al. 1993a, b; Smith et al. 2015). Neurotoxigenic clostridia have in common the requirements for anaerobic growth, complex nutrient needs, formation of resistant endospores, and elicitation of a highly potent neurotoxin. The group *C. botulinum* is artificially designated as a single species due to the common clinical property of producing BoNTs, but this "species" is highly variable from phylogenetic and evolutionary perspective, and in the ability to form the seven known serotypes (A-G) of BoNTs (Lawson and Rainey 2015). Sporadic isolates of *Clostridium butyricum, Clostridium baratii*, and *Clostridium argentinense* have also been shown to produce BoNTs /E /F, and /G, respectively (Hatheway 1993; Suen et al. 1988). Rare strains of *C. sporogenes* possess the genes for BoNT formation (Weigand et al. 2015), but no known human or animal cases of botulism have resulted from this organism. Nonetheless, the acquisition of genes encoding BoNTs by *C. sporogenes*, which often shows higher resistance properties of its spores to heat and vegetative cells to salt and acidity than *C. botulinum*, creates concern about horizontal gene transfer of genes for BoNT to more resistant organisms than *C. botulinum*. The occurrence in *C. botulinum* on plasmids harboring the genes for BoNTs, with some having conjugative properties, was initially identified in our laboratory for type A (Marshall 2007, 2010), and later confirmed (Smith et al. 2007, 2015) and expanded to other *C. botulinum* serotypes and to C. *butyricum* using experimental genetic and genomic approaches (Carter et al. 2016; Franciosa et al. 2003, 2009; Sebaihia et al. 2007).

The initial taxonomic classification of the neurotoxigenic clostridia was based on production of BoNTs and structural and physiological properties, especially a Gram-positive cell wall structure, formation of endospores, saccharolytic and proteolytic abilities, as well as cardinal intrinsic and extrinsic parameters governing growth, including temperature, pH and a_w (Franciosa et al. 2003; Holdeman et al. 1977; Hatheway 1993; Johnson 2005). *C. botulinum* has been classified for several decades into four physiological groups (I-IV) (Hatheway 1993; Holdeman 1977; Johnson 2005; Smith et al. 2015), and *C. butyricum* and *C. baratii* have been considered Groups V and VI by some researchers (Franciosa et al. 2003). Cardinal physiological properties control growth and BoNT formation by the different Groups of *C. botulinum* neurotoxigenic clostridia, and are paramount in formulating and processing various classes of foods to prevent botulism outbreaks (Franciosa et al. 2003; Glass and Johnson 2002; Hauschild 1989; Johnson 2013). Due to the severity of botulism, certain food laws have been implemented mainly to control growth and BoNT formation by *C. botulinum* in foods, including required heat treatments of foods in hermetically sealed containers (12D "bot cook"), low-acid food regulations (pH > 4.6), and critical values of water activity (≤0.93) in food

formulations. From an economic perspective, cases of botulism are among the highest costs per case than other foodborne diseases (Roberts 2000).

Since the 1970s, molecular properties have become the primary criteria for taxonomic delineations within genus *Clostridium.* These have been based primarily on the homologies and sequences of genes encoding 16S rRNA (Collins et al. 1994; Hutson et al. 1993a, b; Johnson and Francis 1975; Lawson and Rainey 2015). However, the genus *Clostridium* over time became a "general depository" for Gram-positive sporeforming bacteria, and eventually expanded to 228 species (Lawson and Rainey 2015). Recently, it was proposed to restrict the genus *Clostridium* Prazmowski to *Clostridium butyricum* (the type species) and related bacteria, designated *Clostridium sensu stricto* (clostridial rDNA group I) (Lawson and Rainey 2015). Group I includes *C. butyricum* (the type species of *Clostridium*), *C. botulinum* serotypes A-G *C. argentinense*, *C. baratii*, and *C. tetani*. Furthermore, *C. botulinum* and other pathogenic clostridia have been analyzed by complete and partial chromosomal gene sequences, particularly for genes encoding BoNTs and associated proteins that complex with the neurotoxin (Williamson et al. 2016). Detailed phylogenomic analyses of *C. botulinum* and related clostridia have been described (e.g. Williamson et al. 2016), and while these genomic studies provide phylogenomic insights, they will require considerable supplemental research to elucidate the biochemistry, physiology, and practical genetics for a better understanding of the biology of the neurotoxigenic clostridia and the functions of their neurotoxins, as well as food safety.

A breakthrough in the understanding of toxigenesis in *C. botulinum* took place during the study of unusual strains in which it was very difficult to neutralize toxicity using single serotype-specific antibodies, while multi-serotype mixtures provided protection against lethality in mice (Ciccarelli and Giménez 1972; Giménez and Ciccarelli 1970; Hatheway et al. 1981). By tedious serological neutralization experiments, these groups showed that the two *C. botulinum* strains (strain 84 and 657Ba) produced more than one serotype of BoNT. Surprisingly, *C. botulinum* strain 84 has recently been shown to produce three BoNTs (Dover et al. 2013). In multi-BoNT producing strains, one of the BoNTs is usually produced in greater quantities compared to the lesser BoNT and the major BoNT is designated in upper case, e.g. Ab. The regulation of the differential expression of the multi-BoNT-gene clusters is not currently understood (Connan and Popoff 2015; Johnson and Bradshaw 2001). Franciosa et al. (1994) expanded upon this work and showed that many strains of *C. botulinum* possess two toxin gene clusters, in which one of the clusters is often "silent" or unexpressed due to mutations in the unexpressed gene encoding an inactive BoNT.

The mobility of BoNT gene clusters was further supported by the isolation from infant botulism cases of strains of *C. butyricum* and *C. baratii* that produced BoNT/E and /F, respectively (Aureli et al. 1986; Hall et al. 1985; McCroskey et al. 1986). Since these initial isolations, several strains of *C. butyricum* and *C. baratii* have been isolated from infant botulism cases, foodborne botulism incidents, and the environment (Franciosa et al. 2003; Hatheway and Johnson 1998; Johnson 2013). Phylogenomic studies have supported that horizontal transfer of genes encoding BoNTs has occurred independently in the evolution of neurotoxigenic clostridia

(Peck 2009; Skarin et al. 2011, 2015; Skarin and Segerman 2011, 2015; Williamson et al. 2016). The food and medical importance of BoNT gene transfer may be substantial, since new neurotoxigenic clostridia could arise, but the mechanisms of molecular transfer and selective advantages of BoNTs for neurotoxigenic clostridia is unclear.

Botulinum Neurotoxins

C. botulinum produces seven serotypes of BoNTs (A-G), and rare neurotoxigenic strains of *C. butyricum*, *C. baratii,* and *C. argentinense* produce serotypes E, F, and G, respectively (Franciosa et al. 2003; Johnson 2013). Human disease is caused predominantly by BoNT serotypes A, B, and E. Serotypes C and F have been associated sporadically with human illness (Fenicia and Anniballi 2009; Koepke et al. 2008; Johnson and Montecucco 2008), whereas types D and G have not been reported to cause human botulism (Hatheway 1995). The specific toxicity of BoNTs measured in mouse LD_{50s} ranges from 3×10^7 to 3×10^8 per mg for the seven serotypes. As described above, seven distinct BoNT serotypes A through G are defined through neutralization by homologous polyclonal antitoxins (Giménez and Giménez 1993; Hatheway 1988). Antibodies for study of BoNTs are usually raised in rabbits, horses, goats, or mice, using intramuscular injections of formalized toxoid, recombinant toxoids, or BoNT fragments, particularly the receptor-binding domain of the HC, which has high immunogenicity compared to other regions of BoNTs (Karalewitz and Barbieri 2012; Webb and Smith 2013). Cross-reactivity with antibodies has been observed between serotypes C and D or E and F (Sugiyama 1980; Giménez and Giménez 1993). Certain BoNTs, particularly C, D, (Nakamura et al. 2013) and the newly described FA (Pellett et al. 2016), are comprised of mosaic BoNTs comprised of domains of different serotypes. These chimeras are usually less efficiently neutralized by standard methods and optimization of the antitoxin assays is required (Maslanka et al. 2016; Pellett et al. 2016). With new molecular biology techniques, the BoNT/B gene was genetically inactivated, which enabled the purification and definitive characterization of BoNT/FA (Pellett et al. 2016).

DNA sequencing of genes, encoding BoNTs have shown that there is a large diversity in the BoNT family (Fig. 2). Within the seven serotypes, more than 40 variants (designated subtypes) of BoNTs have been detected by *bont* gene sequencing (e.g. Williamson et al. 2006) and later, arbitrarily defined as having ≥2.6% differences in amino acid sequence (Hill et al. 2007; Hill and Smith 2013). This arbitrary classification, based on a fixed degree of change in amino acid sequence, does not necessarily represent substantive differences in BoNT biological and toxicological properties, since even a change of one or a few amino acids can affect BoNT characteristics and function (Montecucco and Rasetto 2015; Rossetto et al. 2014). For example, one amino acid change in the LC can destroy catalytic activity (Pier et al. 2008). Structural modeling of subtypes A1-A4 have revealed interesting differences in the location of the variant amino acids (Arndt et al. 2006) and these

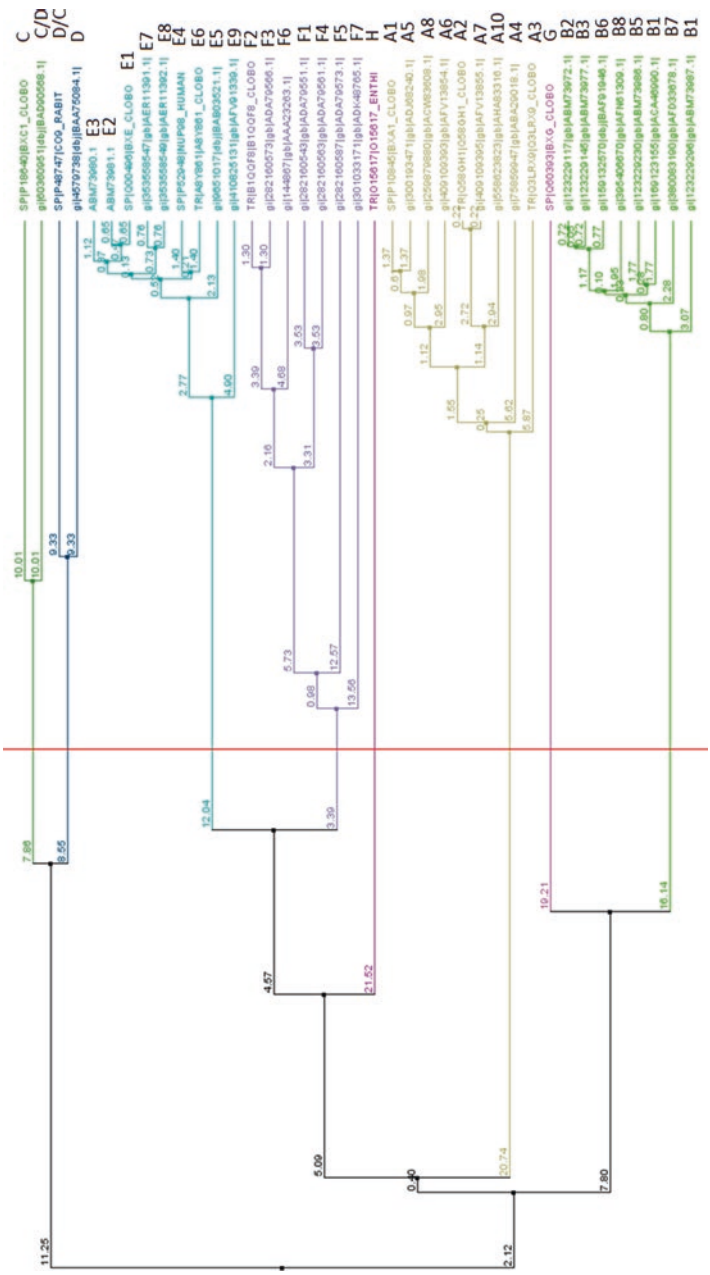

Fig. 2 Phylgenomic diagram of the different serotypes and certain subtypes of *Clostridium botulinum*

differences are likely related to differences in properties of the subtypes (Rossetto et al. 2014). Some of the subtypes within a serotype have distinct and intriguing neurological and pathologic properties in mouse models including rate of onset of symptoms, duration of action, effectiveness of cross-neutralization by antibodies raised against a heterologous subtype, and differences in pathological symptoms (Pellett et al. 2015).

The neurotoxic BoNT component is known to reside in protein complexes (Benefield et al. 2013; Inoue et al. 1996; Lin et al. 2015; Oguma et al. 1995; Sugiyama 1980; Sakaguchi 1983; Sugii and Sakaguchi 1975). The 150-kDa BoNTs exist naturally in two primary types of protein complexes. One class (HA; Type I complex) consists of BoNT associated with hemagglutinins and a protein termed non-toxic non-hemaglutinin (NTNH), and these complexes range in molecular weight from ~500–900 kDa. The second class of BoNT complex (NTNH ("orfX"), Type II) consists of BoNT associated only with NTNH, without the presence of hemagglutinins or other proteins, and has a molecular weight of ~300 kDa. The complexes are also known to contain ribonucleic acid (Schantz and Johnson 1992), but the RNA in the complexes is probably random sequences of rRNA binding through ionic interactions and would not be expected to have a functional role, except for changing the electronic charge of the complex. The complexes are well-known to protect the labile neurotoxins during passage through the gastrointestinal tract, during handling, and likely in the environment (Bonventre 1979; Schantz and Johnson 1992; Ohishi et al. 1977; Simpson 2013; Sugii et al. 1977).

BoNTs are produced as single chain ~150-kDa proteins and undergo post-translational cleavage to the toxic dichain form with HC and LC linked by a disulfide bond (DasGupta and Sugiyama 1972; Sugiyama 1980; Sakaguchi 1983). BoNTs from proteolytic strains of BoNTs A, B, and F are proteolytically cleaved (activated) in native cultures of *C. botulinum*. The inactive 150-kDa single chain BoNT/A is modified to the dichain form by removal of 11 amino acids by native proteases expressed by *C. botulinum* (Krigelstein et al. 1994). BoNTs from proteolytic strains of B and F also undergo native cleavage, but the mechanisms and amino acids removed during conversion of the dichain form remain to be elucidated. In nonproteolytic (physiological Group II) *C. botulinum* types B, E, and F the toxins are not cleaved in culture, and nicking to the dichain form can be artificially achieved by growth in the presence of trypsin or preferably by *in vitro* cleavage to the dichain form (generally with trypsin) (Sugiyama 1980). Following incubation with trypsin, soybean trypsin inhibitor is used in our laboratory to prevent undesired subsequent degradation of BoNT (Pellett et al. 2016). Trypsin treatment cleaves BoNTs at single residues and differs from the native cleavage by clostridial proteases in Group I *C. botulinum* which remove a series of amino acids. This non-native cleavage step by trypsin or other proteases is also necessary in BoNTs produced recombinantly in *E. coli* and other heterologous hosts, and it is not clear whether the differences of in vitro post-translational modification affect biological properties compared to naturally cleaved BoNTs. Cleavage to the dichain form in certain serotypes can result in a 10–100-fold increase in toxicity in mice (Oguma et al. 1995; Sugiyama 1980).

Molecular changes other than cleavage can lead to significant activation and increased potency, but these noncovalent modifications have not been revealed.

Other biochemical and physiological features of BoNTs contribute to their importance as natural intoxicants. BoNT is highly unique compared to most other proteins in that it is stable in the gastrointestinal tract and is absorbed in its intact form into the lymph and blood stream on ingestion (Simpson 2013). In contrast, most other ingested proteins in the diet are degraded in the intestinal tract and are not translocated through the intestinal mucosa to the circulatory system except in the case of sepsis or disruption of intestinal barriers. The stability in the gastric tract and uptake through the intestinal lumen and to the lymph and blood stream may be a common feature of certain microbial enteric protein toxins. The mechanism of intestinal transfer of BoNTs is an active area of study, and current proposed mechanisms have been reviewed (Simpson 2013).

Pathology of Botulism in Humans

The historical basis for the pathology of BoNT was described in section "Brief Historical Description of the Pathology and Microbiology of Botulism: The Foundation for Understanding the Disease and for Control of *C. botulinum* Intoxications", and newer aspects of pathology are described in this section. BoNT affects the neuromuscular system, in which the functional structure is the motor unit (Buchtal et al. 1980). The motor unit emanates from the anterior horn of the spinal cord and the cranial motor nerve nucleus in the brain stem (Ropper et al. 2014). Motor and sensory nerves of the motor unit innervate muscle at the neuromuscular junction (NMJ) (Pytel and Anthony 2015; Sanes and Lichtman 2001). Both striated cranial and skeletal muscle and unstriated smooth muscle are affected by BoNT-intoxicated nerves. Botulinum toxin inhibits vesicular neurotransmission from nerve terminals, resulting in blockage of exocytosis of neurotransmitters (Rizo and Xu 2015; Rossetto et al. 2014). BoNT acts specifically on the nervous system due to its affinity for ganglioside and protein receptors, and by its ability to be internalized into the nerve cytosol by endocytosis and channel formation (Pirazzini et al. 2016), and cleavage of the SNARE proteins SNAP-25, VAMP I and II, and syntaxin (Schiavo and Montecucco 2000; Rossetto et al. 2014). Vesicular neurotransmission also occurs in non-neuronal organs such as the endocrine system for release of hormones, for instance, insulin. However, non-neuronal organs do not possess polysialogangliosides and the protein receptors for BoNT, and some organs may not contain the BoNT-specific SNARE protein substrates (SNAP-25, VAMP I and II, and syntaxin). One example is endocrine cells that contain SNAP-23, which is not cleaved by native BoNTs. When genetic modifications of BoNT are made for cleavage of SNAP-23 (Sikorra et al. 2015), inhibition of release of hormones can occur (Masuyer et al. 2014). The severity of symptoms and duration of botulism in cell and animal models depends on the serotype and subtype of BoNT, generally increasing in the severity

and duration in the order BoNT/A ≥ BoNT/C1 > BoNT/B > BoNT/F > BoNT/E (Foran et al. 2003a, b; Johnson and Montecucco 2008).

Botulism also affects the autonomic nervous system, including, the intestinal musculature, bladder, intestines, sweat glands, and eye pupils, (Dressler and Benecke 2003; Jenzer et al. 1975; Merz et al. 2003; Tintner et al. 2005). BoNT/B intoxication is often associated with autonomic symptoms (Goode and Shearn 1982; Merz et al. 2003). Case reports have described disturbances in the autonomic system, such as dry mouth and throat, postural hypotension, anhydrosis (lack of sweating), urine retention due to absence of bladder contraction, sialorrhea, erectile dysfunction, pupil dilation, and effects on smooth muscle in the esophagus and intestinal tract (Dressler and Benecke 2003; Merz 2003).

Although the effects of BoNT on smooth muscle are less studied than striated muscle, BoNT causes a long lasting paralysis of smooth muscle. Smooth muscle is a major component of the walls of hollow organs, including the gastrointestinal tract, the trachea, bronchi of the respiratory system, blatter, uterus, blood vessels in the cardiovascular system, and the urogenital system. Smooth muscle contracts more slowly than skeletal muscle, thus often exhibits sustained contraction, and also relaxes more slowly than skeletal muscle (Brozovich et al. 2016). Pharmacological treatments of smooth muscle with BoNT can cause long-lasting denervation, often of a year or more (Truong et al. 2013).

The pathology of botulism intoxication and recovery follows these general steps (Montecucco et al. 1994; Schiavo et al. 2000; Foran et al. 2003a, b; Simpson 2013):

1. Absorption of BoNT into the lymphatic system and then into the vasculature through the gastrointestinal tract, wounds, or by inhalation
2. Exit from the vasculature and binding to gangliosides and receptors on neurons
3. Vesicular endocytosis and entry of LC into the neuronal cytosol, leading to cleavage of SNARE protein substrates
4. Catalytic cleavage of the SNARE substrates SNAP-25, VAMP I and II, and syntaxin according to serotype
5. Inhibition of trafficking of vesicles containing acetylcholine (or certain other neurotransmitters) to the membrane and stopping of exocytosis
6. Postsynaptic effects on muscle, including denervation of muscle fibers is followed by atrophy and disruption of ion movement in muscle membranes
7. Eventual recovery of the NMJ block occurs through neurite sprouting and reestablishment of the original NMJ
8. Gradual neuronal and muscle recovery from the intoxication. Since nerve and muscle cells are not killed, the original NMJs are replenished.

Irrespective of the category of botulism, the primary clinical signs are similar (Table 1). The characteristic symptoms of botulism can be principally ascribed to the blockade of acetylcholine transmission at neuromuscular junctions of cranial and skeletal muscle (Dickson 1918; Koenig 1971; Cherington 1998, 2004). The disease initially affects the 12 cranial nerves in the facial region and presents in patients with bilateral vision impairment, ptosis of facial features, dry mouth (due

to lack of production of saliva), slurred speech, and difficulty chewing and swallowing. This pathology is followed by weakness in the neck, shoulders, chest, muscles of respiration, particularly the diaphragm with accompanying labored breathing, and eventually weakness in the legs, arms and hands (Cherington 1998, 2004; Johnson and Montecucco 2008). Although the different forms of botulism show similar clinical signs and symptoms, there are certain distinctions. Before the onset of foodborne paralytic botulism symptoms, patients may have gastrointestinal symptoms such as nausea, vomiting, abdominal cramps and diarrhea (Hughes et al. 1981; Johnson and Montecucco 2008). These symptoms are probably not due directly to BoNT but from components of the spoiled food. Infant botulism differs from other forms of botulism in the ages of the affected individuals and intestinal colonization by neurotoxigenic clostridia Infant botulism often begins with severe constipation, lasting 3 days or longer, that precedes the appearance of other neurologic signs affecting cranial and somatic neuromuscular systems (Arnon 2013; Johnson and Montecucco 2008). BoNT does not usually cause a fever, affect consciousness or alter blood pressure. Physicians often refer to botulism symptoms as a classical triad: bulbar palsy and descending paralysis, lack of fever, and clear mental status (Arnon 2013).

An intriguing question is the progression of symptoms in botulism. Botulism symptoms nearly always first begin with cranial neuropathies and then descend bilaterally to the respiratory diaphragm and skeletal muscle. The initial effects on cranial nerves is likely due to the high level of innervation of cranial muscles and the size of the musculature, i.e. precise and small muscles affecting the eyes (Ruff 2002). The number of muscle fibers within each motor unit throughout the body differ markedly (Ropper and Adams 2014). The degree of innervation is higher for the cranial musculature than for skeletal muscle. Muscles with high precision movement, such as eye muscles, have a high neuron to muscle fiber ratio (ca. 1:10), whereas muscles with relatively extensive and broad movements such as calf muscles, have a much lower neuron to muscle ratio (ca. 1:2000) (Ropper et al. 2014). The high level of nerve to muscle ratio in the cranial motor units probably is the reason that the initial symptoms of botulism always involves cranial nerves, particularly eye movement and vision as well as cranial nerves of the face and mouth, and then is followed by paralysis of the mouth and skeletal muscle of the torso where the ratio of nerve to muscle in the motor units is less. Other factors could be involved in the progression of symptoms in botulism such as differential exit from the blood stream and differences in NMJ structure and composition, but more studies are needed to further understand the progression of symptoms in botulism.

In humans, BoNT does not appear to accumulate in cells or tissues and when exocytosis of neurons is disrupted by intoxication they are generally not sensitive to further uptake of BoNT by primary recycling vesicular transport (Dong et al. 2006; Harper et al. 2016). A minor proportion of BoNT is internalized into nerves by non-recycling vesicles (Harper et al. 2016). Recent evidence supports that similar to tetanus neurotoxin, BoNT can undergo transynpatic transport to the CNS and this is an exciting and active area of study (Mazzochio and Caleo 2015; Wang et al. 2015).

Epidemiology of Botulism

The USA has one of the highest incidences of botulism worldwide, with about 120–150 confirmed cases per year (CDC 2012). The annual occurrence of botulism in the USA usually comprises 10–30 cases of foodborne botulism, 70–130 cases of infant botulism, and 10–30 cases of wound botulism. (www.cdc.gov; www.cdc.gov/nationalsurveillance/pdfs/botulism_cste_2014.pdf). Various countries have different incidences of foodborne and infant botulism (Hauschild 1989, 1993; Johnson and Goodnough 1998), which is related to the level of spores in the environment and in the raw commodity or formulated foodstuff, food processing and handling practices (Dodds 1993; Hauschild 1993). The differences in the incidence of infant botulism worldwide is related to the number of spores in infant foods and the environment (Dodds 1993; Koepke et al. 2008). The food supply for various countries is increasingly global and spores in foods such as honey, which is often formulated from different geographical regions, and caution is needed in interpreting the causative geographic region leading to different incidences of infant and foodborne botulism. The epidemiology of botulism has been described in several reviews (Hauschild 1993; Johnson and Goodnough 1998; Sobel et al. 2004; Koepke et al. 2008), although most of these reviews do not cover the past two decades so updates will be valuable.

Categories of Botulism

Foodborne Botulism

Foodborne botulism is mainly caused by the ingestion of foods containing preformed BoNT. On ingestion, BoNT is absorbed from the small intestine into the lymphatic system and is trafficked to lymph nodes and then into the circulation. BoNT then exits the circulation through capillaries and is bound by nerves in the peripheral nervous system. The period from exposure to BoNTs to onset of symptoms ranges from 4 h to as long as 10 days, but typically presents within 8–36 h (Johnson and Montecucco 2008). The onset period, duration, and severity of foodborne botulism is correlated with the quantity of BoNT ingested (Nishiura 2007). Although infrequently described, it is conceivable in many cases of foodborne botulism that the consumed food contains not only preformed BoNT but a mixture of BoNT, vegetative cells, and spores of *C. botulinu*m. The presence of cells and spores in the ingested foods may permit some growth and BoNT formation in the gastrointestinal tract, potentially prolonging the duration and the severity of botulism.

Most foodborne botulism outbreaks worldwide are due to home-prepared and preserved foods, mainly involving vegetables, fruits, and fish (CDC 1998; Hauschild 1989, 1993; Johnson and Montecucco 2008). Commercial- and restau-

rant-prepared foods have also been responsible for foodborne botulism outbreaks in accordance with a changing epidemiology involving restaurant-associated botulism (MacDonald et al. 1986). Prominent recent commercial examples include canned and frozen chili and carrot juice (Juliao et al. 2013; Sheth et al. 2008). Botulism occurring in prisons from fermented "Pruno" (Walters et al. 2015) illustrates how BoNT can easily be formed in beverages and foods using common food substrates with high levels of spores such as potatoes (Angulo et al. 1998). Fermented potatoes have been involved in all the outbreaks of Pruno botulism (Vugia et al. 2009; Walters et al. 2015).

In nearly all cases in the USA, foodborne botulism is caused by proteolytic *C. botulinum* (Group I) strains due to the heat and chemical resistance of the spores, which can survive home-canning and many commercial food processes and formulations, and can outgrow and produce BoNT in the product (Johnson 2013). Most of the outbreaks in commercial foods have been due to insufficient processing to kill spores of proteolytic strains of serotypes A and B retaining viable spores, and the major contributing factor then is temperature abuse of the food (Glass and Johnson 2002; Setlow and Johnson 2013). In current food production, there is a trend for "natural" foods and "clean labels" with elimination of preservatives and traditional secondary barriers. Many of these foods rely primarily on refrigeration for microbiological safety and do not have secondary barriers to prevent growth during temperature abuse (Glass and Johnson 2002; Johnson 2013). Ensuring safety of minimally processed foods by refrigeration is a particularly susceptible risk factor in food safety (Glass and Johnson 2002).

The level of spores in food commodities is directly related to the incidence of botulism (Hauschild 1989, 1993; ICMSF 2005). Many soil-grown vegetables have higher levels of spores than tree fruits, milk, and meats, including poultry, beef, and pork (ICMSF 2005). Fish and shellfish as well as marine mammals and products such as fish eggs may contain high levels of type E spores (ICMSF 2005). Certain foods grown in soil, including mushrooms, garlic, onions and potatoes or foods that may be cultivated in or fertilized with manure such as *Agaricus* mushrooms can contain high levels of spores and have been responsible for outbreaks of foodborne botulism (Johnson 2013; CDC 1998; Smith and Sugiyama 1988). The intestinal contents of many animals (but not humans) contain spores of *C. botulinum* and it is important during processing that the intestinal contents be separated from the meat to the extent feasible. Although healthy humans are not a known carrier of botulinum spores, it is interesting that gorillas (*Gorilla gorilla*) appear to harbor botulinum spores (Bittar et al. 2014). *C. botulinum* spores have also been reported from an 11th century A.D. pre-Columbian Andean mummy (Santiago-Rodriguez et al. 2015). Microbiome studies have begun to explore the microbiota of colonized infants (Shirey et al. 2015), and this approach will be useful in defining the effect of competitor organisms on the ability of neurotoxigenic clostridia to colonize the intestinal tract of humans of various ages and health.

Infant Botulism

Infant botulism, also known as floppy baby syndrome, is the most common form of botulism in the USA, with 70–130 confirmed cases per year (CDC 2012). Infant botulism is also the predominant form in certain other Western countries (Arnon 2013; Koepke et al. 2008), whereas it is very rare in certain European countries such as the United Kingdom (Johnson et al. 2005). Infant botulism is caused by oral ingestion of spores, their colonization of the large bowel and formation of BoNT that is absorbed into the lymphatic system from the cecum. Infants are susceptible from a few days following birth up to 12 months, although >90% of cases occur within 6 months of age (Arnon 2013). There have been very few known deaths from infant botulism, although it has been suggested that fulminant cases may be the cause of a substantial number of cases of sudden infant death syndrome (Bartram and Singer 2004; Byard et al. 1992; Marx 1978; Nevas et al. 2005). Symptoms of infant botulism usually begin with constipation, and then cranial and somatic nerves are affected, including eye movements, ptosis of the face, weak suck, difficulty feeding, lethargy, and generalized weakness (Arnon 2013). In severe cases, a near complete flaccid paralysis is observed together with respiratory impairment. A higher proportion of infants compared to adults need to receive mechanical ventilation, possibly due to the smaller size of muscles involved in respiration at their age (Johnson and Montecucco 2008).

Nearly all infant botulism cases have been caused by proteolytic *C. botulinum* (Group I) *C. botulinum* strains producing BoNTs A and B (Arnon 2013). Several cases of infant botulism in the USA and Italy have also been caused by *C. butyricum*-producing BoNT/E and *C. baratii*-producing type F (Arnon 2013; Franciosa et al. 2003; Hannett et al. 2014; Hatheway 1995; McCroskey et al. 1991). Rare cases of infant botulism have been caused by *C. botulinum* serotypes C, E, and FA (Barash and Arnon 2014; Lúquez et al. 2010; Oguma et al. 1990). *C. baratii*-producing BoNTs E and F have become more prevalent in causing infant botulism (Arnon 2013; Franciosa et al. 2003). The properties of the BoNTs that cause type E and F infant botulism are similar to their counterparts in foodborne botulism from *C. botulinum* types E and F. The ability for spores to colonize the cecum is likely related to undetermined virulence traits in neurotoxigenic clostridia, including attachment within the intestinal mucus, selective nutrient acquisition, and by the competitive microbiota in the infant intestine (Smith and Sugiyama 1988; Sugiyama personal communication). The main competitive flora affecting *C. botulinum* colonization have not been defined, although studies in animals have revealed that lactic acid bacteria may be important competitors (Moberg and Sugiyama 1979; Wells et al. 1982). As few as 10–100 spores have been suspected to cause infant botulism based on the spore levels in retained foods, including honey (Arnon 2013), and levels of spores colonizing animal models (Wang and Sugiyama 1984). Honey is the only food proven to be a vehicle of infant botulism, while many foods that contain *C. botulinum* spores such as soil-grown vegetables could be potential sources if used as infant foods. However, current evidence suggests that most spores are derived not

from food but from dust and possibly other natural vectors in the environment. Many outbreaks have occurred in sites that are undergoing construction or soil movements, or possibly from persons such as parents that work in jobs where they frequently contact dust (Arnon 2013; Nevas et al. 2005).

Adult Intestinal Botulism

Botulism in patients 1 year of age or older typically results from the ingestion of BoNT in foods or during infection of wounds. The CDC initially had a category "classification undetermined" that included cases in patients over 1 year of age in which it was not possible to implicate a food source (Chia et al. 1986; Morris and Hatheway 1979; Sam and Beynon 2010; Shen et al. 1994; Sheppard et al. 2012). It was postulated that these patients had intestinal infections due to *C. botulinum* with accompanying production of BoNT (Morris and Hatheway 1979). Intestinal infections by *C. botulinum* in adults had been proposed in 1925 (Starin and Dack 1925) and this hypothesis was supported by feeding adult animals large doses of spores of *C. botulinum* (cited in Chia et al. 1986). Adult intestinal botulism resembles infant botulism in that *C. botulinum* colonizes the adolescent or adult intestinal tract and produces BoNTs. Adult intestinal botulism was initially confirmed in a woman following a truncal vagotomy antrectomy, (Chia et al. 1986), with BoNT/A detected in serum and stool by the mouse bioassay and *C. botulinum* but not pre-formed BoNT/A was detected in a jar of cream of coconut in her refrigerator that she had consumed and in her stools. In another intriguing incident, two seemingly unlinked type E botulism cases in Italy occurred in 1995 and 1997 in a 9-year-old child and a 19-year-old woman (Fenicia et al. 1999). These patients had undergone a laparotomy, and the surgery and associated antibiotic use may have permitted colonization by *C. butyricum* type E. The isolates were genetically and phenotypically identical to the *C. butyricum* type E strains isolated from infant botulism in 1984–85 in Italy (Franciosa et al. 2003). *C. botulinum* has not been detected in feces of healthy human adults (Dowell et al. 1977), although spores are present in many animals and possibly in nonhuman primates (Bittar et al. 2014; Shirey et al. 2015; Smith and Sugiyama 1988). C. *botulinum,* but not preformed botulinum toxin, has also been found in certain foods causing botulism, and in some cases these have been reported to be more severe. In an intriguing case, a patient with obstruction of the terminal ileum from Crohn's disease developed complete paralysis, and was suspected of having Guillain-Barré syndrome (Griffin et al. 1996) over several weeks. BoNT/A was detected in serum and stool specimens and a *C. botulinum* type A strain was isolated from stools confirming a diagnosis of botulism. Antibodies to BoNT/A were detected in the patient's serum after 19 weeks and remained at a protective level for more than a year. These cases highlight the presence of intestinal botulism in adolescents and adults, usually following surgery, possibly antibiotic use, or GI tract alteration of the microbiota.

Wound Botulism

Wound botulism, formerly a rare type of botulism, was a suspected form of botulism for many years, and was confirmed in the 1940s and 1950s (Davis 1951; Hatheway 1995; Weber et al. 1993). In the past two decades, wound botulism has become more frequent due to "skin popping" of street drugs and from "snorting" of cocaine (Maselli et al. 1997; Roblot et al. 2006; Werner et al. 2000; Sandrock and Murin 2001; Tucker and Frazee 2014; Yuan et al. 2011). These actions cause skin disruptions that can provide an anaerobic environment for spore exposure, outgrowth and vegetative cell colonization and production of BoNTs.

Latrogenic Botulism

Botulism related to injection of commercial pharmaceutical BoNT preparations and non-approved preparations, has caused serious cases of botulism in recent years (Ghasemi et al. 2012; Johnson and Montecucco 2008). Some of the cases in which approved commercial preparations were used involved treatment of patients with underlying diseases involving the NMJ, especially Myasthenia Gravis and Guillain Barré Syndrome (Dressler 2010; Watts et al. 2015). Counterfeit pharmaceutical botulinum toxin preparations not produced under appropriate GMP facilities and without required quality control have been widely recognized, particularly on the internet, and use of these products could lead to iatrogenic botulism (Pickett and Mewies 2009). The severity of symptoms caused by illicit injection of high quantities of botulinum toxin was vividly illustrated in 4 patients treated with very high doses of nonapproved BoNT for aesthetic purposes (Chertow et al. 2006). The patients were injected with as much as ca. 2857 times the estimated human lethal dose. Serum samples contained 21–43 times the estimated human lethal dose. The clinical findings were typical of other categories of botulism, and the patients required ventilator support for 36–171 days, although administration of antitoxin was helpful in sequestering BoNT in the serum and enhancing recovery. In a concise and well-controlled medical study, high doses of BoNt/A were injected into large muscles in the neck for cervical dystonia without detrimental effects (Dressler et al. 2015), thereby indicating that properly injected BoNT, even at relatively high therapeutic doses, does not usually spread systemically.

Inhalational Botulism

Due to their high toxicity and potential for causing mass casualties, BoNTs have been considered as bioterrroism weapons (Arnon 2001). A potential route of botulism in bioterrorism incidents, analogous to other nerve agents, is by exposure to

aerosols. Animal studies have revealed that intoxication can occur by inhalation and passage through mucosal membranes and entry into the circulatory system (Park and Simpson 2003; Pitt and LeClaire 2005). Since inhalation exposure is not a known natural route of BoNT intoxication, the estimated doses to cause botulism have been based on rodent and primate exposure and little data is available in the public domain. The LD_{50} in rhesus macaques has been estimated at 200–500 and 21,600 LD_{50s} for types A and B, respectively (Franz et al. 1993; Sanford et al. 2010). Inhalational botulism has been extremely rare in the laboratory environment, and only one incident has been attributed to inhalation of BoNT from animal fur in a laboratory accident (Holzer 1962).

Diagnosis of Botulism

The initial diagnosis of botulism is based on detection of characteristic clinical signs (Cherington 1998, 2004; Sobel 2005) that include oculobulbar disturbances initially affecting eyes, face and mouth, followed by generalized weakness, and fatigue (Johnson and Montecucco 2008; Cherington 1998, 2004; Sobel 2005). The diagnosis of botulism is often supported by an epidemiologic survey surrounding the incident, with information including illnesses occurring in persons who consumed food in the same household or restaurant, the different foods consumed, preparation of the foods, temperature profile of the suspect foods, and other relevant aspects that would enable *C. botulinum* to grow and produce BoNT. Clinical diagnosis of botulism can be difficult since the symptoms can mimic other neurological diseases and intoxications affecting the NMJ (Table 2) (Francisco and Arnon 2007; Merriggioli et al. 2004; Shapiro et al. 1998). Being an extremely rare disease, many physicians do not have experience in diagnosing botulism. Electrodiagnostic testing can be valuable in the diagnosis of botulism (Cherington 2004; Merriggioli et al. 2004). Guidelines for electrodiagnostic testing for botulism have been outlined

Table 2 Case definitions of potential botulism and confirmed botulism and their diagnosis

I. Potential botulism. This category includes a person who has myasthenic symptoms typical of botulism and several other diseases. Recognition of clinical symptoms and signs. Neurological examination, including electromyograpy
II. Confirmed botulism. A confirmed case of botulism involves two or more of the symptoms listed in Table 1 and who meet one of the following conditions:
1. The identification of botulinum neurotoxin in an implicated food; or in serum, stool, gastric aspirate, or vomitus collected from the person. The confirmatory test usually utilizes the traditional mouse bioassay but detection of BoNT and the gene encoding BoNT in certain cases can include indirect methods including ELISA, Mass Spectroscopy, and/or PCR
2. The isolation of *C. botulinum* organism from the persons' stool, serum, or gastric aspirate vomitus
3. A history of eating the same implicated (confirmed) food as a person meeting one of the first two conditions

(Cherington 1998, 2004; Maselli and Bakshi 2000; Merriggioli et al. 2004). However, review of botulism case reports has revealed that electrodiagnostic testing may be difficult, variable, and inconsistent in the diagnosis of botulism (Cherington 1998, 2004; Merriggioli et al. 2004). Furthermore, EMG testing capabilities may not be available for botulism outbreaks in many hospitals and in countries having limited neurological diagnostic capabilities.

Laboratory Confirmation of Botulism

Clinical symptoms must be substantiated by laboratory tests for BoNT and/or *C. botulinum* to be confirmed as a case of botulism (Cherington 2004; Hodowanec and Bleck 2015; Maslanka et al. 2013; Sharma and Whiting 2005; Solomon and Lilly 2003; Woodruff et al. 1992). The case definition of botulism has been described by the Centers for Disease Control and Prevention (CDC 2015, (http://www.cdc.gov/mmwr/pdf/rr/rr4610.pdf)). A laboratory-confirmed case of botulism must meet at least 1 of 3 criteria: (1) detection of BoNT (usually by mouse bioassay) in a clinical specimen obtained from the patient, including serum, vomitus and/or stool; (2) the detection of BoNT in the suspect food and/or, (3) isolation of *C. botulinum* from the patient's feces and/or the ingested food (Table 3). Diagnostic tests must be

Table 3 Differential diagnosis of botulism from other disorders (CDC 2006; Caya et al. 2004; Meriggoli et al. 2004)

Differential diagnoses for botulism in adults, adolescents, and infants	
Adults and children	Infants
Meningitis	Sepsis, meningitis
Guillain-Barré Syndrome (GB)	Guillain-Barré Syndrome (GB)
Myasthenia Gravis	Myasthenia Gravis
Lambert-Eaton syndrome	Acute infantile neuropathy
Cerebrovascular accidents	Meningitis/Encephalitis
Acute intermediate porphyria	Metabolic disorders, e.g. electrolyte imbalance
Carcinomatosis of cranial nerves	Reye's syndrome
Neoplasm of CNS	Neoplasm
Tick paralysis	Congenital myopathy
Diphtheritic neuropathy	Enteric virus
Polymyelitis	Poliomyelitis
Miller-Fischer variant of GB	Werdnig-Hoffman disease
Food poisoning (e.g. Saxitoxin)	Leigh disease
Chemical neurotoxin exposure	Chemical neurotoxin exposure
Mushroom poisoning	Food poisoning
Neuronal viral infection	Neuronal viral infection

performed in an approved laboratory according to biological safety and Select Agent requirements (http://www.selectagents.gov/Regulations.html) and laboratory safety guidelines BMBL (http://www.cdc.gov/biosafety/publications/bmbl5/). When animals are used, the facilities must be AALAC approved), and in accordance with appropriate protocols. The laboratory personnel must have expertise for safe handling of BoNT, as well as necessary materials for a confirmatory assay, including reference toxins, validated antitoxins, and standard media and protocols for detection of *C. botulinum*. In certain putative cases of botulism, it can be difficult to detect BoNT in the clinical and food samples, which is often due to delays or inability to obtain clinical specimens and food items, handling of the samples, and the presence of toxicants other than BoNT (Hatheway 1979, 1988; Maslanka et al. 2013; Merson and Dowell 1973). In complex matrices such as foods and stools, the assay method needs to be robust and can tolerate nontoxic and non-botulinum lethal materials that are often present (Hatheway 1988).

Assays used for definitive determination of BoNTs should embody all four physiological steps involved in the intoxication process to reach an unambiguous conclusion of BoNT toxicity related to pathology. Currently the mouse bioassay and neuronal cell-based assays (for BoNT therapeutic preparations) are the only assays approved that require the activity of all four steps. These four steps entail (Johnson and Montecucco 2008):

1. Binding of circulating BoNT to polygangliosides and protein receptors on the surface of nerves or neuronal cells
2. Endocytosis in vesicles into the nerve cytosol
3. Translocation of the light chain from the vesicles
4. Specific enzymatic cleavage of SNARE substrates

The "gold-standard" assay for BoNT detection, and the only method currently accepted by the FDA and CDC, is the intraperitoneal mouse bioassay (Cunniff 1995; Hatheway 1988; Kautter and Solomon 1977; Maslanka et al. 2013; Schantz and Kautter 1978). The mouse assay is extremely sensitive, and the quantity of BoNT for 50% death in a population of ~20-g-mice (MLD_{50}) is generally 5–10 pg, depending on the serotype. For the mouse bioassay, samples are usually diluted in sodium phosphate buffer within a pH range of 6–6.8, and with 0.2% gelatin to stabilize BoNT in the presence of potential interfering compounds and at high dilutions of the toxin. In the standard assay, 0.5 ml is intraperitoneally (ip) injected into mice. Treatment with trypsin or other proteases for BoNT activation is required for certain BoNT serotypes, particularly BoNT/B, /E and /F produced by nonproteolytic strains. Certain controls need to be employed, including heating of samples (e.g., 80 °C for 10 min), to destroy BoNT. A second essential control is antibody neutralization of BoNT activity. Samples are mixed with serotype-specific antibodies to confirm the lethality is due to BoNT and not nonspecific toxic substances often present in food and clinical samples. A toxin standard should also be included in the assays. Mice are observed for 1–4 days for characteristic botulism symptoms and death (Hatheway 1988; Maslanka et al. 2013; and by the FDA method, (http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070879.htm)). Mouse

symptoms depend on the serotype but typically include ruffled fur, labored abdominal breathing, a pinched or wasped waste, weakness of the limbs, dragging of the hind legs, gasping for breath, and spasticity immediately prior to death. Depending on the serotype and quantity of toxin in the samples, mice generally become sick within 4–12 h and fatalities often occur within 12–48 h, while some serotypes and subtypes of BoNT require observation for up to 7 days. The sensitivity of the mouse LD_{50} assay is 5–10 pg of BoNT, with the limit of detection being 0.01 ng per ml (Smith and Sugiyama 1988). A more rapid assay and using fewer mice involves the injection of 0.1 ml by the intravenous route and BoNT titer is determined from a standard curve of LD_{50s} of a specific serotype of BoNT vs. time to death (Boroff and Fleck 1966; Malizio et al. 2000). However, this assay causes massive systemic botulism and confirmation should be performed with an ip assay with appropriate dilutions of the extracted samples.

Certain difficulties are often encountered in the mouse ip bioassay for BoNT, especially nonspecific deaths in mice due to toxic lethal non-BoNT substances present in clinical samples and in foods, and sensitivity of detection in serum and food matrices (Hatheway 1998; Johnson and Montecucco 2008; Wheeler et al. 2009). Non-BoNT substances can show similar signs to those of botulism, and thus death must be reached as an endpoint to confirm the presence of BoNT. To definitively demonstrate the presence of BoNT in complex matrices it is necessary to conduct neutralization assays with serotype-specific antibodies to ensure toxicity is due to BoNT and not to other components in foods and clinical samples. It may be necessary to prepare high dilutions of the samples to exceed the threshold of substances causing nonspecific deaths relative to BoNT. Other potential confounding issues with the mouse bioassay need to be considered, including assay of BoNT toxicity from *C. botulinum* strains that produce more than one serotype of BoNT, often with one of the toxins in excess (Giménez and Giménez 1993; Hatheway 1988). In extracts that contain more than one serotype of BoNT, mixtures of antibodies may be required for neutralization. Reviews of the problems that may be encountered in the mouse bioassay have been expertly described (Hatheway 1988). Other important issues include the time-consuming labor involved, the requirement for animal facilities and highly trained scientists to perform the test, and the ethical use of animals.

Neuronal cell-based assays also require all four steps of intoxication in the measurement of BoNT toxicity (Pellett 2013). Two neuronal cell assays have been approved by the FDA and by regulatory agencies in certain European countries for determination of the concentration of BoNT in pharmaceutical preparations (http:/agn.client.shareholder.com/release detail.cfm?ReleaseID = 587,234; https://www.merz.com/blog/news/botulinum-neurotoxin/). Neuronal cell assays can have greater sensitivity and a smaller variation in results compared to the mouse bioassay (Pellett 2013). Neuronal cell assays have been useful for detection of BoNT in serum (Pellett 2013), but they have not been qualified for detection of BoNT in foods or feces. Neuronal cells may not be as robust as mice injected intraperitoneally with samples in complex matrices, and further research is required to develop and validate neuronal assays for food products and clinical samples. Other limitations

compared to the in vivo mouse bioassay is that cell-based assays do not provide a model for BoNT distribution, clearance, transport and other properties of in vivo assays in mice and other animal models.

The rodent hemidiaphragm assay has been used for determination of BoNT activity, particularly in Europe. Although the assay is sensitive and can determine approx. 1 mouse LD_{50} depending on the serotype, it requires considerable expertise and still uses animals yet at reduced numbers. Generally the sample preparation must possess relatively pure BoNT, and usually the serotype must be known prior to testing.

Another major class of BoNT assays uses antibodies for detection of BoNTs (Capek and Dickerson 2010; Ferreira et al. 2004; Linstrom et al. 2006; Maslanka et al. 2011, 2013; Scotcher et al. 2010). Antibody-based tests have been developed in a variety of platforms (Capek and Dickerson 2010). Some of the most commonly used formats are sandwich ELISA, Western blot, and ELISA coupled to cleavage of BoNT SNARE substrates (Capek and Dickerson 2010; Hallis et al. 1996). Current ELISA platforms generally have sensitivity to detect 0.5–10 mouse LD_{50s}. Other antibody platforms include flow through cells, immuno-PCR, immunoprecipitation coupled with enzymatic assays, Western blot, and electochemiluminescence (Capek and Dickerson 2010; Grenda et al. 2014). ELISA formats can be multiplexed to detect more than one serotype of BoNT (Singh et al. 2015). A highly sensitive antibody-based assay (ALISSA) for BoNTs has been developed by capturing BoNT on beads followed by assay Kalkum and colleagues (Bagramyan et al. 2008). For antibody-based assays to give definitive results, a high signal to background ratio and high reproducibility among different laboratories is required. One of the primary factors affecting sensitivity and specificity is the quality and uniformity of the antibodies used in the assays. ELISA has been useful for screening of the toxicity of BoNT in foods and clinical samples (Lindström and Korkeala 2006). A number of variations of antibody-based assays for enhanced sensitivity and specificity have been described, including increased sensitivities and specificity for BoNT in food systems (Capek and Dickerson 2010). Monoclonal antibodies have been developed for capture which can increase the specificity and discrimination of epitopes of BoNTs (Scotcher et al. 2009; Stanker et al. 2008). There are certain practical drawbacks to ELISA and other antibody-based tests for BoNTs. All antibody-based tests can yield false-positive reactions since inactivated BoNT as well as BoNT fragments can be detected leading to inaccurate results. Components of the food matrix and clinical samples can also interfere with the detection of BoNTs.

A third commonly used group of assays for BoNTs is based on determining single steps in the four-step intoxication process, but these must be interpreted with caution since the full intoxication process is not evaluated. Several platforms have been developed to detect the catalytic step of BoNT acting on its specific SNARE substrates (Capek and Dickerson 2010). A similar class entails binding of BoNTs to protein receptors and/or gangliosides, and this step may be coupled with its catalytic activity, or a combination of both (Capek and Dickerson 2010). A rapid and sensitive test is mass spectroscopy for detection of BoNT substrate cleavage products (Kalb et al. 2015). Mass spectrometry also enables analyses of kinetics and

determination of the cleavage site on the substrate. BoNTs in food and clinical matrices can be captured with specific antibodies and then analyzed by mass spectroscopy. Mass spectroscopy is currently being considered as a replacement for the mouse bioassay in certain academic and governmental laboratories, but as with all SNARE cleavage assays, it has the drawback of measuring the catalytic activity which is only one of the four steps of intoxication, and this could lead to false-positive or negative results. This assay needs to be carefully considered and evaluated before it is widely implemented by governmental and commercial testing laboratories.

Another approach for indirect detection of BoNTs and *C. botulinum* cells is PCR for the detection of genes encoding BoNTs or associated complexing proteins (Lindtröm and Korkeala 2006). PCR has been useful in epidemiologic determinations of the presence of *C. botulinum* in food products and clinical samples (Lindström and Korkeala 2006; Fach et al. 2009). PCR has known drawbacks including inhibition of amplification by substances in complex matrices, including foods and clinical samples. Importantly, the detection of a BoNT gene or gene fragment is not necessarily indicative of the presence of BoNT, and results need to be interpreted with caution.

A future milestone for the rapid detection of BoNTs in clinical samples would occur at the point of care (POC), whereby an assay could be performed in the clinic or hospital, ideally within a few hours of collection of clinical samples (Drancourt et al. 2016). POC detection offers the potential to accurately and efficiently identify pathogens and facilitate rapid diagnosis and initiation of therapy. A POC method would also be useful for distinguishing botulism from other more common myasthenic diseases such as Myasthenia Gravis and Lambert-Eaton syndrome. POC methods have been developed for *Clostridium difficile*, *Staphylococcus aureus* MRSA, *Mycobacterium tuberculosis*, and other pathogens in the clinic (Bomers et al. 2015; Catanzaro and Cirone 2012; Drancourt et al. 2016; Goldenberg et al. 2014). POC methods have the potential to decrease patient morbidity and mortality, enhance health care outcomes, and reduce the high costs associated with botulism cases. Currently, most POC methods utilize nucleic acid amplification (Drancourt et al. 2016; Spencer et al. 2015). Mass spectrometry targeting gas emission (volatile organic compounds) in stool samples is also under evaluation for on-site detection of *C. difficile* and potentially other clostridial pathogens (Bomers et al. 2015) and POC methods for determination of proteins such as BoNT are in development. A POC method for detection of BoNT using the protease activity of BoNT LC as a prototype has been proposed (Park et al. 2016).

Culturing of *Clostridium botulinum*

Several approaches have been used for culturing of *C. botulinum* cells from clinical samples and foods (Hatheway 1988; Holdeman and Moore 1977; Maslanka et al. 2013; Lindström and Korkeala 2006; FDA Bacteriological Analytical Methods,

BAM, http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm). For isolation of strains, an anaerobic environment and nutrient-rich media are required. Commonly used media are Trypticase peptone glucose yeast extract medium (TPGY), cooked meat medium (CMM), reinforced clostridial medium (RCM) (Lindström and Korkeala 2006; Maslanka et al. 2013; BAM) and egg yolk agar which is used for detection of lipolytic and lecithinase reactions (Glasby and Hatheway 1985). Enrichment cultures are often required to recover a suitable number of *C. botulinum* cells from samples for enumeration and toxicity testing. Selective enrichment is also useful to dilute out competitor microbiota and enable the growth of *C. botulinum* since the organism is usually a poor competitor in bacterial populations (Glass and Johnson 2002). Quantitation of viable *C. botulinum* cells can be attained by using the most probable number method. Strains may be distinguished by colony morphology and physiological reactions on agar plates or in customized media (Holdeman and Moore 1977).

Molecular Typing of *C. botulinum*

Molecular typing of neurotoxigenic clostridia is valuable for studies of the epidemiology of botulism outbreaks, including gathering information of suspect food products in foodborne botulism (Gerner-Smidt et al. 2006; Lindström and Korkeala 2006). For example, typing of *C. botulinum* strains isolated from an infant with botulism and infant formula present in the household showed that the two strains were different, hence the infant formula was a less likely source of the illness (Johnson et al. 2005). Currently, the most commonly used method of molecular typing for epidemiologic purposes is pulsed-field gel electrophoresis. PFGE was useful in establishing the source of *C. botulinum* in a case of infant botulism in the United Kingdom (Johnson et al. 2005). Methods for PFGE of genomic and plasmid DNA have been established (Lin and Johnson 1995; Lúquez et al. 2015). Standard methods for PFGE used by the CDC have been developed for use in PulseNet (www.cdc.gov/pulsenet/PDF/c-botulinum-protocol-508c.pdf). Isolation of high quality DNA from neurotoxigenic clostridia and particularly from endospores can be problematic because *C. botulinum* is Gram-positive with thick cell walls and spores are encased in a highly impermeable and lysis-resistant form. Additionally, certain serotypes and strains produce nucleases that can degrade the DNA (Linström and Korkeala 2006). Certain *C. botulinum* strains lack S layer proteins which can facilitate lysis and DNA purification.

In addition to PFGE, other methods have been successfully used for molecular typing of *C. botulinum,* including multi-locus-sequence-typing (MLST), DNA microarrays, ribotyping, and several PCR-based methods, such as amplified fragment length polymorphism (AFLP), repetitive element sequence-based PCR (rep-PCR) (Lindström and Korkeala 2006). These methods have been useful in epidemiologic and phylogenomic studies, and identification of strains involved in foodborne and infant botulism outbreaks (Deng et al. 2016; Grenda et al. 2014).).

However, it is becoming apparent that whole genome sequencing (WGS) by next generation sequencing (NGS) methods will supersede methods that only determine a nominal number of genes (Hasman et al. 2014) and extrachromosomal elements of foodborne pathogens, including *C. botulinum*, and it is anticipated that WGS will likely replace PFGE and be adapted for PulseNet and other epidemiologic systems (Carter and Peck 2015; Deng et al. 2016).

Treatment of Botulism

At present, there are no FDA-approved antidotes or treatments for botulism once the BoNT has entered the nerves (Simpson 2013; Johnson and Montecucco 2008). Untreated botulism has a mortality rate of 40–60%, whereas with intensive supportive care, mechanical ventilation, and serum therapy, the fatality rate has decreased to 5–10% (Dembek et al. 2007; Johnson and Montecucco 2008).

Serum therapy, using antitoxins to BoNT for sequestration of the toxin in serum, has helped to improve the outcome of botulism by preventing entry of circulating toxin into nerves (Manohar et al. 2015; Mayers et al. 2001; Thanongsaksrikul and Chaicumpa 2011). Prompt administration of antitoxin can shorten the duration of hospitalization and the time for recovery. Currently, serum therapy is considered an archaic method for control of pathogens or their toxins and is rarely used for infections or intoxications, except for certain natural toxins in the bloodstream (Manohar et al. 2015). For most diseases that were formerly treated by serum therapy, such as diphtheria and tetanus, vaccination provides early and more efficacious protection. However, it is not practical to vaccinate civilian humans against BoNT since botulism is extremely rare and vaccination would also prevent the use of the toxin as a pharmaceutical. Several domesticated animals, including poultry, cattle, horses, goats, fur-farm animals such as minks, foxes and sheep, and wet wild birds may receive vaccinations to prevent botulism (Anniballi et al. 2013).

Antitoxin treatment has advantages and limitations in the treatment of botulism. Some patients have hypersensitivity reactions to antitoxin administration (Black and Gunn 1980; Tacket et al. 1984), but this is likely to be less common in more current preparations such as HBAT. Antitoxin can only be successfully utilized for a limited time after food ingestion and observation of the first clinical signs, typically within 12–36 h. During this initial period, BoNT is circulating in the bloodstream and can physically react with antibodies. Once BoNT enters nerve terminals it is unavailable physically to react with the antibodies. Passive antibody therapy has shown utility in large foodborne outbreaks, where the onset time is variable among the victims, and the window of onset and circulation duration is widened. In certain foodborne botulism cases where a large quantity of toxin is ingested and remains in the sera, serum therapy can also be very effective (Chertow et al. 2006). In some foodborne cases of botulism, spores and cells are ingested in addition to or independent of toxin, and BoNT may be produced for an extended time in the gastrointestinal tract. Serum therapy would be of utility in bioterrorism events, in which toxin

may be ingested or inhaled by humans or animals at various time intervals and doses, and antioxins can be administered prophylactically.

Several antitoxin preparations have been used over the years (Thanongsaksrikul and Chaicumpa 2011). In the 1920s serum therapy was shown to be effective in monkeys (Dack and Wood 1928). Trivalent antitoxin to serotypes A, B, and E prepared against crude toxin precipitates in the 1960s and 1970s was the mainstay therapy until 2010 and was used successfully in many foodborne botulism outbreaks (Hatheway 1988). In 2010, an immunoglobulin ($F(ab')_2$)) hepatavalent antitoxin (HBAT) prepared against purified BoNTs (A, B, C, D, E, F, G) became available for treatment (CDC 2010). Despeciation of HBAT is expected to significantly reduce the risk of hypersensitivity reactions, anaphylaxis, and serum sickness that was occasionally encountered with trivalent antitoxin (Black and Gunn 1980; Hill et al. 2013). In 2003, the FDA approved human botulinum immune globulin for the treatment of infant botulism (Arnon et al. 2006). This product was derived from pooled plasma of adult humans that had been immunized against pentavalent botulinum toxoid. The human-derived product poses fewer risks of anaphylaxis or sensitivity reactions compared to equine antitoxins (Robinson and Nahata 2003).

Several factors affect the efficacy of serum therapy using antitoxins, particularly the affinity for BoNT and duration of the antitoxins in serum (Fagan et al. 2009; Hatheway et al. 1984). Limited studies are available on the half-life of BoNTs and antitoxins in serum. Hatheway and collaborators (1984) provided preliminary data from one patient that received A, B, and E (trivalent) antitoxin which had a half-life of about 6–7 days in serum. A steady state was reached and then the antitoxin was gradually eliminated. There are limited data on the half-life of HBAT. In serum the half-life of HBAT in serum was estimated to be 7.5–34.2 h for type F botulism (Fagan et al. 2011), but these results need substantiation, and with its current more frequent use, a definitive duration should become established. Monoclonal antibodies (MAbs) are also in development to treat human botulism. A three-MAb mixture prepared with human or humanized domains that target different regions of BoNT/A1 was very effective in neutralizing and clearing BoNT from the system in mouse studies (Nowakowski et al. 2002). The three-MAb mixture was well-tolerated and was detected for a minimum of 4 weeks in humans (Nayak et al. 2014).

Recovery from Botulism

Recovery from botulism can be slow, gradual and tedious (Colebatch et al. 1989; Eleopra et al. 1998; Mann et al. 1981; Wilcox et al. 1990). Presynaptic and postsynaptic functions at the NMJ must be restored and muscle strength needs to be replenished. Patients vary in the duration of paralysis from days to several months and the rate of repair and time to full recovery is correlated with the severity of botulism. Botulism is unique compared to certain other more common myasthenic diseases such as Myasthenia Gravis and Guillain Barré Syndrome, in being an acute and not chronic disease, and neuronal and muscle tissue is fully repaired, and complete

recovery is generally achieved unless there are other underlying disease syndromes. During recovery, nerves sprout neurites that may provide minimal innervation of the NMJ. However, the original presynaptic nerve terminals are gradually repaired and the neurites are eliminated (Foran et al. 2003a, b; Johnson and Montecucco 2008). Similarly, BoNT causes paralysis and atrophy of muscle fibers comprised of myocytes, but these cells are not killed by the actions of BoNTs and they fully recover. During intoxication, muscle tissue can undergo atrophy and temporary loss of ion channel activities (Foran et al. 2003a), but these are restored during recovery. Compared to the presynaptic molecular actions of BoNT, relatively little is known of the molecular mechanisms leading to muscle recovery.

Symptoms generally repair gradually during the extended recovery period and different neuromuscular groups vary in progressive recovery (Johnson and Montecucco 2008). Recovery of speech and the ability to swallow returns relatively early. Torso muscular paralysis and weakness also usually returns relatively early in the recovery from botulism. The oculobulbar disturbances are usually the last symptoms to clear. Some patients continue to experience weakness, fatigue, and symptoms of impaired autonomic nervous system dysfunction such as dry mouth, constipation, and impotence even after 1–2 years following onset of botulism (Johnson and Montecucco 2008). Certain botulism survivors have claimed to have chronic botulism, but these conclusions have been based on questionnaires of symptoms such as weakness, while definitive neurological examinations were not performed to substantiate the chronicity of botulism (Sobel 2014). Evidence strongly indicates that patient nerve and muscle function recover completely following the intoxication and full activity of affected persons is complete.

Prevention of Botulism

The primary technologies for preventing the hazard of *C. botulinum* and many other pathogens in foods are: (a) preventing contamination of the raw food commodity; (b) inactivating pathogens including spores by physical treatments such as an extensive thermal treatment (e.g. 12D botulinum cook); (c) formulating botulinal-safe foods by using inhibitory values of pH, a_w, ORP, temperature control, and (d) use of efficacious antimicrobials and other secondary barriers (Glass and Johnson 2002; Johnson 2013). The different parameters for controlling *C. botulinum* growth and BoNT formation can act in combination or ideally in synergy, and this forms the basis of "hurdle" technology for production of safe foods (Gould and Jones 1989; Leistner 1995). Control of growth of *C. botulinum* and BoNT formation should not depend on refrigeration alone, as temperature is difficult to control in the food supply chain and in the home and epidemiologic data reveals that temperature abuse is the most common contributing factor for commercial outbreaks of botulism (Glass and Johnson 2002; Johnson 2013). The prevention of infant botulism is more difficult. The prevalence of *C. botulinum* spores in the baby's household environment ideally should be minimized, including the feeding of foods that may contain spores

such as honey. Precautionary avoidance of infant foods up to 1 year of age, including home-prepared baby foods from ingredients that are known to contain *C. botulinum* spores such as soil-cultivated vegetables could reduce the incidence of infant botulism. The control of spores in the environment of a child is difficult to prevent, but minimal exposure to dust such as occurs in construction sites or on clothing would be beneficial.

Perspectives and Conclusions

The global impact of foodborne disease is of high significance in affecting the morbidity and mortality of humans and animals, safety and sustainability of the food supply, as well as having an enormous economic impact on society. *C. botulinum* produces a characteristic neurotoxin (BoNT) that is the most poisonous substance known to humankind. Botulism is an acute disease that can cause a long-lasting paralysis of several weeks to months. Severe cases require intensive nursing support, parenteral feeding, and mechanical ventilation. The confirmed diagnosis of botulism involves the recognition of characteristic symptoms of botulism, and detection of BoNT in clinical or food samples. Despite the rare occurrence of the disease, botulism is a continual concern in medicine, the food industry, and by regulatory agencies. Our understanding of the actions of BoNT and its pathophysiology is gradually growing, largely due to its phenomenal success in the treatment of humans for neuronal diseases that has led to many studies of pathology and recovery. Certain aspects *C. botulinum* and BoNT related to foods offers many opportunities for future research and development, including improved assays that depend on all molecular steps of intoxication, a more rapid diagnosis and differentiation from other myasthenic diseases, the development of improved countermeasures to diminish the impact of the disease and enable a more rapid recovery from botulism, and novel processing technologies and formulation strategies to assure food safety related to botulism.

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