Complexity of Botulinum Neurotoxins: Challenges for Detection Technology

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Abstract The detection of botulinum neurotoxins (BoNT) is extremely challenging due to their high toxicity and the multiple BoNT variants. To date, seven serotypes with more than 30 subtypes have been described, and even more subtypes are expected to be discovered. The fact that the BoNT molecules are released as large complexes of different size and composition adds further complexity to the issue. Currently, in the diagnostics of botulism, the mouse bioassay (MBA) is still considered as gold standard for the detection of BoNT in complex sample materials. Over the years, different functional, immunological, and spectrometric assays or combinations thereof have been developed, supplemented by DNA-based assays for the detection of the organism. In this review, advantages and limitations of the current technologies will be discussed, highlighting some of the intricacies of real sample analysis.

Keywords Botulinum neurotoxin · Detection · Activity · ELISA · Endopeptidase assay · Mouse bioassay · Mass spectrometry

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Abbreviations

ALISSA	Assay using a large immuno-sorbent surface area
BoNT	Botulinum neurotoxin
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FRET	Förster resonance energy transfer
GE	Genome equivalents
HA	Hemagglutinin
HC	Heavy chain
LC	Light chain
LC	Liquid chromatography
LFA	Lateral flow assay
mAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MBA	Mouse bioassay
MPN ASSAY	Mouse phrenic nerve hemidiaphragm assay
MS	Mass spectrometry
NTNHA	Non-toxic nonhemagglutinin
orf	Open reading frame
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PTC	Progenitor toxin complex
SNAP-25	Synaptosome-associated protein of 25 kDa
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
TeNT	Tetanus neurotoxin
TOF	Time-of-flight
VAMP	Vesicle-associated membrane protein

Contents

1 Complexity of Botulinum Neurotoxins

Botulism, the disease caused by botulinum neurotoxins (BoNTs), has first been described in 1822 by Kerner as sausage poisoning (*botulus* = Latin: sausage). At the turn of the nineteenth century the causative agent, the anaerobic bacterium *Bacillus botulinus* (since 1923 *Clostridium botulinum*), or more precisely a heat-labile toxin in the culture supernatant, was identified by van Ermengem during his investigation of a botulism outbreak 1895 in Ellezelles, Belgium (van Ermengem 1897). The idea that botulism is caused by a single toxin produced by a single species (*Clostridium botulinum*) was shaken already some years later when in 1904 another strain of *C. botulinum* was isolated from bean salad in Darmstadt, Germany (Landmann 1904). Landmann was the first one to notice differences in optimal growth temperature and culture characteristics between the very first strains isolated. These differences were confirmed by Leuchs who also showed that the toxin produced by the Ellezelles strain was of a different serotype than the one produced by the Darmstadt strain (Leuchs 1910). Unfortunately, both strains were lost, but it seems likely that the Ellezelle strain belonged to a nonproteolytic strain of serotype B, whereas the Darmstadt strain was probably a proteolytic strain of serotype A (Meyer and Gunnison 1929). Today, the species *C. botulinum* is recognized as being inhomogeneous and is divided into four groups (I–IV) with distinct physiological characteristics (growth temperature, spore heat-resistance, salt tolerance etc.). The analysis of 16S rRNA sequences separates the four groups and places them together with other nonneurotoxic clostridia (Collins and East 1998; Peck 2009). Apart from *C. botulinum*, some strains of *C. baratii* and *C. butyricum* harbor the botulinum neurotoxin (*bont*) gene; thus, botulism can be caused by six distinct neurotoxin-producing species: *C. botulinum* group I–IV, *C. baratii*, and *C. butyricum*.

Since the beginning of the twentieth century, it has become clear that not only the BoNT-producing clostridia represents a heterogeneous family, but also that the neurotoxin is more divergent than originally anticipated. Some years after the discovery of BoNT/B and A the serotype C was identified in the 1920s (Bengtson 1922; Seddon 1922; Theiler and Robinson 1927). The group of serotypes was extended by D, E, F, and finally G between 1935 and 1969 (Gunnison et al. 1936; Hazen 1937; Møller and Scheibel 1960; Giménez and Cicarelli 1970), (Hill and Smith 2012). The serotypes show between 32 and 65 % identity at the amino acid level. It became apparent that certain serotypes can be produced by different *C. botulinum* groups and other clostridia species. For instance, serotype F can be produced by *C. botulinum* of group I, group II, and *C. baratii* (Peck 2009). In particular for serotype C, some of the controversies observed could be resolved by the identification of the toxins C2 and C3, which add to toxicity of the producing strains without being neurotoxins but belonging to the ADP-ribosyltransferase superfamily (Aktories et al. 2011; Just et al. 2011).

Many riddles remained unsolved before the era of modern molecular biology allowed for a more detailed analysis and interpretation of the *bont* gene locus. The comparison of *bont* sequences revealed a great degree of heterogeneity at the

nucleotide and amino acid levels (Hill and Smith 2012). This heterogeneity led to the introduction of different subtypes for a given serotype, which vary up to 36 % at the amino acid level. Until today, six subtypes of serotype A have been described (A1–A6), seven subtypes of serotype B (B1–B7), eight subtypes of serotype E (E1–E8), and seven subtypes of serotype F (F1–F7), and more are still to be expected (Hill et al. 2007; Lúquez et al. 2009; Umeda et al. 2009; Raphael et al. 2010a; Macdonald et al. 2011; Kalb et al. 2012a). The differences within the subtypes of a given serotype are greatest in serotype A (16 %) and serotype F (36 %) as compared to serotype B (7 %) and serotype E (6 %) at the amino acid level. For serotypes C and D, mosaic toxins named C/D and D/C, respectively, have been described (Moriishi et al. 1996), shedding some light on cross-inhibition of these two particular serotypes by certain antisera. No subtypes have been identified yet for serotype G, which to date has not been linked with natural disease.

The differences observed among the subtypes at the nucleotide and amino acid levels have major implications for detection methods relying on either protein epitopes (e.g., enzyme-linked immunosorbent assay [ELISA]-based detection) or sequence recognition (e.g., polymerase chain reaction [PCR]-based assays). The issue is made even more complex by the fact that in bacterial supernatants the neurotoxins are not found as holotoxins, but are associated with a nontoxic non-hemagglutinin (NTNHA), and additionally—depending on serotype and subtype—with up to three different hemagglutinins (HA1 of 33 kDa, HA2 of 17 kDa, HA3 of 70 kDa (East and Collins 1994; Fujinaga et al. 1994; Inoue et al. 1996)). The composition of the progenitor toxin complex (PTC) out of BoNT, NTNHA, and other accessory proteins depends on the genes located within the neurotoxin gene cluster and other yet unidentified factors. Two different neurotoxin clusters have been described. One is named the *ha*$^{+}$*orfX*$^{-}$ cluster and consists of the bicistronically expressed *bont* and *ntnha* genes, and three genes coding for the hem-agglutinins in reverse orientation, separated by *botR*, an alternative sigma factor as regulatory element. Alternatively, a second cluster is known which is called *ha*$^{-}$*orfX*$^{+}$ cluster in which the *ha* genes are replaced by three open reading frames called *orfX1*, *orfX2* and *orfX3*, and *p21* that codes for a positive regulatory protein analogous to *botR* (Gu and Jin 2012) of this issue of CTMI. In many cases, the type of neurotoxin cluster is unique for a given subtype; however, some BoNT/A1-producing strains which occur mostly in an *ha*$^{+}$*orfX*$^{-}$ cluster can also be found associated with an *ha*$^{-}$*orfX*$^{+}$ cluster (Raphael et al. 2008; Lúquez et al. 2009). When present in an *ha*$^{+}$*orfX*$^{-}$ cluster, the complex consisting of BoNT and NTNHA, also called M-PTC ($\sim$300 kDa; 12S), can associate with hemagglutinins leading to higher molecular weight complexes (L-PTC, $\sim$600 kDa; 16S); the subtype A1 is able to form even larger complexes of about 900 kDa (LL-PTC; 19S).

While the crystal structure of the M-PTC has just been resolved ((Gu et al. 2012); (Gu and Jin 2012) of this issue of CTMI), the exact stoichiometry and structure of L- and LL-PTC are still under investigation (Inoue et al. 1996; Hasegawa et al. 2007). When BoNT and NTNHA are expressed within an *ha*$^{-}$*orfX*$^{+}$ cluster, only the M-PTC has been purified. Eventually, minor amounts of OrfX proteins have been identified in association with the M-PTC or have been

observed in crude toxin preparations (Lin et al. 2010). So far the function of the OrfX proteins remains unclear.

2 Mode of Action

In terms of function, the seminal work of Burgen, Jahn, Montecucco, and others made it clear that from the different components of the neurotoxin complex the exquisite specificity and neurotoxicity is mediated by the 150 kDa BoNT molecule (Burgen et al. 1949; Jahn and Niemann 1994; Montecucco and Schiavo 1994). BoNTs act as endopeptidases at the neuromuscular junction, cleaving components involved in neurotransmitter release, thus leading to neuromuscular paralysis.

Active BoNTs are dichain molecules consisting of a 50 kDa *N*-terminal light chain (LC) that is responsible for enzymatic activity and a 100 kDa *C*-terminal heavy chain (HC) that is involved in receptor binding and cellular uptake (Rummel 2012; Bercseny et al. 2012; Fischer 2012; Binz 2012 and Ahnert-Hilger et al. 2012 of this issue of CTMI). After oral uptake into the body, BoNT molecules are protected from the harsh conditions in the gastrointestinal tract by NTNHA which shields the toxin in a pH-dependent manner (Gu et al. 2012). Within the small intestine, BoNT complexes bind to microvilli mediated by the hemagglutinins (Fujinaga 2006, 2010). The mechanism by which the BoNT complex crosses the epithelial barrier is still under debate. Two mechanisms have been suggested: receptor-mediated endocytosis with subsequent transcytosis and ternary HA complex mediated destruction of the intercellular junctions, followed by paracellular influx (Maksymowych and Simpson 1998, 2004; Fujinaga et al. 2009; Fujinaga 2010), (Fujinaga et al. 2012). Directly after absorption of the BoNT complex, it dissociates before it reaches the blood circulation that takes it to its neuronal target cells (Sakaguchi 1982). At the neuromuscular junctions, the BoNT binds to serotype-specific ganglioside and glycoprotein receptors on the presynaptic membrane of neurons and is internalized through endocytosis (Montecucco and Schiavo 1994; Brunger and Rummel 2009). In a pH-dependent process, the BoNT HC confers translocation of the BoNT LC into the cytoplasm (Koriazova and Montal 2003). After the disulfide bond that links LC und HC is reduced, the released LC acts as a zinc-dependent endopeptidase. It selectively cleaves proteins of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex which normally mediates the release of acetylcholine from synaptic vesicles (Montecucco and Schiavo 1994). The SNARE complex is formed by the assembly of the proteins synaptosome-associated protein (SNAP)-25, syntaxin and vesicle-associated membrane protein (VAMP)/synaptobrevin. While BoNT/A, C, and E cleave at different sites of SNAP-25, BoNT/C also targets syntaxin. BoNT/B, D, F, G, and the closely related tetanus neurotoxin (TeNT) cleave at distinct sites of VAMP (Montecucco and Schiavo 1994). After cleavage of any of the above-mentioned SNARE proteins, the formation of the SNARE complex is inhibited, resulting in the blockage of neurotransmitter release. This leads to the classical paralytic symptoms

of botulism. The estimated lethal dose of complexed crystalline serotype A for humans is 1 μg/kg, 10–13, and 1–2 ng/kg for oral, inhalational, and intravenous exposure, respectively (Arnon et al. 2001).

3 Challenges in BoNT Detection

Generally, there are two different fields in BoNT research which require highly sensitive detection of BoNT molecules:

(i) Analysis of suspected cases of botulism in humans and animals (the focus of this review);
(ii) Potency testing of highly purified pharmacological products used for medical or cosmetic (Pellett 2012 of this issue of CTMI)

The two fields have quite contrary requirements for diagnostic approaches:

In the case of botulism diagnostics, the focus lies on the detection of all serotypes and subtypes, including known and unknown subtypes. The detection methods have to be compatible with different clinical, food, and environmental matrices (see below). It is critical to get a timely response, since therapeutic measures have to be taken in due time. On the other hand, it is sufficient to get a rough estimate of the toxin's functional activity.

In the case of potency testing, however, the correct recognition of the serotype is second-tier since it is already defined through the industrial production process. Pharmacological products are composed of highly pure toxins or toxin complexes plus additives and stabilizing proteins suspended in physiological buffer; therefore, matrix effects are usually not critical for their detection. With respect to the length of the whole production process, the time to result is not important for the detection assay. The main focus, however, is on the highly precise, statistically valid potency determination of the neurotoxin, which has to reflect all four steps of the BoNT action: binding to specific surface receptors, internalization into neurons, translocation of the LC into the cytoplasm, and finally proteolytic cleavage of SNARE proteins.

Botulism occurs in three major forms: (i) Food-borne botulism is caused by ingestion of food contaminated with BoNT; (ii) Wound botulism occurs after uptake and growth of *C. botulinum* in wounds with parallel production of BoNT; (iii) Infant botulism is caused by colonization of the intestinal tract and toxin production (Johnson and Montecucco 2008). Depending on the form of botulism, different sample materials are usually analyzed: in the case of food-borne botulism serum, feces, and suspected food; in wound botulism wound swabs, pus, tissue, and serum; and in infant botulism feces, serum, intestinal contents, suspected food, and environmental samples (Lindström and Korkeala 2006). With respect to diagnostics of suspicious botulism samples, attention has to be paid to the fact that the toxin occurs in different forms in different sample matrices. As discussed above, botulinum toxin is not a single protein but in fact a heterogeneous family of

neurotoxins, comprising seven serotypes and more than 30 subtypes, thus challenging modern detection methods. In food matrices and bacterial culture supernatants, the toxin is usually present in one of its complexed forms. While the complex is stable at acidic pH, it dissociates spontaneously at physiological pH and high ionic strength (Sakaguchi 1982; Simpson 2004); this is of relevance for the analysis of food samples. In serum samples, however, free BoNT is found and the exact fate of the associated complex proteins is not clear to date. Due to the high toxicity of BoNT, their detection methods should be (i) highly sensitive down to the low pg/mL (fM–pM) range; (ii) able to detect all serotypes and subtypes including both the free neurotoxins and the high-molecular weight complexes; and (iii) compatible with a range of complex matrices. Additional points of interest are the simplicity of the assay, the potential for automation, and the robust assay performance including a high precision and accuracy.

4 Activity-Based Detection Assays

4.1 In Vivo and Ex Vivo Tests

Already in the 1920s, the mouse bioassay (MBA) for toxin detection was proposed: a BoNT-containing solution (e.g., patient serum, culture supernatant, or food extract) is injected intraperitoneally into mice and symptoms are observed for several hours up to 4 days. Characteristically, mice sequentially show ruffled fur, labored but not rapid breathing, a characteristic wasp-like abdomen with narrowed waist caused by increased respiratory effort due to paralysis of the diaphragm, weakness of limbs that progresses to total paralysis, and gasping for breath followed by death as a result of respiratory failure (Bengtson 1921). Mostly, the symptoms can be distinguished from symptoms caused by other toxins, e.g., TeNT which causes spastic paralysis. However, it should be noted that large doses of TeNT have been shown to initially mimic botulism symptoms (Matsuda et al. 1982). This phenomenon has been observed in a human patient, too ((König et al. 2007); TeNT and BoNT/B cleave VAMP at the very same position, but in different anatomic locations: while TeNT is retrogradely transported to the central nervous system, BoNT acts at the neuromuscular junction. One possible explanation for a sequential change from botulism to tetanus symptoms is that in the presence of high amounts of TeNT not all toxin is retrogradely transported from the neuromuscular junction to the central nervous system, thus inducing VAMP-cleavage at the neuromuscular junction similar to BoNT serotype B; Bercsenyi et al. 2012). Death of mice in the absence of neurological symptoms is not an acceptable indication of botulism (or tetanus), because it may be nonspecifically caused by other microorganisms, chemicals present in the test fluids, or injection trauma (Kautter and Solomon 1977). It is important to perform a number of specificity controls. Since the BoNT molecules are heat labile, a heat treatment of the sample material (15 min at 95 °C) should render it nontoxic, thus

failing to induce any clinical symptoms. Furthermore, a trypsin activation step may be required for the detection of toxins of weak or nonproteolytic strains. Confirmation and neurotoxin typing is performed by mouse-protection tests using polyvalent or, even better, monovalent neutralizing antibodies: on simultaneous application of toxin-containing material and the respective neutralizing antibodies, the mice are rescued and no symptoms occur. To estimate the quantity of BoNT in a sample, different dilutions are injected into mice and symptoms are followed as described above. The quantity of toxin in the sample is then estimated by relating the maximum dilution killing the mice to the known mouse lethal dose: 10 pg for serotype A (Ferreira 2001).

The MBA is currently the only widely accepted method, the "gold standard", for confirmation of active BoNT molecules and is included in official methods and national guidelines (e.g., AOAC Official Method 977.26 or the German Standard DIN 10102). While the performance of the test is of serious ethical concern, it still has several advantages over other methods:

(i) It has an exquisite sensitivity of between 10 and 100 pg/mL, depending on the serotype and subtype analyzed (Ohishi and Sakaguchi 1980; Sugiyama 1980; Sharma et al. 2006);
(ii) The MBA displays all four steps of BoNT action and the physiological outcome: binding, uptake, translocation, and target cleavage resulting in inhibition of neurotransmitter release and muscle paralysis;
(iii) All serotypes and subtypes can be detected in their free and complexed form; and
(iv) The assay is compatible with the use of complex matrices like serum, feces, gastric content, wound samples, food samples, and bacterial cultures (after clarification and pH adjustment).

Apart from the ethical concern, however, the MBA has a number of technical disadvantages. Depending on the amount of toxin present in the sample, the assay takes 1–4 days to yield a result, and a precise quantitation of BoNT in a sample requires many animals. Inter-laboratory comparisons have shown that MBA results and precise quantitation may be variable depending on the age and strain of mice and other factors (McLellan et al. 1996; Sesardic et al. 2003). Additionally, recent work has shown that the potency of, e.g., BoNT/B cannot be directly transferred from mice to man due to differences in protein receptor amino acid sequences in both species (Strotmeier et al. 2012).

The ethical concerns of the MBA encouraged the development of different alternative assays. They are still refined in vivo assays, but nonlethal and with greatly reduced suffering of animals. These assays measure the local paralysis induced by BoNT, e.g., flaccid paralysis (Sesardic et al. 1996; Jones et al. 2006), abdominal ptosis (Takahashi et al. 1990), hind limp paralysis (Sugiyama et al. 1975; Pearce et al. 1994; Aoki 2001), grip strength (Meyer et al. 1979; Torii et al. 2011), and toe-spread reflex (Wilder-Kofie et al. 2011). Other assays use an electromyographic measurement of the compound action muscle potential to quantify BoNT activity or anti-BoNT antibodies (Sakamoto et al. 2009; Torii et al.

2010a, b). Generally, these in vivo assays have not been used frequently for the detection of BoNT out of complex food, environmental, or clinical samples. Rather they have been used to quantify BoNT from pharmaceutical-grade toxin preparations (Huber et al. 2008) or for the detection of antibodies against BoNT (Sesardic et al. 2004; Jones et al. 2006). However, these assays still require several days to perform and are in vivo tests with more or less objective readouts.

To avoid suffering of animals, replacement methods for the MBA have been described. Most widely used is an ex vivo test, the rat or mouse phrenic nerve hemidiaphragm (MPN) assay (Burgen et al. 1949). In this test, the phrenic nerve connected with the hemidiaphragm muscle is prepared from sacrificed mice or rats and transferred to a culture bath. The phrenic nerve is electrically stimulated and the resulting muscle twitches are measured. Upon addition of BoNT, the time required to decrease the amplitude to 50 % of the starting value, the paralytic half-time, is measured as a function of the dose of BoNT applied (Simpson and Tapp 1967; Simpson 1973, 1974; Habermann et al. 1980). Serotyping is performed in a way similar to the MBA, using monovalent antibodies. Apart from quantitation of BoNT activity, the hemidiaphragm assay has also been used to detect antibodies against BoNT (Dressler et al. 2005; Rasetti-Escargueil et al. 2009, 2011). Like the MBA, the MPN assay has the advantage to measure all four steps of BoNT action and the physiological endpoint (muscle paralysis). The duration of the assay is much shorter (<4 h) while its sensitivity is similar to the one of the MBA. However, less animals are needed and their suffering is greatly reduced; nevertheless, animals are still required. A disadvantage of the method is that it is sensitive to matrix interference with components of real samples.

As pure in vitro tests, cell culture-based assays have been developed which are also able to display four steps in BoNT action. These assays are mainly suited to quantify the activity of purified BoNT preparations (Pellett 2012 of this issue of CTMI).

4.2 Endopeptidase Assays

In vitro, activity assays focusing on the endopeptidase activity of the LC of BoNT have been developed and improved since the identification of their substrates. Basically, endopeptidase assays display the serotype-specific proteolytic cleavage of SNARE proteins in conjunction with technically different readouts.

One of the most straightforward ways to detect cleavage of SNARE proteins is by immunoblotting. Very soon after the elucidation of the endopeptidase activity of BoNT toward SNARE proteins, immuno blots of toxin-treated synaptosomes or neuronal cells were probed with anti-SNAP-25 or anti-VAMP antibodies to visualize the substrate cleavage (Poulain et al. 1993; Schiavo et al. 1993). Twenty years later, this is still a useful technique in the field of basic research, e.g., to study cellular uptake kinetics (Pier et al. 2011). This type of endopeptidase assay is particularly useful for deducing BoNT activity by analyzing cell lysates for

SNARE cleavage, e.g., to show the persistence of BoNT activity ex vivo or to demonstrate the anterograde axonal transport and transcytosis of catalytically active BoNT/A (Keller et al. 1999a; Restani et al. 2011).

In 1996, Shone and colleagues started to use immobilized peptides derived from SNAP-25 and VAMP which are cleaved by serotypes A and B, respectively. The newly generated amino acid terminus, the neoepitope, was then recognized specifically by enzyme-labeled antibodies, thus allowing the quantitation of the enzymatic activity in conjunction with a BoNT standard curve (Hallis et al. 1996). Antibody-based detection of the neoepitope was also used by the group of Sesardic to detect and quantify substrate cleavage by serotypes A, E, and C in purified toxin solutions (Jones et al. 2008, 2009). In a converse approach, the uncleaved SNARE substrate was detected by an antibody, which does not recognize the cleaved products: after coating of the VAMP substrate to microtiter plates, BoNT activity was correlated with the loss of detectable substrate (Keller et al. 1999b).

Another widely used endopeptidase technology applies the use of Förster resonance energy transfer (FRET, (Förster 1948)): here a SNARE peptide harboring the specific BoNT cleavage site is labeled with a fluorescence donor and a fluorescence acceptor. As long as the fluorescence donor–acceptor pair is located in close vicinity in the uncleaved substrate molecule, the fluorescence of the excited donor is absorbed (quenched) by the fluorescence acceptor molecule. Upon substrate cleavage, the two fluorophores are separated, so that the fluorescence of the donor is no longer quenched and can be measured. This principle was used by different groups to detect the activity of different BoNT serotypes with sensitivities between 35 pg/mL and 150 ng/mL depending on serotype, FRET substrate, and assay time used (Anne et al. 2001; Dong et al. 2004; Rasooly and Do 2008; Rasooly et al. 2008; Pires-Alves et al. 2009; Poras et al. 2009; Gilmore et al. 2011; Ruge et al. 2011). The principle has also been implemented into portable devices with sensitivities in the ng/mL range (Sapsford et al. 2008; Kostov et al. 2009; Sun et al. 2010; Balsam et al. 2011) and is the basis for commercial substrates like SNAPtide® (Shine et al. 2002).

However, many of the assays mentioned have not been tested on complex matrices yet and are indeed aiming at inhibitor or potency testing rather than detection of BoNT dissolved in complex matrices. As enzymatic assays, endopeptidase assays are relatively sensitive toward changes in reaction conditions, and testing of complex matrices can dramatically affect assay performance (Rasooly and Do 2008; Rasooly et al. 2008). To reduce matrix interference, an immunoaffinity enrichment step has been introduced, where the toxin is captured from the matrix using antibody-coated magnetic microbeads prior to performing the endopeptidase reaction, resulting in assay sensitivities similar to those of the MBA (Wictome et al. 1999a, b; Rasooly and Do 2008; Rasooly et al. 2008; Piazza et al. 2011). Immunoaffinity enrichment is advantageous since it separates the toxin from other proteases which might cleave the SNARE peptide unspecifically, preventing false-positive results. Of particular concern is the protease trypsin which is present in the gastrointestinal tract of vertebrates and in feces. Trypsin cleaves SNAP-25 at exactly the same position as BoNT/C. The problem of

unspecific cleavage by other proteases is more substantial in endopeptidase assays recognizing proteolytic cleavage via FRET or other readouts as compared to assays which detect the precise cleavage product by a neoepitope-specific antibody or by mass spectrometry (see below). As a control for unspecific cleavage, Tucker and co-workers used a mutated SNAP-25 that could not be cleaved by serotypes A, C, and E (Piazza et al. 2011). Nevertheless, immunoaffinity enrichment combined with an FRET-based endopeptidase assay can be highly sensitive. The group of Kalkum developed an assay using a large immunosorbent surface area (ALISSA) for extraction of the toxins, resulting in excellent sensitivities of about 0.5 fg/mL for the detection of BoNT/A and E; matrices like serum, juice, and milk have been successfully analyzed (Bagramyan et al. 2008; Bagramyan and Kalkum 2011). Depending on the antibody used for immunoaffinity enrichment, the endopeptidase assay detects the LC activity only. Therefore, it can be advantageous to combine HC-specific antibodies for extraction with neoepitope-specific antibodies for the cleaved SNAP-25 in a single reaction (Liu et al. 2012).

To include cell binding in the in vitro activity assay, Shone and colleagues included a ganglioside-binding step in their endopeptidase method. To capture BoNT, they used synaptosomes, which contain gangliosides, followed by an endopeptidase assay, resulting in assay sensitivities in the range of the MBA (Evans et al. 2009).

4.3 Endopeptidase-Mass Spectrometry (Endopep-MS) Assay

Another variation of the endopeptidase format was developed by Barr and colleagues who coupled the endopeptidase format with a precise mass spectrometric detection and identification of the cleavage products, a method known as Endopeptidase-Mass Spectrometry (Endopep-MS) (Barr et al. 2005; Boyer et al. 2005). The detection and identification of the cleavage products can be performed either by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS or by liquid chromatography (LC) electrospray ionization (ESI) tandem MS (MS/MS). After implementing an immunoaffinity enrichment step, the Endopep-MS approach turned out to be very useful for the detection of BoNT/A, B, E and F activity out of serum, feces and organ homogenates. This was done either by using serotype-specific antibodies (Kalb et al. 2005, 2006, 2008; Gaunt et al. 2007; Parks et al. 2011; Wang et al. 2011) or by using a pan-reactive antibody directed against a common epitope present in the H_N of all four toxins (Kalb et al. 2010). In addition, BoNT/C, D, and G can also be detected by the Endopep-MS assay (Moura et al. 2011; Terilli et al. 2011). In the case of BoNT/C and D, the Endopep-MS method is even more precise in discriminating the two serotypes than the MBA, since the latter often suffers from a substantial cross-reactivity of antisera against serotypes C and D used for functional blockade due to the occurrence of BoNT/CD and DC mosaics (Hedeland et al. 2011).

Using an internal isotope-labeled standard with identical composition as one of the product peptides, but a different mass, Endopep-MS is able to precisely quantify the activity of toxin present in a sample. Depending on the serotype and complexity of the matrix analyzed, sensitivities between 0.05 and 50 pg/mL can be reached, similar to or even better than those reached by using the MBA (Boyer et al. 2005; Kalb et al. 2006, 2010; Moura et al. 2011). In contrast to endopeptidase assays using FRET or neoepitope recognition, the combination of immunoaffinity enrichment, endopeptidase reaction, and MS-based detection allows for simultaneous measurement of activity plus unambiguous identification of the corresponding immunocaptured toxin (Kalb et al. 2005, 2006, 2011a; Wang et al. 2011).

While the MBA is sensitive to all known and unknown BoNT serotypes and subtypes, in vitro endopeptidase assays have to include specific substrates for the BoNT serotypes aimed at. Until recently, all subtypes within a given serotype have been reported to share the same cleavage site on their respective SNARE protein. However, this principle was recently shaken by the finding that BoNT/F5 cleaves VAMP-2 at a different site from that of all other BoNT/F subtypes known. The cleavage site is located four amino acids upstream and has been identified by Endopep-MS (Kalb et al. 2012b). Also, not all subtypes of a serotype must recognize a given substrate equally well, as the detailed comparison of BoNT/F subtypes elucidated (Kalb et al. 2011a). It is also notable that the catalytic activity depends on the nature of the substrate and on the assay conditions used. For example, it has been shown that the catalytic activity of the BoNT subtypes A1–A4 tested on an SNAP-25 peptide was different from their activity on a longer recombinant SNAP-25 fragment. In particular, BoNT/A3, which showed 50 % of BoNT/A1 activity on a longer recombinant SNAP-25 fragment (aa 141–206), cleaved the peptide substrate at a much faster rate than subtype A1 (Henkel et al. 2009). Some of the discrepancies can be explained by the fact that SNAP-25 is not only bound around the active site of BoNT, but also by so-called α- and β-exosites upstream and downstream of the active site (Breidenbach and Brunger 2004; Brunger et al. 2008; Henkel et al. 2009). With respect to assay conditions, it has been shown that certain buffer components such as NaCl can reduce or even abolish cleavage of SNAP-25 or VAMP (Ferracci et al. 2011; Jones et al. 2011; Piazza et al. 2011).

In contrast to other in vitro methods, e.g., immunological assays which detect the presence of the protein, endopeptidase assays detect the functional activity of BoNT molecules in vitro. This is a clear advantage and is relevant, e.g., in the field of food safety. Endopeptidase assays inherently amplify the signal intensity by the catalytic reaction, since one BoNT molecule is able to cleave several substrate molecules. This results in excellent sensitivities, even beyond the sensitivity of the MBA. However, like most enzymatic assays, endopeptidase assays are quite sensitive to interference with matrix components. Therefore, immunoaffinity enrichment is performed to analyze real samples. Depending on the capture antibody or reagent used, the assay usually detects the activity of the LC plus the presence of the HC. Endopep-MS has been proven to be very useful to simultaneously measure the activity of BoNT molecules in parallel with an unambiguous identification of the immunocaptured toxin (Fig. 1). The only disadvantage is that

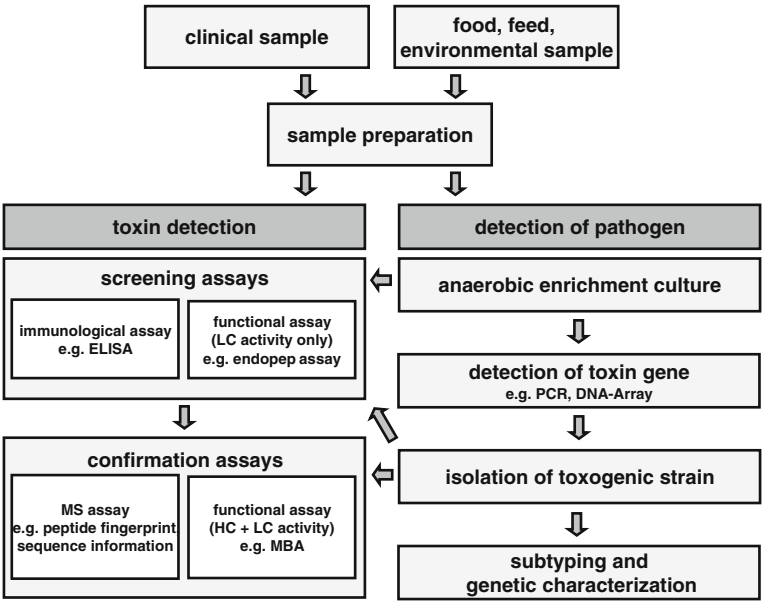

Fig. 1 Laboratory diagnostics of botulism. Depending on the type of botulism, clinical, food or environmental samples are analyzed for BoNT (*left*) and for the BoNT-producing pathogen (*right*), either directly or after anaerobic enrichment culture. A successful strategy combines fast and easy screening methods with confirmation assays providing information on the activity and/or identity of the holotoxin. The detection, isolation and genetic characterization of the toxin-producing strain deliver important additional information in an epidemiological investigation

this technology requires expensive equipment and a highly specialized technical expertise, and is therefore less common in clinical routine laboratories.

5 Immunological Assays

5.1 Classical Sandwich ELISA

ELISA-based technologies are by far the in vitro methods most commonly employed for BoNT detection. This is due to their generally high sensitivity, the simplicity of the assay which can be easily done in clinical routine laboratories, short assay times, and a robust assay performance including high precision and accuracy. After thorough validation, ELISA-based techniques can be applied to detect BoNT in a range of complex matrices. However, quality and validity of obtained data strictly depend on the quality and combination of the antibodies used.

In sandwich ELISA-based assays, a capture antibody is immobilized onto a solid support to capture BoNT. In the classical assay format these are plastic microtiter plates; other supports like silica microbeads, affinity columns, magnetic

microbeads, glass surfaces, and biochips are possible. The bound analyte is usually detected via a secondary hapten-coupled detector antibody plus an enzyme conjugate to develop a measurable readout. Different assay variations can be performed, e.g., using indirect or direct antibody–antigen interactions, using different haptens (e.g., biotin, digoxigenin), using different read-out systems (e.g., UV/VIS absorption, fluorescence, and chemiluminescence), and using different signal amplification strategies (e.g. polymeric enzyme conjugates, tyramide amplification, and immuno-PCR (see below)). In order to quantify BoNT in a real sample, a pure BoNT standard is analyzed in parallel in defined concentrations.

The use of classical immunoassays dates back to the late 1970s when the first sandwich ELISA systems for the detection of BoNT/A, B, and E were introduced (Notermans et al. 1978, 1979; Kozaki et al. 1979). They were soon complemented by ELISA for the detection of the other serotypes (Lewis et al. 1981; Lee and Yang 1982; Notermans et al. 1982b). Results were routinely obtained within 4–6 h, a clear advancement in comparison to the MBA. However, when using polyclonal antibodies (pAb) generated against purified BoNT or BoNT complexes, the ELISAs were clearly less sensitive than the MBA with detection limits usually down to a few ng/mL (Thomas 1991; Doellgast et al. 1993, 1994; Szílagyi et al. 2000; Ferreira 2001; Ferreira et al. 2004; Sharma et al. 2006). Later, in a collaborative effort, sandwich ELISAs based on pAb against BoNT/A, B, E, and F have been validated and compared to the MBA, resulting in assay sensitivities of 0.1–1 ng/mL. The method was approved as AOAC Official Method 2002.08 by The Scientific Association Dedicated to Analytical Excellence (Ferreira 2001; Ferreira et al. 2003). Sharma and colleagues developed an amplified immunoassay based on digoxigenin-labeled pAb to detect the same serotypes in food matrices with assay sensitivities of 60–176 pg/mL (Sharma et al. 2006). Even more sensitive and specific immunoassays have been developed with the accessibility of monoclonal antibodies (mAb) (Köhler and Milstein 1975): sandwich ELISAs incorporating mAb resulted in assay sensitivities of around 1–100 pg/mL from culture supernatants, beverages, and complex food matrices (Shone et al. 1985; Ferreira et al. 1987, 1990; Gibson et al. 1987, 1988; Ekong et al. 1995; Varnum et al. 2006; Chiao et al. 2008b; Stanker et al. 2008; Volland et al. 2008; Brooks et al. 2010; Scotcher et al. 2010; Weingart et al. 2010).

5.2 *Selected Examples of Different ELISA Formats*

Albeit the sensitivity and specificity of BoNT-specific sandwich ELISA systems have been improved, the detection from complex matrices is still challenging. In *bead-based immunoassays* the capture antibody is immobilized onto microbeads, allowing to separate the toxin from the matrix, a step which is not possible in conventional plate-bound ELISA formats. Especially, magnetic microbeads have proved to be useful for extracting BoNT from complex and even colloidal matrices (Gessler et al. 2006; Pauly et al. 2009; Garber et al. 2010). This immunoaffinity

enrichment step is not only useful for ELISAs, but is also often used in combination with other techniques (e.g., endopeptidase assays, mass spectrometry, cp. 12.4.2) (Bagramyan et al. 2008; Parks et al. 2011; Piazza et al. 2011; Liu et al. 2012).

In this context, flow cytometry has been shown to be a useful technique to detect and to quantify bead-bound BoNT molecules through a fluorescent readout (Anderson and Taitt 2008; Ozanich et al. 2009; Warner et al. 2009). A further development is *suspension array technologies* (e.g., the Luminex® xMAP technology). Fluorescent suspension arrays use either polystyrene or magnetic microspheres which are embedded with precise ratios of either two or three different fluorescent dyes, thus yielding arrays of 100 or 500 bead sets, respectively. The different bead sets are spectrally unique and are individually addressed by the measurement instrument. When immobilizing different antibodies to different bead sets, simultaneous and miniaturized immunoassays can be performed out of a single sample, thus reducing materials as well as cost and effort. Pauly and colleagues used color coded, magnetic Luminex beads to set up a pentaplex immunoassay for different biological toxins, among them BoNT/A and B. The detection limits were 21 and 73 pg/mL, respectively, out of 50 µL sample volume and could be further improved by magnetic immunoaffinity enrichment. Additionally, this method worked well to detect the toxins from different food matrices (Pauly et al. 2009). A similar approach was followed by Garber and colleagues for the detection of BoNT/A and five additional toxins with sensitivities of 1 ng/mL in spiked food samples (Garber et al. 2010). They took advantage of the multiplex approach by using different antibody pairs for each toxin, some recognizing different epitopes or displaying different binding kinetics, and implemented up to eight control assays. By doing so, they were able to reduce the likelihood of false-positive and false-negative results when testing toxins in complex matrices. In practice, fluorescent suspension arrays with up to 20–30 analytes in parallel are possible using a sandwich-ELISA principle.

Electrochemiluminescence-based immunoassays are usually performed on magnetic beads, too. They use a secondary reporter antibody, which is covalently coupled to a ruthenium (II) tris (bipyridyl) complex that becomes luminescent in the presence of an electric potential. The method has been used to detect BoNT/A, B, E, and F from clinical samples and food matrices with sensitivities of 50 pg/mL to 5 ng/mL, depending on the assay conditions used (Guglielmo-Viret et al. 2005; Rivera et al. 2006; Phillips and Abbott 2008). An advantage of this technique is its high signal-to-noise ratio due to the absence of optical background signals. However, the sensitivity boost compared to other ELISA readouts is limited and the technology is quite expensive, thus a widespread application is less likely.

Immuno-PCR is a further modified ELISA format using DNA-labeled detection antibodies (Wu et al. 2001; Chao et al. 2004; Adler et al. 2008; Rajkovic et al. 2012). Upon binding of the detection antibody to its target molecule, the oligonucleotide tag is amplified by PCR, resulting in assay sensitivities of 1 pg/mL for BoNT/A in buffer (Chao et al. 2004). In a different approach, Mason and colleagues described an ultrasensitive immunoassay based on liposomes with encapsulated DNA reporters and gangliosides embedded in the lipid bilayer as

detection reagent (Mason et al. 2006). After binding of a BoNT to a specific immobilized capture antibody and subsequent binding of the ganglioside to the toxin, the liposomes were ruptured to release the reporter DNA for amplification by real-time-PCR. The assay combined the detection of the toxin with detection of ganglioside binding as part of the functional HC activity. Thereby the assay reached a sensitivity so far unmatched of 0.02 fg/mL for BoNT/A in water (Mason et al. 2006). However, this method has not been tested on complex matrices yet, so its wider applicability will have to be worked out in the future.

While the above-mentioned examples showed that current ELISA-based technologies can technically reach the sensitivity of the MBA and beyond, the main problem when analyzing unknown samples is still to discriminate true-positive from false-positive signals and to exclude false-negative results. It is per se difficult to anticipate the degree of cross-reactivity between the antibodies used and the matrix components in an unknown sample. Therefore, a thorough validation of the ELISA is necessary that includes determination of recovery rates from the most important matrices. Generally, ELISA systems based on pAb show a higher tendency for cross-reactivity compared to ELISAs using mAb. Especially, if the pAb has been generated against BoNT complexes or bacterial supernatants rather than the pure holotoxins, high titers against accessory proteins or unrelated proteins have been observed which can result in substantial cross-reactivity (Sakaguchi et al. 1974; Sugiyama et al. 1974; Betley and Sugiyama 1979; Notermans et al. 1982b; Sakaguchi 1982; Dezfulian et al. 1984; Kumar et al. 1994; Ferreira 2001). It has also been noticed that culture supernatants of *C. botulinum* shared antigens found in supernatants of other clostridia (Poxton 1984; Poxton and Byrne 1984) and gave rise to cross-reactivity with nontoxogenic clostridia (Lewis et al. 1981; Thomas 1991).

Certain matrices have been recognized as being difficult to analyze for the presence of BoNT. Especially, fecal specimens were shown to be problematic (Dezfulian et al. 1984; Viscidi et al. 1984). By diluting infant botulism stool samples in 40 % fetal bovine serum, Dezfulian and colleagues were able to decrease interference with fecal specimens and obtained a good correlation between MBA and their ELISA format (Dezfulian et al. 1984). Certain food matrices are rich in avidin (e.g., egg white); therefore, they might cause problems when employing ELISA formats using biotinylated detection antibodies. In this case, detection via digoxigenin/anti-digoxigenin amplification offers an alternative with low background signals due to the absence of endogenous digoxigenin in all prokaryotic and eukaryotic cells (except for *Digitalis purpurea* (Dorner et al. 2003)).

MAb are generally more specific and less sensitive to cross-reactivity than pAb, but their supreme specificity can be a pitfall in the light of the different subtypes of BoNT serotypes described. Optimally, ELISAs based on mAb have to be tested against all the different subtypes of a given serotype. Indeed, a variation or lack in recognition of a certain subtype has been observed for some mAb and mAb-based ELISAs (Gibson et al. 1987, 1988; Smith et al. 2005; Kalb et al. 2009, 2011b; Brunt et al. 2010).

Failure of a mAb to recognize individual subtypes of a serotype has implications for all of those assays in which this mAb is used, e.g., for immunoaffinity

enrichment strategies. This problem is usually less pronounced with pAb recognizing numerous epitopes on a target protein, but also pAb have been shown to neutralize BoNT subtypes differently (Kozaki et al. 1977) or in extreme fail to recognize a certain subtype: Brunt and colleagues showed that a particular pAb directed against BoNT/F1 failed to recognize culture supernatants of a nonproteolytic F strain (Brunt et al. 2010). Notably, the highest divergence among the subtypes is found in BoNT/F (36 %) (Raphael et al. 2010a).

With respect to the detection of active versus inactive BoNT, it should be noted that only very few ELISA formats based on mAb have been shown to be able to discriminate the active toxin from its denatured form (Weingart et al. 2010). This might be relevant in thermal inactivation studies when comparing ELISA results with activity-based results.

In the light of the different sources of error connected with ELISA-based technologies, it is recommended to use this method for screening purposes to detect BoNT in complex matrices. Results should be confirmed by MBA or by other independent technologies having a high confirmatory power like mass spectrometry (Fig. 1; (Ferreira 2001; Ferreira et al. 2003)). Additionally, the detection, isolation, and characterization of the toxin-producing organism delivers important information.

5.3 Rapid Detection Tools Based on ELISA Formats

In a clinical case of botulism, first ELISA results can be obtained within several hours. Together with the characteristic clinical picture of acute botulism, this is timely enough in most cases to start medical treatment. However, in certain situations it is desirable to obtain results within 1 h, e.g., in a suspected case of intentional food poisoning in a bioterrorism scenario. Due to the fact that BoNTs are ranked among the category A agents of highest priority in the field of bioterrorism by the *Centers for Disease Control and Prevention* (Atlanta, USA), a number of on-site detection technologies have been developed over the last decade.

Among them, *lateral flow assays* (*LFA*) have been developed for commercial use, best known from pregnancy test kits (Posthuma-Trumpie et al. 2009). LFAs are hand-held devices based on immunochromatography on paper strips such as nitrocellulose. The sample is applied to one end of the strip and migrates by capillary action to the opposite end of the strip. While migrating along the strip the sample molecules first bind to an immobilized detection antibody conjugated to gold nanoparticles or dyes. The antibody–antigen complex further continues to migrate along the strip and is captured in the detection zone by a capture antibody, resulting in a visible change of color. Several LFAs for the detection of different BoNT serotypes have been developed with detection limits usually between 0.3 and 250 ng/mL (Chiao et al. 2004, 2008a; Klewitz et al. 2006; Attrée et al. 2007; Han et al. 2007). Sharma and co-workers evaluated two commercial products on spiked food samples and found detection limits of above 20 ng/mL for BoNT

complexes of serotypes A, B, and E (Sharma et al. 2005). Others reported that some commercial tests were unable to recognize the purified holotoxin but detected the toxin complexes only (Gessler et al. 2007).

Although the sensitivity of LFA is clearly lower than laboratory-based ELISA technologies, they offer several advantages: they are inexpensive, easy to use without any sophisticated equipment, and have a rapid read-out time (usually 20 min). This makes them a good tool for field use by untrained personnel. It is often argued that in a case of a bioterrorism incident high levels of toxin are likely to be found; hence LFAs with their limited sensitivity might nevertheless be useful.

As an alternative, *column-based immunochromatography* tests requiring only few handling steps have been developed (ABICAP® technology). In this technique, the capture antibody is immobilized on a 3-dimensional immunofiltration column made of sintered material providing a large inner surface. For a colorimetric readout the technology uses the increased sensitivity of polymeric enzyme conjugates covalently attached to a streptavidin conjugate. Using this rather straightforward technology, BoNT/C and D have been detected from culture supernatants within 40 min with sensitivities close to that of the MBA (Gessler et al. 2005). Peck and colleagues recently expanded the method to detect BoNT/B, E, and F (Brunt et al. 2010). BoNT/A has been detected from different clinical samples, food, or powder materials with a detection limit in the low pg/mL-range, similar to that of the MBA (Attrée et al. 2007).

Apart from these technically simple devices, a number of sophisticated biosensor technologies based on different principles have been established. Most of them detect BoNT in the ng/mL-range (Ogert et al. 1992; Shriver-Lake et al. 1993; Kumar et al. 1994; O'Brien et al. 2000; Varnum et al. 2006; Grate et al. 2009; Ren and Pearton 2012). Only few of them have been tested with complex matrices like food, clinical, or environmental samples (Ganapathy et al. 2008; Weingart et al. 2012).

6 BoNT Detection by Mass Spectrometry

Mass spectrometry (MS) is a powerful tool to detect and to unambiguously identify analytes.

The principle is that charged ions are generated by an ion source, separated on the basis of their mass-to-charge (*m*/z) ratio, and finally recorded on a detector (Boyer et al. 2011). Different types of ionization methods are commonly used for biological substances, e.g., MALDI and electrospray ionization (ESI). In tandem (MS/MS) mass spectrometry, multiple cycles of MS analysis are performed, usually in conjunction with a fragmentation or dissociation process. This allows for protein sequencing of the analyte.

Per se the molecular mass of a protein is not a unique characteristic; therefore, large proteins are usually enzymatically fragmented by proteases (e.g., trypsin, chymotrypsin), delivering a characteristic peptide fingerprint. By searching in protein databases, the peptide fragments are then assigned to an individual protein.

The first characterization of BoNT serotypes A–F by enzymatic digest followed by MALDI-TOF MS and ESI–MS/MS has been described by van Baar and colleagues (van Baar et al. 2002, 2004). The approach was extended to detect all BoNT serotypes together with their nontoxic accessory proteins from the high molecular weight complexes with nano-Liquid Chromatography (LC)-ESI–MS/MS (Hines et al. 2005). For this approach, μg-amounts of pure BoNT or BoNT complex were necessary.

Compared to other in vitro methods described so far, the significance of data obtained is higher for MS-based methods, since they allow for unambiguous identification of the toxins by a unique peptide fingerprint pattern or a protein sequence. However, MS methods are usually not as sensitive as other in vitro methods and require pre-enrichment or purification steps to be compliant with complex matrices.

To this end, Klaubert and colleagues presented a method to detect and identify complexes of BoNT/A, B, E, and F out of bacterial culture supernatants (Klaubert et al. 2009): starting with a culture volume of 1 mL, they used a peptic sample pretreatment strategy combined with 2D-nano-LC–ESI–MS/MS to identify the toxins; around 30 fmol toxin could be detected.

In a different approach, a multiplex immunoaffinity enrichment strategy for BoNT/A and B, their respective complexes, and other biological toxins has been used, followed by tryptic digest and MALDI-TOF MS-based detection of characteristic peptide fingerprints (Kull et al. 2010). The approach turned out to be successful at detecting the toxins out of beverages with a detection limit of 300–500 fmol. Starting from an anaerobic enrichment culture of a suspected specimen in a real case of botulism, the multiplex approach correctly identified the BoNT serotype and subtype involved.

An interesting proteomics approach was recently published by Barr and coworkers: in order to identify new BoNT subtypes, they created an amino acid substitution database in which every position of the BoNT protein sequence was substituted against all other possible amino acids. The database allowed for the precise identification of multiple BoNT/B subtypes including the novel subtype BoNT/B7 with no DNA required (Kalb et al. 2012a).

As described above, Endopep-MS has been proven to be very useful for simultaneously measuring the activity of BoNT molecules in parallel to an unambiguous identification of the immunocaptured toxin. The method has been successfully applied to a number of clinical and food matrices (Kalb et al. 2005, 2006, 2011a; Hedeland et al. 2011; Parks et al. 2011; Wang et al. 2011).

7 DNA-Based Detection of BoNT-Producing Bacteria

In parallel to methods aiming at detection of the neurotoxins, most laboratories engaged in botulism diagnostics use technically independent DNA-based methods to screen suspect samples for BoNT-producing organisms and to perform an epidemiological investigation.

By far, the most commonly employed methods are PCR-based techniques (Mullis et al. 1986; Saiki et al. 1988), many of which aim at detecting *bont* genes by conventional or quantitative amplification reactions (Szabo et al. 1992, 1993; Franciosa et al. 1994, 1996; Fach et al. 1995, 2009; Takeshi et al. 1996; Aranda et al. 1997; Braconnier et al. 2001; Kimura et al. 2001; Craven et al. 2002; Popoff and Walker 2003; Akbulut et al. 2004; Takeda et al. 2005; Yoon et al. 2005; Lindström and Korkeala 2006; Artin et al. 2007; Fenicia et al. 2007; Heffron and Poxton 2007; Prévot et al. 2007; Sánchez-Hernández et al. 2008; Sakuma et al. 2009; Hill et al. 2010; Lindberg et al. 2010; Takahashi et al. 2010). Since conventional PCR is difficult to quantify and requires a post-PCR step to visualize and to verify the PCR product, many modern approaches use quantitative PCR (qPCR) formats. Inclusion of a fluorogenic probe in qPCR assays was shown to increase specificity and to allow simultaneous detection of a number of genes via differently labeled probes. Using qPCR, 10–100 genome equivalents (GE) can be readily detected in about 2 h. Including DNA purification and dilution steps, this has led to detection limits of 10^3–10^5 GE/mL (Fach et al. 2009; Kirchner et al. 2010).

Since in botulism diagnosis more than one serotype is of concern, multiplex reactions covering several serotypes simultaneously have been reported in recent years. In particular, assays able to detect *bont*/*a*, /*b*, /*e*, and /*f*, which are known to be pathogenic to humans, in a single reaction have been developed (Lindström et al. 2001; Shin et al. 2007; Kirchner et al. 2010; Satterfield et al. 2010; Fach et al. 2011). Assays covering *bont*/*c*, /*d*, and their mosaic forms have been developed to meet the needs of veterinary medicine (Anniballi et al. 2012; Woudstra et al. 2012).

From a diagnostic point of view, assays including an internal amplification control allow for a more accurate evaluation of results, which is mandatory under certain quality control schemes, and procedures have thus been implemented accordingly (Braconnier et al. 2001; Akbulut et al. 2004; Messelhäusser et al. 2007; De Medici et al. 2009; Kirchner et al. 2010; Fach et al. 2011; Fenicia et al. 2011; Anniballi et al. 2012).

The differences observed among the subtypes at the genetic level have, of course, major implications on PCR-based assays. It has been noted that some PCR assays fail to detect certain subtypes due to sequence variations (De Medici et al. 2009). Thus, whenever new subtypes are reported it is important to re-analyze the capacity of the assay used, and, if necessary, to amend primer and probe sequences. Conversely, differences between subtypes have also been used to specifically differentiate them (Umeda et al. 2009, 2010).

In addition to assays focusing on the detection of the *bont* genes, PCR approaches have been described that amplify the *ntnha* gene located directly upstream of *bont* within the toxin gene cluster (Raphael and Andreadis 2007; Hill et al. 2010). The *ntnha* gene is present in all *bont*-containing gene clusters but is less divergent than the *bont* itself (Peck 2009). Thus, it has been used as a valuable surrogate marker for *bont*-positive clostridia (Raphael and Andreadis 2007; Hill et al. 2010).

With respect to botulism cases, a caveat is that the detection of *bont* (i) cannot account for the amount of toxin produced; and (ii) does not necessarily discriminate between intact and silent genes (Franciosa et al. 1994).

As an alternative to a deeper analysis of the genome of toxin-producing clostridia, DNA-microarrays have been used to differentiate serotypes (Gauthier et al. 2005) and to reveal information on the different neurotoxin clusters (Raphael et al. 2008, 2010b). These and other methods described (e.g. amplified fragment length polymorphism, pulsed-field gel electrophoresis, multilocus sequence typing, and whole-genome sequencing) are valuable for characterizing the genome of BoNT-producing clostridia and help to complement epidemiological investigations, but—except for whole genome approaches—do not necessarily deduce the BoNT sero- or subtype (Lindström and Korkeala 2006).

8 Laboratory Diagnostics of Botulism: Conclusion and Perspectives

As botulism is a potentially life-threatening illness, a rapid diagnosis is important to start medical treatment in due time. In this context, reliable laboratory diagnostics is essential to support and to confirm the suspected diagnosis. Starting from clinical samples (serum, feces) and, if appropriate, also food, feed, or environmental samples, the detection of the toxin itself remains the standard method. Supporting information is obtained from the detection of the toxin-producing pathogen, either directly out of the sample material or after anaerobic enrichment culture. Both pathways together provide important and technically independent pieces of information in an epidemiological investigation (Fig. 1).

While the MBA is still seen as the "gold standard" in BoNT detection, numerous in vitro methods have been established based on different functional, immunological, and spectrometric principles or combinations thereof. Technically challenging is the fact that BoNT is not a single molecule but occurs in different serotypes and subtypes. Ideally, a BoNT detection method is able to detect them all, providing a similar or better sensitivity than the MBA. Furthermore, it should be fast and easy to perform with a high precision and accuracy. Finally, an ideal BoNT detection method should be compatible with the analysis of complex matrices.

Immunological techniques offer the advantage of being highly sensitive and specific, and are easy to perform in routine laboratories. A number of assay formats have been tested with complex matrices and reached sensitivities close to the MBA and beyond, also in a multiplex format (Table 1). However, the main difficulty remains the discrimination of true-positive from false-positive signals and the exclusion of false-negative results when analyzing unknown samples. Thorough validation of an ELISA for BoNT detection in different matrices is indispensable, because the extent of cross-reactivity between the antibodies used and the matrix components in an unknown sample is difficult to predict. Also when

Table 1 An overview of common BoNT detection assays

Method[a]	Serotype detected	Detection limit	Analysis time	Matrices tested[b]	References
In vivo/ex vivo activity assays					
MBA	A, B, C, D, E, F, G	10–100 pg/mL	0.5–4 days	cs, se, fo, fe	Bengtson (1921), Kautter and Solomon (1977), Hatheway and McCroskey (1987), Sesardic et al. (2003)
Phrenic nerve hemidiaphragm assay	A, B, E	20–600 pg/mL	2–4 h	cs	Simpson (1973, 1974), Rasetti-Escargueil et al. (2009)
Endopeptidase assays					
Neoepitope detection	A, B, C, E	0.04–200 pg/mL	4–24 h	cs, se, fo	Hallis et al. (1996), Wictome et al. (1999a, b), Jones et al. (2008, 2009), Evans et al. (2009), Liu et al. (2012)
Fluorescence/FRET detection	A, B, D, E, F, G	0.035–150 ng/mL	15 min–20 h	se	Anne et al. (2001), Schmidt et al. (2001), Dong et al. (2004), Poras et al. (2009), Ruge et al. (2011), Joshi (2012)
Immunocapture, FRET detection	A, B, E	0.5–500 fg/mL 0.5–38 ng/mL	3–6 h	cs, se, fo	Bagramyan et al. (2008), Bagramyan and Kalkum (2011), Rasooly and Do (2008, 2010), Rasooly et al. (2008), Piazza et al. (2011)
Portable endopeptidase assay	A	25–62.5 ng/mL	2–3 h	–	Sapsford et al. (2005, 2008), Kostov et al. (2009), Sun et al. (2009, 2010), Balsam et al. (2011)
Endopep-MS	A, B, C, D, E, F, G	0.05–50 pg/mL	4–17 h	cs, se, fo, fe	Barr et al. (2005), Boyer et al. (2005), Kalb et al. (2005, 2006, 2010, 2011a), Hedeland et al. (2011), Moura et al. (2011), Terilli et al. (2011), Wang et al. (2011)

(continued)

Table 1 (continued)

Method[a]	Serotype detected	Detection limit	Analysis time	Matrices tested[b]	References
Immunoassays					
ELISA (pAb)	A, B, C, D, E, F, G	0.1–100 ng/mL	5 h-2 days	cs, se, fo, fe	Notermans et al. (1978), Kozaki et al. (1979), Notermans et al. (1979), Lewis et al. (1981), Lee and Yang (1982), Notermans et al. (1982a, b), Dezfulian et al. (1984), Michalik et al. (1986), Thomas (1991), Doellgast et al. (1993), Potter et al. (1993), Doellgast et al. (1994), Szílagyi et al. (2000), Ferreira (2001), Ferreira et al. (2001, 2004), Poli et al. (2002), Sharma et al. (2006), Rajkovic et al. (2012)
ELISA (mAb)	A, B, C, D, E, F	1–1000 pg/mL	5–7 h	cs, se, fo	Shone et al. (1985), Ferreira et al. (1987, 1990), Gibson et al. (1987, 1988), Ekong et al. (1995), Guglielmo-Viret et al. (2005), Gessler et al. (2006), Varnum et al. (2006), Stanker et al. (2008), Volland et al. (2008), Brooks et al. (2010), Scotcher et al. (2010)
Electrochemiluminescence immunoassay	A, B, E, F	50–5000 pg/mL	2–3 h	se, fo	Guglielmo-Viret et al. (2005), Rivera et al. (2006), Phillips and Abbott (2008)
Immuno-PCR	A	0.02–4000 fg/mL	3–10 h	fo	Wu et al. (2001), Chao et al. (2004), Mason et al. (2006), Warner et al. (2009)
Flow cytometric assay/ suspension arrays	A, B	10–5000 pg/mL	15 min–4 h	cs, fo	Varnum et al. (2006), Grate et al. (2009), Ozanich et al. (2009), Pauly et al. (2009), Warner et al. (2009), Garber et al. (2010)
LFA	A, B, D, E	0.3–250 ng/mL	10–30 min 2–4 h	cs, se, fo, fe	Chiao et al. (2004, 2008a), Sharma et al. (2005), Klewitz et al. (2006), Attrée et al. (2007), Gessler et al. (2007), Han et al. (2007), Yamashiro et al. (2007)

(continued)

Table 1 (continued)

Method[a]	Serotype detected	Detection limit	Analysis time	Matrices tested[b]	References
Immunochromatography columns	A, B, C, D, E, F	0.01–50 ng/ml	40 min	cs, se, fo	Gessler et al. (2005), Attrée et al. (2007), Brunt et al. (2010)
Different biosensor technologies	A, B, F	1–5 ng/ml	1–25 min	–	Ogert et al. (1992), Shriver-Lake et al. (1993, Kumar et al. (1994), O'Brien et al. (2000), Ladd et al. (2008), Ren and Pearton (2012)
Protein identification					
Mass spectrometry	A, B, C, D, E, F, G	49–375 ng/ml	8–14 h	cs, se, fo, fe	van Baar et al. (2002, 2004), Hines et al. (2005), Kalb et al. (2005, 2008, 2010, 2011a, 2012a), Gaunt et al. (2007), Klaubert et al. (2009), Kull et al. (2010), Hedeland et al. (2011), Moura et al. (2011), Parks et al. (2011), Terilli et al. (2011), Wang et al. (2011)
DNA-based detection					
PCR	A, B, C, D, E, F, G	10^3–10^5 GE/mL 10–100 GE	0.5–4 h	cs, se, fo, fe	Szabo et al. (1994), Braconnier et al. (2001), Lindström et al. (2001), Akbulut et al. (2004), Messelhäusser et al. (2007), De Medici et al. (2009), Kirchner et al. (2010), Fach et al. (2011), Fenicia et al. 2011), Anniballi et al. (2012)

[a] *ELISA* enzyme-linked immunosorbent assay; *PCR* polymerase chain reaction; *LFA* lateral flow assay; *FRET* Förster resonance energy transfer; *ALISSA* assay with a large immuno-sorbent surface area

[b] Complex matrices tested—buffer only; *cs* culture supernatant; *se* serum; *fo* food/feed; *fe* feces

using validated ELISA, it is still recommended to confirm results by either functional or mass spectrometric methods with a high confirmative power.

Compared to immunological methods detecting the presence of BoNT, functional in vitro assays like endopeptidase assays or the MBA offer the advantage of detecting the activity of BoNT. Endopeptidase assays focus on the activity of the BoNT LC only, but can include the presence of the HC depending on the antibodies employed for immunocapture. Since endopeptidase assays inherently amplify the signal intensity by the catalytic reaction, they reach very good sensitivities even beyond the MBA (Table 1). However, as enzymatic assays they can be quite sensitive to interference with matrix components, in particular other proteases. Hence, results obtained should be confirmed by MBA or by MS-based methods.

Generally, MS-based methods have the advantage to deliver unambiguous results, although they are still somewhat less sensitive and more time consuming than other methods. However, in combination with immunoaffinity enrichment, Endopep-MS and the parallel identification of the immunocaptured BoNT by peptide fingerprint or protein sequencing has proven to be very sensitive and enabled analysis of BoNT in clinical and food matrices (Table 1). In summary, a suitable combination of modern BoNT detection methods based on different technical approaches—functional, immunological, or spectrometric—is necessary and able to deliver confirmed results in a reasonable amount of time.

Tremendous progress has been made in the development of in vitro BoNT detection assays. However, more needs to be done. Highly specific and affine BoNT antibodies as central tools for enrichment strategies are not easily available, and only few of them have been commercialized. Along the same line, there is a lack of commercially available in vitro tests (e.g., ELISA, endopeptidase assays) which have been thoroughly validated on a broad range of complex matrices. Another problem is that currently there is no certified BoNT reference material available which can be used to compare different analytical approaches in expert laboratories. In the future, it will be important to strengthen quality assurance for the detection of BoNT and BoNT-producing clostridia by organizing regular proficiency tests.

On a technical level, it is anticipated that in vitro BoNT detection methods will be further multiplexed and miniaturized to detect and to differentiate the growing number of BoNT subtypes. Array-based platforms for protein detection and genetic characterization will become more important and replace singleplex detection methods. With the rising sequencing capabilities and the associated drop in cost it is expected that whole-genome sequencing will more and more replace classical genetic typing methods currently in use to characterize strains. At the same time, the gain of knowledge will allow a deeper understanding of physiological processes within BoNT-producing clostridia.

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References

Adler M, Wacker R, Niemeyer CM (2008) Sensitivity by combination: immuno-PCR and related technologies. Analyst 133:702–718

Akbulut D, Grant KA, McLauchlin J (2004) Development and application of real-time PCR assays to detect fragments of the *Clostridium botulinum* types A, B, and E neurotoxin genes for investigation of human foodborne and infant botulism. Foodborne Pathog Dis 1:247–257

Aktories K, Lang AE, Schwan C et al (2011) Actin as target for modification by bacterial protein toxins. FEBS J 278:4526–4543

Anderson GP, Taitt CR (2008) Amplification of microsphere-based microarrays using catalyzed reporter deposition. Biosens Bioelectron 24:324–328

Anne C, Cornille F, Lenoir C et al (2001) High-throughput fluorogenic assay for determination of botulinum type B neurotoxin protease activity. Anal Biochem 291:253–261

Anniballi F, Auricchio B, Delibato E et al (2012) Multiplex real-time PCR SYBR green for detection and typing of group III *Clostridium botulinum*. Vet Microbiol 154:332–338

Ahnert-Hilger G, Münster-Wandowski A, Höltje M (2012) Synaptic vesicle proteins: Targets and routes for Botulinum nurotoxins. doi:10.1007/978-3-642-33570-9-8

Aoki KR (2001) A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice. Toxicon 39:1815–1820

Aranda E, Rodriguez MM, Asensio MA et al (1997) Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe. Lett Appl Microbiol 25:186–190

Arnon SS, Schechter R, Inglesby TV et al (2001) Botulinum toxin as a biological weapon: medical and public health management. J Am Med Assoc 285:1059–1070

Artin I, Bjorkman P, Cronqvist J et al (2007) First case of type E wound botulism diagnosed using real-time PCR. J Clin Microbiol 45:3589–3594

Attrée O, Guglielmo-Viret V, Gros V et al (2007) Development and comparison of two immunoassay formats for rapid detection of botulinum neurotoxin type A. J Immunol Methods 325:78–87

Bagramyan K, Kalkum M (2011) Ultrasensitive detection of botulinum neurotoxins and anthrax lethal factor in biological samples by ALISSA. Methods Mol Biol 739:23–36

Bagramyan K, Barash JR, Arnon SS et al (2008) Attomolar detection of botulinum toxin type A in complex biological matrices. PLoS ONE 3:e2041

Balsam J, Ossandon M, Kostov Y et al (2011) Lensless CCD-based fluorometer using a micromachined optical Söller collimator. Lab Chip 11:941–949

Barr JR, Moura H, Boyer AE et al (2005) Botulinum neurotoxin detection and differentiation by mass spectrometry. Emerg Infect Dis 11:1578–1583

Bengtson I (1921) Direct inoculation test for *B. botulinus* toxin. Public Health Rep 36:1665–1671

Bengtson I (1922) Preliminary note on a toxin-producing anaerobe isolated from the larvae of *Lucilia caesar*. Public Health Rep 37:164–170

Bercsenyi k, Giribaldi F, Schiavo G (2012) The elusive compass of clostridial Neurotoxins: Deciding when and where to go. doi:10.1007/978-3-642-33570-9-5

Betley MJ, Sugiyama H (1979) Noncorrelation between mouse toxicity and serologically assayed toxin in *Clostridium botulinum* type A culture fluids. Appl Environ Microbiol 38:297–300

Binz T (2012) Clostridial Neurotoxin light chains:Devices for SNARE cleavage Mediated blockde of neurotransmission. doi:10.1007/978-3-642-33570-9-7

Boyer AE, Moura H, Woolfitt AR et al (2005) From the mouse to the mass spectrometer: detection and differentiation of the endoproteinase activities of botulinum neurotoxins A–G by mass spectrometry. Anal Chem 77:3916–3924

Boyer AE, Gallegos-Candela M, Lins RC et al (2011) Quantitative mass spectrometry for bacterial protein toxins—a sensitive, specific, high-throughput tool for detection and diagnosis. Molecules 16:2391–2413

Braconnier A, Broussolle V, Perelle S et al (2001) Screening for *Clostridium botulinum* type A, B, and E in cooked chilled foods containing vegetables and raw material using polymerase chain reaction and molecular probes. J Food Prot 64:201–207

Breidenbach MA, Brunger AT (2004) Substrate recognition strategy for botulinum neurotoxin serotype A. Nature 432:925–929

Brooks CE, Clarke HJ, Finlay DA et al (2010) Culture enrichment assists the diagnosis of cattle botulism by a monoclonal antibody based sandwich ELISA. Vet Microbiol 144:226–230

Brunger AT, Rummel A (2009) Receptor and substrate interactions of clostridial neurotoxins. Toxicon 54:550–560

Brunger AT, Jin R, Breidenbach MA (2008) Highly specific interactions between botulinum neurotoxins and synaptic vesicle proteins. Cell Mol Life Sci 65:2296–2306

Brunt J, Webb MD, Peck MW (2010) Rapid affinity immunochromatography column-based tests for sensitive detection of *Clostridium botulinum* neurotoxins and *Escherichia coli* O157. Appl Environ Microbiol 76:4143–4150

Burgen ASV, Dickens F, Zatman LJ (1949) The action of botulinum toxin on the neuro-muscular junction. J Physiol 109:10–24

Chao H-Y, Wang Y-C, Tang S-S et al (2004) A highly sensitive immuno-polymerase chain reaction assay for *Clostridium botulinum* neurotoxin type A. Toxicon 43:27–34

Chiao DJ, Shyu RH, Hu CS et al (2004) Colloidal gold-based immunochromatographic assay for detection of botulinum neurotoxin type B. J Chromatogr B Analyt Technol Biomed Life Sci 809:37–41

Chiao DJ, Wey JJ, Shyu RH et al (2008a) Monoclonal antibody-based lateral flow assay for detection of botulinum neurotoxin type A. Hybridoma (Larchmt) 27:31–35

Chiao DJ, Wey JJ, Tang SS (2008b) Monoclonal antibody-based enzyme immunoassay for detection of botulinum neurotoxin type A. Hybridoma (Larchmt) 27:43–47

Collins MD, East AK (1998) Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. J Appl Microbiol 84:5–17

Craven KE, Ferreira JL, Harrison MA et al (2002) Specific detection of *Clostridium botulinum* types A, B, E, and F using the polymerase chain reaction. J AOAC Int 85:1025–1028

De Medici D, Anniballi F, Wyatt GM et al (2009) Multiplex PCR to detect botulinum neurotoxin-producing clostridia in clinical, food and environmental samples. Appl Environ Microbiol 75:6457–6461

Dezfulian M, Hatheway CL, Yolken RH et al (1984) Enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* type A and type B toxins in stool samples of infants with botulism. J Clin Microbiol 20:379–383

Doellgast GJ, Triscott MX, Beard GA et al (1993) Sensitive enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay. J Clin Microbiol 31:2402–2409

Doellgast GJ, Beard GA, Bottoms JD et al (1994) Enzyme-linked immunosorbent assay and enzyme-linked coagulation assay for detection of *Clostridium botulinum* neurotoxins A, B, and E and solution-phase complexes with dual-label antibodies. J Clin Microbiol 32:105–111

Dong M, Tepp WH, Johnson EA et al (2004) Using fluorescent sensors to detect botulinum neurotoxin activity in vitro and in living cells. Proc Natl Acad Sci U S A 101:14701–14706

Dorner BG, Steinbach S, Hüser MB et al (2003) Single-cell analysis of the murine chemokines MIP-1alpha, MIP-1beta, RANTES and ATAC/lymphotactin by flow cytometry. J Immunol Methods 274:83–91

Dressler D, Lange M, Bigalke H (2005) Mouse diaphragm assay for detection of antibodies against botulinum toxin type B. Mov Disord 20:1617–1619

East AK, Collins MD (1994) Conserved structure of genes encoding components of botulinum neurotoxin complex M and the sequence of the gene coding for the nontoxic component in nonproteolytic *Clostridium botulinum* type F. Curr Microbiol 29:69–77

Ekong TAN, McLellan K, Sesardic D (1995) Immunological detection of *Clostridium botulinum* toxin type A in therapeutic preparations. J Immunol Methods 180:181–191

Evans ER, Skipper PJ, Shone CC (2009) An assay for botulinum toxin types A, B and F that requires both functional binding and catalytic activities within the neurotoxin. J Appl Microbiol 107:1384–1391

Fach P, Gibert M, Griffais R et al (1995) PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium* spp. and evaluation in food samples. Appl Environ Microbiol 61:389–392

Fach P, Micheau P, Mazuet C et al (2009) Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*. J Appl Microbiol 107:465–473

Fach P, Fenicia L, Knutsson R et al (2011) An innovative molecular detection tool for tracking and tracing *Clostridium botulinum* types A, B, E, F and other botulinum neurotoxin producing Clostridia based on the GeneDisc cycler. Int J Food Microbiol 145:S145–S151

Fenicia L, Anniballi F, De MD et al (2007) SYBR green real-time PCR method to detect *Clostridium botulinum* type A. Appl Environ Microbiol 73:2891–2896

Fenicia L, Fach P, van Rotterdam BJ et al (2011) Towards an international standard for detection and typing botulinum neurotoxin-producing Clostridia types A, B, E and F in food, feed and environmental samples: a European ring trial study to evaluate a real-time PCR assay. Int J Food Microbiol 145:S152–S157

Ferracci G, Marconi S, Mazuet C et al (2011) A label-free biosensor assay for botulinum neurotoxin B in food and human serum. Anal Biochem 410:281–288

Ferreira JL (2001) Comparison of amplified ELISA and mouse bioassay procedures for determination of botulinal toxins A, B, E, and F. J AOAC Int 84:85–88

Ferreira JL, Hamdy MK, Herd ZL et al (1987) Monoclonal antibody for the detection of *Clostridium botulinum* type A toxin. Mol Cell Probes 1:337–345

Ferreira JL, Hamdy MK, McCay SG et al (1990) Monoclonal antibody to type F *Clostridium botulinum* toxin. Appl Environ Microbiol 56:808–811

Ferreira JL, Eliasberg SJ, Harrison MA et al (2001) Detection of preformed type A botulinal toxin in hash brown potatoes by using the mouse bioasssay and a modified ELISA test. J AOAC Int 84:1460–1464

Ferreira JL, Maslanka S, Johnson E et al (2003) Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked immunosorbent assay: collaborative study. J AOAC Int 86:314–331

Ferreira JL, Eliasberg SJ, Edmonds P et al (2004) Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type A botulinal toxin in food. J Food Prot 67:203–206

Fischer A (2012) Synchronized Chapterone function of Botulinum neurotoxin domain Mediates light chain translocation into neutrons.doi:10.1007/978-3-642-33570-9-6

Förster T (1948) Zwischenmolekulare Energiewanderung und Fluoreszenz. Ann Phys 437:55–75

Franciosa G, Ferreira JL, Hatheway CL (1994) Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. J Clin Microbiol 32:1911–1917

Franciosa G, Fenicia L, Caldiani C et al (1996) PCR for detection of *Clostridium botulinum* type C in avian and environmental samples. J Clin Microbiol 34:882–885

Fujinaga Y (2006) Transport of bacterial toxins into target cells: pathways followed by cholera toxin and botulinum progenitor toxin. J Biochem 140:155–160

Fujinaga Y (2010) Interaction of botulinum toxin with the epithelial barrier. J Biomed Biotechnol 2010:974943

Fujinaga Y, Inoue K, Shimazaki S et al (1994) Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. Biochem Biophys Res Commun 205: 1291–1298

Fujinaga Y, Matsumura T, Jin Y et al (2009) A novel function of botulinum toxin-associated proteins: HA proteins disrupt intestinal epithelial barrier to increase toxin absorption. Toxicon 54:583–586

Fujinaga Y, Sugawara Y,Mtsumura T (2012) Uptake of Botulinum Neurotoxin in the intestine.doi: 10.1007/978-3-642-33570-9-3

Ganapathy R, Padmanabhan S, Eric YP et al (2008) Rapid detection of botulinum neurotoxins A, B, E, and F by optical immunoassay. Front Biosci 13:5432–5440
Garber EA, Venkateswaran KV, O'Brien TW (2010) Simultaneous multiplex detection and confirmation of the proteinaceous toxins abrin, ricin, botulinum toxins, and Staphylococcus enterotoxins A, B, and C in food. J Agric Food Chem 58:6600–6607
Gaunt PS, Kalb SR, Barr JR (2007) Detection of botulinum type E toxin in channel catfish with visceral toxicosis syndrome using catfish bioassay and endopep mass spectrometry. J Vet Diagn Invest 19:349–354
Gauthier M, Cadieux B, Austin JW et al (2005) Cloth-based hybridization array system for the detection of *Clostridium botulinum* type A, B, E, and F neurotoxin genes. J Food Prot 68:1477–1483
Gessler F, Hampe K, Böhnel H (2005) Sensitive detection of botulinum neurotoxin types C and D with an immunoaffinity chromatographic column test. Appl Environ Microbiol 71:7897–7903
Gessler F, Hampe K, Schmidt M et al (2006) Immunomagnetic beads assay for the detection of botulinum neurotoxin types C and D. Diagn Microbiol Infect Dis 56:225–232
Gessler F, Pagel-Wieder S, Avondet MA et al (2007) Evaluation of lateral flow assays for the detection of botulinum neurotoxin type A and their application in laboratory diagnosis of botulism. Diagn Microbiol Infect Dis 57:243–249
Gibson AM, Modi NK, Roberts TA et al (1987) Evaluation of a monoclonal antibody-based immunoassay for detecting type A *Clostridium botulinum* toxin produced in pure culture and an inoculated model cured meat system. J Appl Bacteriol 63:217–226
Gibson AM, Modi NK, Roberts TA et al (1988) Evaluation of a monoclonal antibody-based immunoassay for detecting type B *Clostridium botulinum* toxin produced in pure culture and an inoculated model cured meat system. J Appl Bacteriol 64:285–291
Gilmore MA, Williams D, Okawa Y et al (2011) Depolarization after resonance energy transfer (DARET): a sensitive fluorescence-based assay for botulinum neurotoxin protease activity. Anal Biochem 413:36–42
Giménez DF, Cicarelli AS (1970) Studies on strain 84 of *Clostridium botulinum*. Zentralbl Bakteriol Parasitenkd Infektionskr Abt 1 Orig Reihe A 215:212–220
Grate JW, Warner MG, Ozanich RM Jr et al (2009) Renewable surface fluorescence sandwich immunoassay biosensor for rapid sensitive botulinum toxin detection in an automated fluidic format. Analyst 134:987–996
Gu S, Rumpel S, Zhou J et al (2012) Botulinum neurotoxin is shielded by NTNHA in an interlocked complex. Science 335:977–981
Gu S, Jin R (2012) Assembly and function of the Botulinum neurotoxin progenitor complex. doi: 10.1007/978-3-642-33570-9-2
Guglielmo-Viret V, Attree O, Blanco-Gros V et al (2005) Comparison of electrochemiluminescence assay and ELISA for the detection of *Clostridium botulinum* type B neurotoxin. J Immunol Methods 301:164–172
Gunnison JB, Cummings JR, Meyer KF (1936) *Clostridium botulinum* Type E. Proc Soc Exp Biol Med 35:278–280
Habermann E, Dreyer F, Bigalke H (1980) Tetanus toxin blocks the neuromuscular transmission in vitro like botulinum A toxin. Naunyn Schmiedebergs Arch Pharmacol 311:33–40
Hallis B, James BA, Shone CC (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. J Clin Microbiol 34:1934–1938
Han SM, Cho JH, Cho IH et al (2007) Plastic enzyme-linked immunosorbent assays (ELISA)-on-a-chip biosensor for botulinum neurotoxin A. Anal Chim Acta 587:1–8
Hasegawa K, Watanabe T, Suzuki T et al (2007) A novel subunit structure of *Clostridium botulinum* serotype D toxin complex with three extended arms. J Biol Chem 282:24777–24783
Hatheway CL, McCroskey LM (1987) Examination of feces and serum for diagnosis of infant botulism in 336 patients. J Clin Microbiol 25:2334–2338
Hazen EL (1937) A strain of *B. botulinus* not classified as type A, B, or C. J Infect Dis 60:260–264
Hedeland M, Moura H, Baverud V et al (2011) Confirmation of botulism in birds and cattle by the mouse bioassay and Endopep-MS. J Med Microbiol 60:1299–1305

Heffron A, Poxton IR (2007) A PCR approach to determine the distribution of toxin genes in closely related *Clostridium* species: *Clostridium botulinu*m type C and D neurotoxins and C2 toxin, and *Clostridium novyi* α toxin. J Med Microbiol 56:196–201

Henkel JS, Jacobson M, Tepp W et al (2009) Catalytic properties of botulinum neurotoxin subtypes A3 and A4. Biochemistry 48:2522–2528

Hill KK, Smith TJ, Helma CH et al (2007) Genetic diversity among botulinum neurotoxin-producing clostridial strains. J Bacteriol 189:818–832

Hill KK, Smith TJ (2012) Genetic diversity within Clostridium botulinum serotypes, botulinum neurotoxin Gene. Cluster and toxin subtypes. doi: 10.1007/978-3-642-33570-9-1

Hill BJ, Skerry JC, Smith TJ et al (2010) Universal and specific quantitative detection of botulinum neurotoxin genes. BMC Microbiol 10:267

Hines HB, Lebeda F, Hale M et al (2005) Characterization of botulinum progenitor toxins by mass spectrometry. Appl Environ Microbiol 71:4478–4486

Huber A, France RM, Riccalton-Banks L et al (2008) The intercostal NMJ assay: a new alternative to the conventional LD50 assay for the determination of the therapeutic potency of botulinum toxin preparations. Altern Lab Anim 36:141–152

Inoue K, Fujinaga Y, Watanabe T et al (1996) Molecular composition of *Clostridium botulinum* type A progenitor toxins. Infect Immun 64:1589–1594

Jahn R, Niemann H (1994) Molecular mechanisms of clostridial neurotoxins. Ann N Y Acad Sci 733:245–255

Johnson EA, Montecucco C (2008) Botulism. Handb Clin Neurol 91:333–368

Jones RG, Alsop TA, Hull R et al (2006) Botulinum type A toxin neutralisation by specific IgG and its fragments: a comparison of mouse systemic toxicity and local flaccid paralysis assays. Toxicon 48:246–254

Jones RG, Ochiai M, Liu Y et al (2008) Development of improved SNAP25 endopeptidase immuno-assays for botulinum type A and E toxins. J Immunol Methods 329:92–101

Jones RG, Liu Y, Sesardic D (2009) New highly specific botulinum type C1 endopeptidase immunoassays utilising SNAP25 or syntaxin substrates. J Immunol Methods 343:21–27

Jones RG, Liu Y, Halls C et al (2011) Release of proteolytic activity following reduction in therapeutic human serum albumin containing products: detection with a new neoepitope endopeptidase immunoassay. J Pharm Biomed Anal 54:74–80

Joshi SG (2012) Detection of biologically active botulinum neurotoxin-A in serum using high-throughput FRET-assay. J Pharmacol Toxicol Methods 65:8–12

Just I, Rohrbeck A, Hülsenbeck SC et al (2011) Therapeutic effects of *Clostridium botulinum* C3 exoenzyme. Naunyn Schmiedebergs Arch Pharmacol 383:247–252

Kalb SR, Goodnough MC, Malizio CJ et al (2005) Detection of botulinum neurotoxin A in a spiked milk sample with subtype identification through toxin proteomics. Anal Chem 77:6140–6146

Kalb SR, Moura H, Boyer AE et al (2006) The use of endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. Anal Biochem 351:84–92

Kalb SR, Smith TJ, Moura H et al (2008) The use of endopep-MS to detect multiple subtypes of botulinum neurotoxins A, B, E, and F. Int J Mass Spectrom 278:101–108

Kalb SR, Lou J, Garcia-Rodriguez C et al (2009) Extraction and inhibition of enzymatic activity of botulinum neurotoxins/A1,/A2, and/A3 by a panel of monoclonal anti-BoNT/A antibodies. PLoS ONE 4:e5355

Kalb SR, Garcia-Rodriguez C, Lou J et al (2010) Extraction of BoNT/A,/B,/E, and/F with a single, high affinity monoclonal antibody for detection of botulinum neurotoxin by endopep-MS. PLoS ONE 5:e12237

Kalb SR, Pirkle JL, Barr JR (2011a) Mass spectrometric detection of botulinum neurotoxin by measuring its activity in serum and milk. In: Banoub J (ed) Detection of biological agents for the prevention of bioterrorism. Springer, Dordrecht, pp 115–129

Kalb SR, Santana WI, Geren IN et al (2011b) Extraction and inhibition of enzymatic activity of botulinum neurotoxins/B1,/B2,/B3,/B4, and/B5 by a panel of monoclonal anti-BoNT/B antibodies. BMC Biochem 12:58

Kalb SR, Baudys J, Rees JC et al (2012a) De novo subtype and strain identification of botulinum neurotoxin type B through toxin proteomics. Anal Bioanal Chem 403:215–226
Kalb SR, Baudys J, Webb RP et al (2012b) Discovery of a novel enzymatic cleavage site for botulinum neurotoxin F5. FEBS Lett 586:109–115
Kautter DA, Solomon HM (1977) Collaborative study of a method for the detection of *Clostridium botulinum* and its toxins in foods. J Assoc Off Anal Chem 60:541–545
Keller JE, Neale EA, Oyler G et al (1999a) Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett 456:137–142
Keller JE, Nowakowski JL, Filbert MG et al (1999b) Rapid microplate assay for monitoring botulinum neurotoxin B catalytic activity. J Appl Toxicol 19:S13–S17
Kimura B, Kawasaki S, Nakano H et al (2001) Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. Appl Environ Microbiol 67:206–216
Kirchner S, Krämer KM, Schulze M et al (2010) Pentaplexed quantitative real-time PCR assay for the simultaneous detection and quantification of botulinum neurotoxin-producing clostridia in food and clinical samples. Appl Environ Microbiol 76:4387–4395
Klaubert B, Vujtovic-Ockenga N, Wermter R et al (2009) Determination of botulinum toxins after peptic sample pre-treatment by multidimensional nanoscale liquid chromatography and nano-electrospray ion-trap mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 877:1084–1092
Klewitz T, Gessler F, Beer H et al (2006) Immunochromatographic assay for determination of botulinum neurotoxin type D. Sens Actuators B Chem 113:582–589
Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497
König K, Ringe H, Dorner BG et al (2007) Atypical tetanus in a completely immunized 14-year-old boy. Pediatrics 120:e1355–e1358
Koriazova LK, Montal M (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat Struct Biol 10:13–18
Kostov Y, Sergeev N, Wilson S et al (2009) A simple portable electroluminescence illumination-based CCD detector. Methods Mol Biol 503:259–272
Kozaki S, Miyazaki S, Sakaguchi G (1977) Development of antitoxin with each of two complementary fragments of *Clostridium botulinum* type B derivative toxin. Infect Immun 18:761–766
Kozaki S, Dufrenne J, Hagenaars AM et al (1979) Enzyme linked immunosorbent assay (ELISA) for detection of *Clostridium botulinum* type B toxin. Jpn J Med Sci Biol 32:199–205
Kull S, Pauly D, Störmann B et al (2010) Multiplex detection of microbial and plant toxins by immunoaffinity enrichment and matrix-assisted laser desorption/ionization mass spectrometry. Anal Chem 82:2916–2924
Kumar P, Colston JT, Chambers JP et al (1994) Detection of botulinum toxin using an evanescent wave immunosensor. Biosens Bioelectron 9:57–63
Ladd J, Taylor AD, Homola J et al (2008) Detection of botulinum neurotoxins in buffer and honey using a surface plasmon resonance (SPR) sensor. Sens Actuators B Chem 130:129–134
Landmann G (1904) Ueber die Ursache der Darmstädter Bohnenvergiftung. Hyg Rundsch 14:449–452
Lee JK, Yang KH (1982) Enzyme-linked immunosorbent assay (ELISA) for detection of *Clostridium* type F toxin. Kor J Appl Microbiol Bioeng 10:205–209
Leuchs J (1910) Beiträge zur Kenntnis des toxins und antitoxins des *Bacillus botulinus*. Z Hyg 65:55–84
Lewis GE Jr, Kulinski SS, Reichard DW et al (1981) Detection of *Clostridium botulinum* type G toxin by enzyme-linked immunosorbent assay. Appl Environ Microbiol 42:1018–1022
Lin G, Tepp WH, Pier CL et al (2010) Expression of the *Clostridium botulinum* A2 neurotoxin gene cluster proteins and characterization of the A2 complex. Appl Environ Microbiol 76:40–47

Lindberg A, Skarin H, Knutsson R et al (2010) Real-time PCR for *Clostridium botulinum* type C neurotoxin (BoNTC) gene, also covering a chimeric C/D sequence-application on outbreaks of botulism in poultry. Vet Microbiol 146:118–123

Lindström M, Korkeala H (2006) Laboratory diagnostics of botulism. Clin Microbiol Rev 19:298–314

Lindström M, Keto R, Markkula A et al (2001) Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. Appl Environ Microbiol 67:5694–5699

Liu YY, Rigsby P, Sesardic D et al (2012) A functional dual-coated (FDC) microtiter plate method to replace the botulinum toxin LD(50) test. Anal Biochem 425:28–35

Lúquez C, Raphael BH, Maslanka SE (2009) Neurotoxin gene clusters in *Clostridium botulinum* type Ab strains. Appl Environ Microbiol 75:6094–6101

Macdonald TE, Helma CH, Shou Y et al (2011) Analysis of *Clostridium botulinum* serotype E strains by using multilocus sequence typing, amplified fragment length polymorphism, variable-number tandem-repeat analysis, and botulinum neurotoxin gene sequencing. Appl Environ Microbiol 77:8625–8634

Maksymowych AB, Simpson LL (1998) Binding and transcytosis of botulinum neurotoxin by polarized human colon carcinoma cells. J Biol Chem 273:21950–21957

Maksymowych AB, Simpson LL (2004) Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. J Pharmacol Exp Ther 310:633–641

Mason JT, Xu L, Sheng ZM et al (2006) Liposome polymerase chain reaction assay for the sub-attomolar detection of cholera toxin and botulinum neurotoxin type A. Nat Protoc 1:2003–2011

Matsuda M, Sugimoto N, Ozutsumi K et al (1982) Acute botulinum-like intoxication by tetanus neurotoxin in mice. Biochem Biophys Res Commun 104:799–805

McLellan K, Das RE, Ekong TA et al (1996) Therapeutic botulinum type A toxin: factors affecting potency. Toxicon 34:975–985

Messelhäusser U, Zucker R, Ziegler H et al (2007) Nachweis von *Clostridium botulinum* Typ A, B, E und F mittels real-time-PCR. J Verbr Lebensm 2:198–201

Meyer KF, Gunnison JB (1929) European strains of *Cl. botulinum.* XXXVI. J Infect Dis 45:96–105

Meyer OA, Tilson HA, Byrd WC et al (1979) A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav Toxicol 1:233–236

Michalik M, Grzybowski J, Ligięza J et al (1986) Enzyme-linked immunosorbent assay (ELISA) for the detection and differentiation of *Clostridium botulinum* toxins type A and B. J Immunol Methods 93:225–230

Møller V, Scheibel I (1960) Preliminary report on the isolation of an apparently new type of *Cl. botulinum*. Acta Pathol Microbiol Scand 48:80

Montecucco C, Schiavo G (1994) Mechanism of action of tetanus and botulinum neurotoxins. Mol Microbiol 13:1–8

Moriishi K, Koura M, Abe N et al (1996) Mosaic structures of neurotoxins produced from *Clostridium botulinum* types C and D organisms. Biochim Biophys Acta 1307:123–126

Moura H, Terilli RR, Woolfitt AR et al (2011) Studies on botulinum neurotoxins type/C1 and mosaic/DC using endopep-MS and proteomics. FEMS Immunol Med Microbiol 61:288–300

Mullis K, Faloona F, Scharf S et al (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 51:263–273

Notermans S, Dufrenne J, Schothorst M (1978) Enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* toxin type A. Jpn J Med Sci Biol 31:81–85

Notermans S, Dufrenne J, Kozaki S (1979) Enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* type E toxin. Appl Environ Microbiol 37:1173–1175

Notermans S, Dufrenne J, Kozaki S (1982a) The relation between toxicity and toxin-related-antigen contents of *Clostridium botulinum* types C and D cultures as determined by mouse bioassay and ELISA. Jpn J Med Sci Biol 35:203–211

Notermans S, Hagenaars AM, Kozaki S (1982b) The enzyme-linked immunosorbent assay (ELISA) for the detection and determination of *Clostridium botulinum* toxins A, B, and E. Methods Enzymol 84:223–238

O'Brien T, Johnson LH 3rd, Aldrich JL et al (2000) The development of immunoassays to four biological threat agents in a bidiffractive grating biosensor. Biosens Bioelectron 14:815–828

Ogert RA, Brown JE, Singh BR et al (1992) Detection of *Clostridium botulinum* toxin A using a fiber optic-based biosensor. Anal Biochem 205:306–312

Ohishi I, Sakaguchi G (1980) Oral toxicities of *Clostridium botulinum* type C and D toxins of different molecular sizes. Infect Immun 28:303–309

Ozanich RM Jr, Bruckner-Lea CJ, Warner MG et al (2009) Rapid multiplexed flow cytometric assay for botulinum neurotoxin detection using an automated fluidic microbead-trapping flow cell for enhanced sensitivity. Anal Chem 81:5783–5793

Parks BA, Shearer JD, Baudys J et al (2011) Quantification of botulinum neurotoxin serotypes A and B from serum using mass spectrometry. Anal Chem 83:9047–9053

Pauly D, Kirchner S, Störmann B et al (2009) Simultaneous quantification of five bacterial and plant toxins from complex matrices using a multiplexed fluorescent magnetic suspension assay. Analyst 134:2028–2039

Pearce LB, Borodic GE, First ER et al (1994) Measurement of botulinum toxin activity: evaluation of the lethality assay. Toxicol Appl Pharmacol 128:69–77

Peck MW (2009) Biology and genomic analysis of *Clostridium botulinum*. Adv Microb Physiol 55(183–265):320

Pellett S (2012) Progress in cell based assays for botulinum neurotoxin detection. doi: 10.1007/978-3-642-33570-9-12

Phillips RW, Abbott D (2008) High-throughput enzyme-linked immunoabsorbant assay (ELISA) electrochemiluminescent detection of botulinum toxins in foods for food safety and defence purposes. Food Addit Contam 25:1084–1088

Piazza TM, Blehert DS, Dunning FM et al (2011) In vitro detection and quantification of botulinum neurotoxin type E activity in avian blood. Appl Environ Microbiol 77:7815–7822

Pier CL, Chen C, Tepp WH et al (2011) Botulinum neurotoxin subtype A2 enters neuronal cells faster than subtype A1. FEBS Lett 585:199–206

Pires-Alves M, Ho M, Aberle KK et al (2009) Tandem fluorescent proteins as enhanced FRET-based substrates for botulinum neurotoxin activity. Toxicon 53:392–399

Poli MA, Rivera VR, Neal D (2002) Development of sensitive colorimetric capture ELISAs for *Clostridium botulinum* neurotoxin serotypes E and F. Toxicon 40:797–802

Popoff MR, Walker JM (2003) Detection of toxigenic *Clostridia*. Methods Mol Biol 216:137–152

Poras H, Ouimet T, Orng SV et al (2009) Detection and quantification of botulinum neurotoxin type a by a novel rapid in vitro fluorimetric assay. Appl Environ Microbiol 75:4382–4390

Posthuma-Trumpie GA, Korf J, van Amerongen A (2009) Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. Anal Bioanal Chem 393:569–582

Potter MD, Meng JH, Kinsey P (1993) An ELISA for detection of botulinal toxin types A, B, and E in inoculated food samples. J Food Protect 56:856–861

Poulain B, Rossetto O, Deloye F et al (1993) Antibodies against rat brain vesicle-associated membrane protein (synaptobrevin) prevent inhibition of acetylcholine release by tetanus toxin or botulinum neurotoxin type B. J Neurochem 61:1175–1178

Poxton IR (1984) Demonstration of the common antigens of *Clostridium botulinum*, *C. sporogenes* and *C. novyi* by an enzyme-linked immunosorbent assay and electroblot transfer. J Gen Microbiol 130:975–981

Poxton IR, Byrne MD (1984) Demonstration of shared antigens in the genus *Clostridium* by an enzyme-linked immunosorbent assay. J Med Microbiol 17:171–176

Prévot V, Tweepenninckx F, van Nerom E et al (2007) Optimization of polymerase chain reaction for detection of *Clostridium botulinum* type C and D in bovine samples. Zoonoses Public Health 54:320–327

Rajkovic A, El Moualij B, Fikri Y et al (2012) Detection of *Clostridium botulinum* neurotoxins A and B in milk by ELISA and immuno-PCR at higher sensitivity than mouse bio-assay. Food Anal Methods 5:319–326

Raphael BH, Andreadis JD (2007) Real-time PCR detection of the nontoxic nonhemagglutinin gene as a rapid screening method for bacterial isolates harboring the botulinum neurotoxin (A–G) gene complex. J Microbiol Methods 71:343–346

Raphael BH, Luquez C, McCroskey LM et al (2008) Genetic homogeneity of *Clostridium botulinum* type A1 strains with unique toxin gene clusters. Appl Environ Microbiol 74:4390–4397

Raphael BH, Choudoir MJ, Luquez C et al (2010a) Sequence diversity of genes encoding botulinum neurotoxin type F. Appl Environ Microbiol 76:4805–4812

Raphael BH, Joseph LA, McCroskey LM et al (2010b) Detection and differentiation of *Clostridium botulinum* type A strains using a focused DNA microarray. Mol Cell Probes 24:146–153

Rasetti-Escargueil C, Jones RG, Liu Y et al (2009) Measurement of botulinum types A, B and E neurotoxicity using the phrenic nerve-hemidiaphragm: improved precision with in-bred mice. Toxicon 53:503–511

Rasetti-Escargueil C, Liu Y, Rigsby P et al (2011) Phrenic nerve-hemidiaphragm as a highly sensitive replacement assay for determination of functional botulinum toxin antibodies. Toxicon 57:1008–1016

Rasooly R, Do PM (2008) Development of an in vitro activity assay as an alternative to the mouse bioassay for *Clostridium botulinum* neurotoxin type A. Appl Environ Microbiol 74:4309–4313

Rasooly R, Do PM (2010) *Clostridium botulinum* neurotoxin type B Is heat-stable in milk and not inactivated by pasteurization. J Agric Food Chem 58:12557–12561

Rasooly R, Stanker LH, Carter JM et al (2008) Detection of botulinum neurotoxin-A activity in food by peptide cleavage assay. Int J Food Microbiol 126:135–139

Ren F, Pearton SJ (2012) Sensors using AlGaN/GaN based high electron mobility transistor for environmental and bio-applications. Phys Status Solidi C 9:393–398

Restani L, Antonucci F, Gianfranceschi L et al (2011) Evidence for anterograde transport and transcytosis of botulinum neurotoxin A (BoNT/A). J Neurosci 31:15650–15659

Rivera VR, Gamez FJ, Keener WK et al (2006) Rapid detection of *Clostridium botulinum* toxins A, B, E, and F in clinical samples, selected food matrices, and buffer using paramagnetic bead-based electrochemiluminescence detection. Anal Biochem 353:248–256

Ruge DR, Dunning FM, Piazza TM et al (2011) Detection of six serotypes of botulinum neurotoxin using fluorogenic reporters. Anal Biochem 411:200–209

Rummel A (2012) Double Receptor ancharage of botulinum neurotoxins accounts for thier exquisite neurospecificity. doi: 10.1007/978-3-642-3-570-9-4

Saiki RK, Gelfand DH, Stoffel S et al (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Sakaguchi G (1982) *Clostridium botulinum* toxins. Pharmacol Ther 19:165–194

Sakaguchi G, Sakaguchi S, Kozaki S et al (1974) Cross reaction in reversed passive hemagglutination between *Clostridium botulinum* type A and B toxins and its avoidance by the sue of anti-toxic component immunoglobulin isolated by affinity chromatography. Jpn J Med Sci Biol 27:161–172

Sakamoto T, Torii Y, Takahashi M et al (2009) Quantitative determination of the biological activity of botulinum toxin type A by measuring the compound muscle action potential (CMAP) in rats. Toxicon 54:857–861

Sakuma T, Kurosaki Y, Fujinami Y et al (2009) Rapid and simple detection of *Clostridium botulinum* types A and B by loop-mediated isothermal amplification. J Appl Microbiol 106:1252–1259

Sánchez-Hernández L, Cifuentes A, Jiménez B et al (2008) Detection of *Clostridium botulinum* neurotoxin coding genes: analysis of PCR products by real time versus capillary gel electrophoresis methods. Eur Food Res Technol 227:495–502

Sapsford KE, Taitt CR, Loo N et al (2005) Biosensor detection of botulinum toxoid A and staphylococcal enterotoxin B in food. Appl Environ Microbiol 71:5590–5592

Sapsford KE, Sun S, Francis J et al (2008) A fluorescence detection platform using spatial electroluminescent excitation for measuring botulinum neurotoxin A activity. Biosens Bioelectron 24:618–625

Satterfield BA, Stewart AF, Lew CS et al (2010) A quadruplex real-time PCR assay for rapid detection and differentiation of the *Clostridium botulinum* toxin genes A, B, E and F. J Med Microbiol 59:55–64

Schiavo G, Rossetto O, Catsicas S et al (1993) Identification of the nerve terminal targets of botulinum serotypes A, D, and E. J Biol Chem 268:23784–23786

Schmidt JJ, Stafford RG, Millard CB (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. Anal Biochem 296:130–137

Scotcher MC, Cheng LW, Stanker LH (2010) Detection of botulinum neurotoxin serotype B at sub mouse LD_{50} levels by a sandwich immunoassay and its application to toxin detection in milk. PLoS ONE 5:e11047

Seddon HR (1922) Bulbar paralysis in cattle due to the action of toxicogenic bacillus, with a discussion on the relationship of the condition to forage poisoning (botulism). J Comp Pathol Therapeut 35:147–190

Sesardic D, McLellan K, Ekong TA et al (1996) Refinement and validation of an alternative bioassay for potency testing of therapeutic botulinum type A toxin. Pharmacol Toxicol 78:283–288

Sesardic D, Leung T, Gaines Das R (2003) Role for standards in assays of botulinum toxins: international collaborative study of three preparations of botulinum type A toxin. Biologicals 31:265–276

Sesardic D, Jones RG, Leung T et al (2004) Detection of antibodies against botulinum toxins. Mov Disord 19:S85–S91

Sharma SK, Eblen BS, Bull RL et al (2005) Evaluation of lateral-flow *Clostridium botulinum* neurotoxin detection kits for food analysis. Appl Environ Microbiol 71:3935–3941

Sharma SK, Ferreira JL, Eblen BS et al (2006) Detection of type A, B, E, and F *Clostridium botulinum* neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. Appl Environ Microbiol 72:1231–1238

Shin NR, Yoon SY, Shin JH et al (2007) Development of enrichment semi-nested PCR for *Clostridium botulinum* types A, B, E, and F and its application to Korean environmental samples. Mol Cells 24:329–337

Shine N, Eaton L, Crawford K (2002) A continuous fluorimetric assay for high throughput screening for BoNT/A inhibitors. Naunyns Schmiedebergs Arch Pharmacol 365(Suppl. 2), Abstract R128

Shone C, Wilton-Smith P, Appleton N et al (1985) Monoclonal antibody-based immunoassay for type A *Clostridium botulinum* toxin is comparable to the mouse bioassay. Appl Environ Microbiol 50:63–67

Shriver-Lake LC, Ogert RA, Ligler FS (1993) A fiber-optic evanescent-wave immunosensor for large molecules. Sens Actuators B Chem 11:239–243

Simpson LL (1973) The interaction between divalent cations and botulinum toxin type A in the paralysis of the rat phrenic nerve-hemidiaphragm preparation. Neuropharmacology 12:165–176

Simpson LL (1974) Studies on the binding of botulinum toxin type A to the rat phrenic nerve-hemidiaphragm preparation. Neuropharmacology 13:683–691

Simpson LL (2004) Identification of the major steps in botulinum toxin action. Annu Rev Pharmacol Toxicol 44:167–193

Simpson LL, Tapp JT (1967) Actions of calcium and magnesium on the rate of onset of botulinum toxin paralysis of the rat diaphragm. Int J Neuropharmacol 6:485–492

Smith TJ, Lou J, Geren IN et al (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun 73:5450–5457

Stanker LH, Merrill P, Scotcher MC et al (2008) Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type A and their use in analysis of milk by sandwich ELISA. J Immunol Methods 336:1–8

Strotmeier J, Willjes G, Binz T et al (2012) Human synaptotagmin-II is not a high affinity receptor for botulinum neurotoxin B and G: increased therapeutic dosage and immunogenicity. FEBS Lett 586:310–313

Sugiyama H (1980) Clostridium botulinum neurotoxin. Microbiol Rev 44:419–448

Sugiyama H, Oishi I, Dasgupta BR (1974) Evaluation of type A botulinal toxin assays that use antitoxin to crystalline toxin. Appl Microbiol 27:333–336

Sugiyama H, Brenner SL, Dasgupta BR (1975) Detection of *Clostridium botulinum* toxin by local paralysis elicited with intramuscular challenge. Appl Microbiol 30:420–423

Sun S, Ossandon M, Kostov Y et al (2009) Lab-on-a-chip for botulinum neurotoxin a (BoNT-A) activity analysis. Lab Chip 9:3275–3281

Sun S, Francis J, Sapsford KE et al (2010) Multi-wavelength Spatial LED illumination based detector for in vitro detection of botulinum neurotoxin A activity. Sens Actuators B Chem 146:297–306

Szabo EA, Pemberton JM, Desmarchelier PM (1992) Specific detection of *Clostridium botulinum* type B by using the polymerase chain reaction. Appl Environ Microbiol 58:418–420

Szabo EA, Pemberton JM, Desmarchelier PM (1993) Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. Appl Environ Microbiol 59: 3011–3020

Szabo EA, Pemberton JM, Gibson AM et al (1994) Polymerase chain reaction for detection of *Clostridium botulinum* types A, B and E in food, soil and infant faeces. J Appl Bacteriol 76:539–545

Szílagyi M, Rivera VR, Neal D et al (2000) Development of sensitive colorimetric capture ELISAs for *Clostridium botulinum* neurotoxin serotypes A and B. Toxicon 38:381–389

Takahashi M, Kameyama S, Sakaguchi G (1990) Assay in mice for low levels of *Clostridium botulinum* toxin. Int J Food Microbiol 11:271–277

Takahashi H, Takakura C, Kimura B (2010) A quantitative real-time PCR method for monitoring *Clostridium botulinum* type A in rice samples. J Food Prot 73:688–694

Takeda M, Tsukamoto K, Kohda T et al (2005) Characterization of the neurotoxin produced by isolates associated with avian botulism. Avian Dis 49:376–381

Takeshi K, Fujinaga Y, Inoue K et al (1996) Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by ploymerase chain reaction. Microbiol Immunol 40:5–11

Terilli RR, Moura H, Woolfitt AR et al (2011) A historical and proteomic analysis of botulinum neurotoxin type/G. BMC Microbiol 11:232

Theiler A, Robinson EM (1927) Der Botulismus der Haustiere. Z Infektionskr Haustiere 31: 165–220

Thomas RJ (1991) Detection of *Clostridium botulinum* types C and D toxin by ELISA. Aust Vet J 68:111–113

Torii Y, Goto Y, Takahashi M et al (2010a) Quantitative determination of biological activity of botulinum toxins utilizing compound muscle action potentials (CMAP), and comparison of neuromuscular transmission blockage and muscle flaccidity among toxins. Toxicon 55:407–414

Torii Y, Takahashi M, Ishida S et al (2010b) Quantification of potency of neutralizing antibodies to botulinum toxin using compound muscle action potential (CMAP). Toxicon 55:662–665

Torii Y, Kiyota N, Sugimoto N et al (2011) Comparison of effects of botulinum toxin subtype A1 and A2 using twitch tension assay and rat grip strength test. Toxicon 57:93–99

Umeda K, Seto Y, Kohda T et al (2009) Genetic characterization of *Clostridium botulinum* associated with type B infant botulism in Japan. J Clin Microbiol 47:2720–2728

Umeda K, Seto Y, Kohda T et al (2010) A novel multiplex PCR method for *Clostridium botulinum* neurotoxin type A gene cluster typing. Microbiol Immunol 54:308–312

van Baar BL, Hulst AG, de Jong AL et al (2002) Characterisation of botulinum toxins type A and B, by matrix-assisted laser desorption ionisation and electrospray mass spectrometry. J Chromatogr A 970:95–115

van Baar BL, Hulst AG, de Jong AL et al (2004) Characterisation of botulinum toxins type C, D, E, and F by matrix-assisted laser desorption ionisation and electrospray mass spectrometry. J Chromatogr A 1035:97–114

van Ermengem E (1897) Ueber einen neuen anaëroben Bacillus und seine Beziehungen zum Botulismus. Z Hyg 26:1–56

Varnum SM, Warner MG, Dockendorff B et al (2006) Enzyme-amplified protein microarray and a fluidic renewable surface fluorescence immunoassay for botulinum neurotoxin detection using high-affinity recombinant antibodies. Anal Chim Acta 570:137–143

Viscidi R, Laughon BE, Hanvanich M et al (1984) Improved enzyme immunoassays for the detection of antigens in fecal specimens. Investigation and correction of interfering factors. J Immunol Methods 67:129–143

Volland H, Lamourette P, Nevers M-C et al (2008) A sensitive sandwich enzyme immunoassay for free or complexed *Clostridium botulinum* neurotoxin type A. J Immunol Methods 330:120–129

Wang D, Baudys J, Kalb SR et al (2011) Improved detection of botulinum neurotoxin type A in stool by mass spectrometry. Anal Biochem 412:67–73

Warner MG, Grate JW, Tyler A et al (2009) Quantum dot immunoassays in renewable surface column and 96-well plate formats for the fluorescence detection of botulinum neurotoxin using high-affinity antibodies. Biosens Bioelectron 25:179–184

Weingart OG, Schreiber T, Mascher C et al (2010) The case of botulinum toxin in milk: experimental data. Appl Environ Microbiol 76:3293–3300

Weingart OG, Gao H, Crevoisier F et al (2012) A bioanalytical platform for simultaneous detection and quantification of biological toxins. Sensors (Basel) 12:2324–2339

Wictome M, Newton K, Jameson K et al (1999a) Development of an in vitro bioassay for *Clostridium botulinum* type B neurotoxin in foods that is more sensitive than the mouse bioassay. Appl Environ Microbiol 65:3787–3792

Wictome M, Newton KA, Jameson K et al (1999b) Development of in vitro assays for the detection of botulinum toxins in foods. FEMS Immunol Med Microbiol 24:319–323

Wilder-Kofie TD, Lúquez C, Adler M et al (2011) An alternative in vivo method to refine the mouse bioassay for botulinum toxin detection. Comp Med 61:235–242

Woudstra C, Skarin H, Anniballi F et al (2012) Neurotoxin gene profiling of *Clostridium botulinum* types C and D native to different countries within Europe. Appl Environ Microbiol 78:3120–3127

Wu HC, Huang YL, Lai SC et al (2001) Detection of *Clostridium botulinum* neurotoxin type A using immuno-PCR. Lett Appl Microbiol 32:321–325

Yamashiro S, Sano Y, Komano A et al (2007) Detection of proteinous toxins using the bio-threat alert system, part 4. Differences in detectability according to manufactural lots and according to toxin subtypes. Forensic Toxicol 25:80–83

Yoon SY, Chung GT, Kang DH et al (2005) Application of real-time PCR for quantitative detection of *Clostridium botulinum* type A toxin gene in food. Microbiol Immunol 49:505–511