

Recent Advances in Detection and Identification of Botulinum Neurotoxins (BoNTs)

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ABSTRACT

Botulinum neurotoxins (BoNTs) are metalloproteases of strong bacterial toxins causing heavy neuromuscular poisoning in humans and animals. Botulism is the disease caused by eating food that contains the toxins, BoNTs, produced by *Clostridium botulinum*. BoNTs are also considered the most dangerous biological weapons and terrorist agents. These bacterial toxins are also used as therapeutic agents for neurospastic illnesses, in research examining various endocrine secretions and other molecular functions and, of course, in cosmetic treatments. The identification and detection of these toxins are critical to determining the sources of illnesses, including possible terrorist agent use. A fast, reliable method to identify and detect these toxins is important for diagnosis of the disease and critical in the event of a bioterrorist attack using these agents. In this review, we discuss several recent molecular and instrumental approaches for detection and identification of these toxins.

INTRODUCTION

The botulinum neurotoxins (BoNTs) are zinc-containing metalloproteases produced by bacteria, which cause neuromuscular poisoning (botulism), and which can be harmful to humans and animals. BoNTs are considered among the most dangerous (group A) biological weapons [Caya 2004]. However, in addition to being potential bioterrorist agents, BoNTs are often used as therapeutic and cosmetic pharmaceuticals [Patocka 2005]. The toxins are produced by anaerobic, sporulating bacteria such as *Clostridium botulinum* (types A to G), *Clostridium butyricum* and *Clostridium barati*, which are often found in soil and mud [Caya 2004]. The neurotoxins produced by *Clostridium*, including BoNTs and tetanus toxin (TeNT), are endoproteases, which inactivate specific functional proteins in the nervous system thus blocking neurotransmitter release and neuronal conductivity. This blocking action causes the appearance of flaccid paralysis in botulism (inhibits acetylcholine release), as well as the spastic paralysis in tetanus (inhibits GABA or glycine release). After separation from a large hemagglutinin complex, BoNT has been identified as a single chain

protein that is cleaved into two protein chains, one heavy chain (H) 100 kilo Dalton, and one light chain polypeptide (L) 50 kilo Dalton, stabilized by a tetrahedral Zn⁺⁺ motif and disulfide bond [Figure 1]. BoNT types A and B pass across cell membranes reaching neurotransmitters within the nervous system [Singh 2000].

The intracellular function of BoNTs is associated with proteolytic inactivation of cellular proteins responsible for the release of acetylcholine neurotransmitter in neuro-muscular synapses (SNARE; soluble N-ethylmaleimide sensitive factor attachment proteins receptor) [Caya 2004].

In a three-dimensional complex, BoNT has been shown to surround SNARE molecules as a python ensnares its prey [Breidenbach and Brunger, 2004; Schiavo 1992]. The SNARE complexes, which enable presynaptic exocytosis, are extremely specific for their targets, i.e., each particular toxin, including BoNT, recognizes and reacts with only one of the three different neuronal proteins of the SNARE complex: SNAP-25 (synaptosomal-associated protein-25), synaptobrevin-2 (or VAMP-2 - vesicle associated membrane protein-2), and syntaxin. SNAP-25 is required to release neurotransmitters from the endings of axons. Structural analyses revealed that different neurotoxins wrap around different segments of SNARE by attaching/recognizing multiple domains. Therefore, it is possible that different neurotoxins may attack different specific sites on SNAREs [Breidenbach and Brunger 2004]. In particular, the BoNT type A, as well as types C and E, inactivate SNAP-25, however BoNT types B, D, F and G inactivate synaptobrevin-2. Among the botulinum neurotoxins, only BoNT type C attacks more than one receptor complex, both SNAP-25 and syntaxin. Interestingly, TeNT inactivates synaptobrevin [Schiavo 2000].

NEW ANALYTICAL APPROACHES TO NEUROTOXINS

The Mouse Neutralization Assay is the most common diagnostic test for botulinum toxins and is very sensitive. This biological test is performed in mice and can detect as little as 0.03 ng of the toxin, but the test takes days. Although this method goes back to the very beginning of studies on botulinum poisoning, it is still frequently used in diagnostic laboratories and hospitals. The test allows not only detection of toxic properties of the poison, but also identifies type-specific antiserum for the medical treatment [Rymkiewicz 1971]. The test is still recommended in Sentinel Laboratory Guidelines for Suspected Agents of Bioterrorism published by the American Society for Microbiology and dated July 30, 2003.

<http://www.asm.org/ASM/files/LeftMarginHeaderList/DOWNLOADFILENAME/000000000522/Botulism.pdf>

Despite these advantages, animal or in vivo tests, including the Mouse Neutralization Assay, are inconvenient because they take several days to complete and require the use of live animals. Several in vitro tests have also been developed, including Immunofluorescence-Adsorption Test (IFAT) [Wojtyla 1973], Indirect Hemagglutination Inhibition, Indirect Hemagglutination Blocking Reaction (Hap) [Ligieza and Reiss 1986; Reiss 1983], Immunoenzymatic Test, including ELISA (Michalik 1986), and Immunochemiluminescent Test (ICL) [Ligieza 1986; Ligieza 1994]. Polymerase chain reaction (PCR) methods have been used as a diagnostic tool for the detection of *Clostridium botulinum* in food and stool samples. Although these tests can be used as an extremely sensitive test for the detection of the genome for the four different types of neurotoxins, it does not distinguish between living or dead bacteria and does not detect any of the toxins themselves [Craven 2002; Dahlenborg 2001]. This diagnostic limitation of PCR method mean that they cannot be used to detect the actual toxins in poisoned patients. In recent years, several new experimental methods for the detection and identification of botulinum toxins have been established and these are described below.

Fluorescent Tests

1. Time-Resolved Fluorescence.

Time-Resolved Fluorescence, TRF, assays have been developed for BoNTs types A and B and are based on a typical ELISA (enzyme-linked immunosorbent assay). The assays utilize a specific biotinylated capture antibody pre-bound to a streptavidin-coated, 96-wells, plate and a lanthanide, Eu(III), labeled detector antibody. The bound Eu(III)-labeled detector antibody produces a fluorescent signal upon the addition of an enhancement solution. The signal results from the dissociation of the Eu(III) from the antibody, which creates a micelle, and thus amplifies the signal nearly 106-fold. The sensitivity achieved by this assay is between 4-20 pg/mL. This test, according to the authors, is more sensitive and quicker than a regular ELISA and can provide early detection of the toxins in clinical and environmental samples [Peruski 2002].

2. Fluorometric Biosensor.

Fluorometric biosensor for detection of BoNT, types A - G, and TeNT activities. To detect neurotoxin activity, fragments of the specific toxin substrate, synaptobrevin or SNAP25, are linked with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The cleavage of these fusion proteins by BoNT or TeNT eliminates fluorescence resonance energy transfer (FRET) between CFP and YFP, providing a very sensitive indicator for the detection of toxin activity in real time in vitro and in vivo [Dong 2004; Parpura and Chapman 2005].

Non-Fluorescent Tests

1. Modified ELISA Methods.

A novel modification of ELISA, based on immobilized synthetic substrates for BoNTs, such as SNAP-25 and VAMP, can distinguish between BoNT types A and B. The method uses enzyme-labeled antibodies. Importantly, the tests are very specific and do not cross-react with other *Clostridium* neurotoxins. Their sensitivities range between 0.6 and 4.5 ng/mL and could be increased significantly by the assay amplification system based on catalyzed reporter deposition. In addition, trypsin treatment of BoNT samples, which converts the single chain toxin to an active double-chain form, was found to increase the sensitivity of the assay up to 10-fold [Hallis 1996]. Several other modified ELISA-based detection methods for botulinum toxins have been also described [Doellgast 1994; Doellgast 1993; Ferreira 2004; Ferreira 2003; Sharma 2006].

2. Micromechanosensor Methods.

This unique and extremely fast technology (minutes) requires atomic force microscope (AFM), and a mechanosensor in which nickel-coated agarose beads are conjugated to the C-terminal portion of synaptobrevin-2. This part of the mechanosensor is linked to a nickel-coated cantilever with C-terminally attached syntaxin-1A. In this assay, N-terminally bound synaptobrevin-2 and syntaxin-1A represent the substrate for BoNT type B (BoNT/B) action. In the presence of Zn²⁺, BoNT/B cleaves synaptobrevin-2 in such a manner that a short fragment of synaptobrevin-2 remaining on the bead no longer has the ability to interact with syntaxin-1A on the cantilever, and the bead detaches. Subsequently, the movement of the released cantilever is detected by AFM and the signal is proportional to the amount of BoNT/B present in the reaction [Liu 2003; Parpura and Chapman 2005].

3. Mass spectrometry (MS) Methods.

This extremely sensitive and rapidly evolving technique, which is used for protein identification, can be also adapted for the detection of bacterial toxins. The BoNTs and their direct substrates, SNAP-25 and VAMP-2, have been analyzed by matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) mass spectrometry. In this method, the protein sample (in this case BoNT digested specific substrates) is incorporated into energy-absorbing matrix (prepared from small molecules such as dihydrobenzoic acid and deposited on metal substrate along with the

sample). Ions are generated when the matrix is struck and excited by laser light. The MALDI generates the single charged ion species. There is a direct correlation between mass spectra and concentrations of the protein in the sample. In the next step, the TOF analyzer measures time of the travel of gas-phase ions from the ionization source to the detector, which is specific to each of the ions. Importantly, MALDI-TOF MS is capable of detecting and differentiating between the different botulinum toxins, A, B, E and F, at concentrations starting from 5 pg/mL [Barr 2005].

Diagnostic methods based on MS require special consideration, not only because of their high sensitivity, but also because their principles are not based on the traditional recognition by specific antibodies. Some of MS methods are based on the detection of the SNARE complexes (SNAP-25, synaptobrevin-2, syntaxin), which have been specifically digested and inactivated by Zn-dependent endoproteases of BoNTs; the method is referred to as Endopeptidase-MS. The SNARE-based indicators open a novel approach for the detection and differentiation of a wide variety of these bacterial toxins, which are similar with respect to their pharmaco-dynamic action, but are quite different in their distinct sites of synaptic interaction [Barr 2005]. As described by Barr et al. [Barr 2005], synthetic SNARE substrates (SNAP-25, VAMP) have been used in MS-based detection of the peptide fragments generated as a result of BoNT-mediated degradation. This new approach is extremely sensitive (10-100 fold more sensitive than the Mouse Neutralization Assay), and is considered quite rapid (16 hours), despite the preparation steps. In addition, a complex MS analysis with the involvement of multiple substrates, in a similar manner to the previously mentioned hemagglutination tests [Ligieza and Reiss 1986; Reiss 1983], is capable of distinguishing among the four serotypes BoNT/A, B, E and F. This method can also be used in the analysis of various clinical, environmental and food samples [Barr 2005].

Other studies have contributed to the modification and adaptation of mass spectrometry (liquid chromatography with matrix-assisted laser desorption ionisation and electrospray mass spectrometry) to detect and characterize TeNT, as well as identify the different BoNTs types A, B, C, D, E and F [van Baar, 2002a, 2004; van Baar 2002b]. In this particular methodology, the authors used liquid chromatographic-mass spectrometry (LC-MS) and analyzed purified toxin preparations and subsequent cellular lysates from the liquid cultures of various strains of *Clostridium tetani*. Two distinct MS methods were used to characterize purified and enzymatically digested toxins: (i) accurate mass measurement and (ii) tandem mass spectrometry (MS/MS) amino acid sequencing of isolated peptides. Importantly, the authors strongly suggested that the first method was inadequate for bacterial supernatants because of contamination with other unrelated peptides.

As discussed above, MS methods have also been applied to BoNTs type A and B, and BoNT complexes, which usually consist of neurotoxins (NTs), hemagglutinin (HAs) and particles that are neither toxins nor hemagglutinins (non-toxins non-hemagglutinins; NTNHS). Of interest, results obtained from BoNT MS analyses almost completely matched the information from genomic sequences for the examined strain [van Baar 2002a]. In a similar manner, other types of botulinum complexes including BoNT type C, D, E and F were examined, and similar conclusions have been reached regarding the identification, sensitivity and usefulness of the MS methods [van Baar 2004].

DISCUSSION

Botulinum neurotoxins (BoNTs), which are known to cause profound neuromuscular poisoning in humans and animals, are also classified as the most dangerous biological weapon, group A [Caya 2004]. Therefore, it is increasingly important to develop and employ adequate methodologies for quick and specific detection, and possibly neutralization of BoNTs. Although the Mouse Neutralization Assay is still considered the standard test for the detection of BoNTs, there has been a remarkable progress in the development of alternative tests during the past decade [Lindstrom and Korkeala 2006]. These new techniques have mostly focused on the improvement of the sensitivity and specificity of the test, and on the decrease of time necessary for detection. The ideal analysis would use widely

accessible technology and require basic laboratory equipment, provide simultaneous detection of all known BoNTs at picogram concentrations, and complete the analysis within hours or fractions of hours. With respect to these requirements, the Mouse Neutralization Assay can detect as little as 20-30 pg/mL of the neurotoxin in serum, food, stool and environmental samples. However, the analysis takes about 3 to 4 days, and requires a large number of experimental animals [Rymkiewicz 1971].

In comparison to the Mouse Neutralization Assay, the immunoassays are technically simpler and faster [Ekong 1995]. However, many of these early assays, including radioimmunoassay [Ashton 1985], gel diffusion assay [Ferreira 1981], and ELISA are less sensitive and less specific [Dezfulian and Bartlett 1984]. Using recent techniques in signal amplification, sensitivities similar to those of Mouse Neutralization Assay can be achieved. However, these immunological assays require high-quality antibodies, which are not generally available. In addition, genetic variations within the different serotypes of the neurotoxin may lead to decreased affinity to antibodies, causing false-negative results [Smith 2005].

Also described in this review are methods for cleaved BoNT peptides by mass spectrometry, MS, which further enhances the sensitivity and specificity of detection. However, because of the requirement of very expensive equipment, as well as the necessity for highly trained personnel, all MS-based technologies are available only for a very few highly specialized laboratories.

Endopeptidase-MS is a rapid test to detect, differentiate and quantify BoNTs type A, B, E and F. It can be used for a quick detection of specific serotypes of BoNTs in a variety of materials including food, clinical and environmental samples. Importantly, it is about 100-times more sensitive than the Mouse Neutralization Assay and the detection time can be less than 5 hours [Barr 2005].

Simple endopeptidase assays (based on SNARE complex) have the potential to replace the Mouse Neutralization Assay, as they detect only biologically active neurotoxins, and are generally more sensitive than the Mouse Neutralization Assay. In this respect, endopeptidase modified ELISA is a novel, rapid in vitro assay capable of determining biological activity of type A and type B BoNTs. The test has no cross-reactivity with other clostridial neurotoxins, and its sensitivity ranges from 0.6 to 4.5 ng/mL, or in some amplification variants from 0.1 to 1.2 ng/mL [Hallis 1996]. These SNARE-based assays have the potential to be adapted for use in moderately equipped microbiological and toxicological laboratories because they are not overly expensive or difficult to operate. These assays may also be adapted to specialty field diagnostics and field laboratories.

CONCLUSIONS

Our review of BoNTs, which include structure and molecular interactions with synaptic proteins, describes new possibilities for the detection, identification, and treatments of the BoNTs-mediated neuronal dysfunctions. Given the current environment and threat of bio-terrorism, it is increasingly important to use quick, sensitive and dependable methods for the detection of BoNT poisoning and in order to provide the correct treatment of patients affected by these fatal neurotoxins.

TABLES

Table 1. Comparison of selected diagnostic and detection assays for BoNTs - application for field laboratories.

Assay	Type of toxin	Time of the assay	Detection limit	Potential for field diagnostics*
Mouse neutralization assay	A, B, C, D, E, F, G	1-4 days	20-30 pg/mL	++
TRF [Peruski 2002]	A, B	2 hours	20-200 pg/mL.	+
Fluorometric Biosensor [Dong 2004]	A, B	uncertain	?	+/-
Modified ELISA [Hallis 1996]	A, B	2-3 hours	0.6 and 4.5 ng/mL	++
Micromechanosensor [Liu 2003]	B	15 minutes	>8 nM	++
Mass Spectrometry MALDI-TOF-MS/ Endopeptidase-MS [Barr 2005]	A, B, E, F	4 - 16 hours	5 pg/mL or lower	+/-

*Potential for field diagnostics: ++ high, +intermediate, +/- low

FIGURES

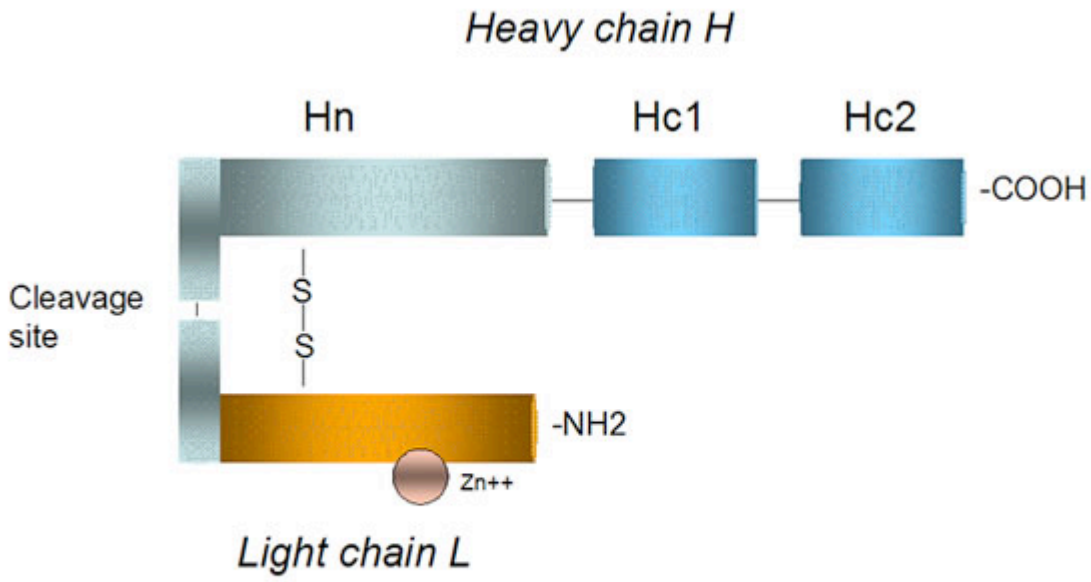


Figure 1. Schematic illustration of the cleavage site of BoNT [according to Singh 2000].

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