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Mass Spectrometric Identification and Differentiation of Botulinum Neurotoxins through Toxin Proteomics

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Abstract

Botulinum neurotoxins (BoNTs) cause the disease botulism, which can be lethal if untreated. There are seven known serotypes of BoNT, A-G, defined by their response to antisera. Many serotypes are distinguished into differing subtypes based on amino acid sequence and immunogenic properties, and some subtypes are further differentiated into toxin variants. Toxin characterization is important as different types of BoNT can respond differently to medical countermeasures for botulism, and characterization of the toxin can aid in epidemiologic and forensic investigations. Proteomic techniques have been established to determine the serotype, subtype, or toxin variant of BoNT. These techniques involve digestion of the toxin into peptides, tandem mass spectrometric (MS/MS) analysis of the peptides, and database searching to identify the BoNT protein. These techniques demonstrate the capability to detect BoNT and its neurotoxin-associated proteins, and differentiate the toxin from other toxins which are up to 99.9% identical in some cases. This differentiation can be accomplished from toxins present in a complex matrix such as stool, food, or bacterial cultures and no DNA is required.

Introduction

Botulism is a disease caused by intoxication with any one of the highly toxic proteins known as botulinum neurotoxins (BoNTs). BoNT are composed of a heavy chain, which binds to receptors on the neuron, and a light chain which is a protease. *In vivo*, the BoNT light chain cleaves proteins necessary for nerve signal transmission resulting in flaccid paralysis.

Botulinum neurotoxins are currently classified into seven serotypes, labeled A-G.

BoNT/A, /C, and /E cleave SNAP-25 (synaptosomal-associated protein) [1–6] whereas BoNT/B, /D, /F, and /G cleave synaptobrevin-2 (also known as VAMP-2) [7–12]. BoNT/C is unique in that it targets more than one protein; it is also known to cleave syntaxin [13]. BoNT is produced as a protein complex in conjunction with neurotoxin-associated proteins (NAPs).

The seven different serotypes of BoNT are defined by their ability to be neutralized by an antiserum produced using a specific BoNT type. Therefore, identification of the serotype of BoNT is important as each serotype is neutralized by a different antiserum. Nucleotide or

amino acid sequence variation within BoNTs in strains of a serotype has led to the designation of subtypes. Historically, subtypes have been defined by cultural/biochemical characteristics [14], functional differences [15], or differential binding of monoclonal antibodies (mAbs) [16–18]. Amino acid variation within a subtype can vary by as much as 30%, in the case of BoNT/F1 and /F5 [19], although amino acid variance within a serotype is typically less than 10%. This variation could be due to a new strain (*Clostridium* organism) or toxin variant (neurotoxin protein), with some of the neurotoxins of some strains having as few as a single amino acid difference, or 0.08% difference.

Identification of the subtype of BoNT is important for several reasons. First, one definition of a subtype of BoNT indicates that different subtypes of toxin may have differential binding to monoclonal antibodies, and perhaps some polyclonal antibodies [15, 20]. This is important while researchers search for an alternative treatment to the currently used equine immunoglobulin approach to treat botulism. Various mAb are proposed as immunoglobulin treatments for botulism; however, if there is differential binding of these antibodies to different subtypes, care must be taken in choosing which antibodies to use as treatment, as the antibodies might not be effective at neutralizing all subtypes of BoNT within a serotype. Secondly, identification of the BoNT subtype could be important to epidemiologic and forensic investigations attempting to trace the origin of the toxin, its spread in a botulism incident, and commonality/differences in concurrent botulism outbreaks.

Subtype identification is typically accomplished through DNA sequencing of the toxin's genes [21]. Other DNA analysis techniques such as pulsed field gel electrophoresis [22], randomly amplified polymorphic DNA analysis [23], amplified fragment-length polymorphism analysis [24], *flaA* variable-region sequencing [25], multilocus sequence typing [26], multiple locus variable-number tandem repeat analysis [27], and a comparative genomic hybridization microarray [28] have also been used to differentiate strains. All of these methods depend on the presence of bacterial DNA in the sample material. However, in some botulism cases, BoNT is present but the bacterium cannot be isolated or cultured. In such a situation, toxin subtype identification or strain characterization is difficult, but perhaps possible, using traditional DNA-based methods [27, 29] as samples can still contain small amounts of DNA. In cases that no DNA is found, then the DNA-based methods cannot be used for subtype identification. Therefore, an alternate method which does not rely upon the presence of DNA may be the only option for subtype/toxin variant identification. Additionally, proteomic techniques are very rapid and have been used to quickly answer critical questions in the required time frame of a botulism outbreak investigation. In this review, we detail proteomic methods which yield information on the amino acid sequence of the toxin in question and thus reveal its serotype or subtype/toxin variant.

Mass Spectral Identification of Botulinum Neurotoxin Serotype

The seven known serotypes of BoNT are 34–64% identical to each other at the amino acid sequence level [30]. Because these proteins contain hundreds of amino acid differences, it is possible to distinguish these serotypes from each other through analysis of their amino acid sequences. Mass spectrometry has become a popular technique for identification of the amino acid sequence of a protein in question, and in 2002, van Baar et al reported on their

mass spectral analysis of BoNT/A and /B [31]. In this work, they digested the BoNT/A and /B protein complexes with trypsin, resulting in a series of peptides. The peptides were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to obtain a peptide mass fingerprinting (PMF) map, and in some selected cases, peptides were sequenced using MALDI with post-source decay (PSD). Peptides were also separated by liquid chromatography (LC) and then analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a quadrupole time-of-flight (QTOF) instrument. PMF data or MS/MS were then searched against the NCBI database containing BoNT protein sequences. Through this work, they reported a sequence coverage of greater than 35% for BoNT/A and 25% for BoNT/B. These sequence coverages are sufficient to identify the protein as BoNT and to differentiate at the serotype level, as BoNT/A or /B.

In 2004, van Baar et al extended this work to encompass serotypes /C, /D, /E, and /F [32]. This work used the same analysis techniques as their previous report [29]. Through this work, they reported sequence coverages of 11% for BoNT/C, 49% for BoNT/D, 30% for BoNT/E, and 53% for BoNT/F. Because these proteins are 36% or more dissimilar, these sequence coverages were sufficient to identify the protein as BoNT and to differentiate the serotype of BoNT. It should be noted that there are other published, non-proteomic mass spectrometric methods which detect BoNT and differentiate at the serotype level [33, 34]. These methods detect and identify BoNT by detecting the specific enzymatic activity of the neurotoxin. Because each serotype of toxin has a different cleavage site on synaptobrevin-2 or SNAP-25, mass spectrometric detection of the cleavage of synaptobrevin-2 or SNAP-25 at that particular cleavage site will identify the presence of BoNT and differentiate the serotype. While these methods are good at detecting the presence of active BoNT and differentiating the serotype, such a method is inadequate at differentiating beyond the serotype levels as all BoNT subtypes within a serotype cleave synaptobrevin-2 or SNAP-25 in the same location [35], with the exception of BoNT/F5 [7].

Mass Spectral Identification of Neurotoxin-Associated Proteins

BoNTs are produced by *Clostridium botulinum*, *C. butyricum*, *C. baratii*, and *C. argentinense*, and are produced as a protein complex also known as the progenitor toxin, consisting of the neurotoxin and neurotoxin-associated proteins (NAPs). The composition of the progenitor toxin can differ between serotypes. The role of these NAPs has not been completely deduced; however, it is likely that the NAPs serve to protect the neurotoxin from harsh conditions found in the stomach and digestive tract, including low pH and digestive enzymes [36]. Additionally, it has been proposed that these NAPs assist with translocation of the neurotoxin across the intestinal epithelium [37], and the NAPs may assist with the immunogenicity of BoNT/A [38]. Identification and characterization of the NAPs may also assist with BoNT serotype identification as these proteins differ between serotypes.

Mass spectral identification of the NAPs associated with BoNT/A-G was first reported in 2005 [39]. In this work, the protein complexes of BoNT/A-G were digested with trypsin, and the resultant peptides were separated by LC and introduced into a QTOF for analysis in the MS and MS/MS modes. Using Mascot, a mass spectrometric software search engine, mass spectral data were searched against the NCBI database for protein identification.

Through this work, the neurotoxins could be identified and differentiated from each other with sequence coverage of 18%, 23.5%, 33.3%, 18.6%, 36.5%, 21.9%, and 16.1% for BoNT/A-G respectively. These sequence coverages are similar to what was previously reported by van Baar [31, 32]. However, this work also reported for the first time, mass spectral identification of the NAPs, and reported that the NAPs varied between the serotypes.

BoNT/A was reported in a complex with nontoxic-nonhemagglutinin and hemagglutinin proteins; specifically, NTNH, HA-70, HA-33, HA-17, and flagellin, with sufficient sequence coverage to definitely identify those proteins [39]. BoNT/B was also reported in a complex with NTNH, HA-70, HA-33, HA-17 and flagellin, but in all cases except HA-70, those NAPs have different amino acid sequences, making it possible to distinguish serotypes. BoNT/C combines with NTNH, HA-70, HA-33, and HA-17; BoNT/D combines with NTNH, HA-70, HA-33, and flagellin; BoNT/E with NTNH, ORF-X1, and NBP; BoNT/F with NTNH only; and BoNT/G was reported in a complex with NTNH, HA-70, and HA-17. In all cases, the serotype of toxin complex could be differentiated based on at least one of the associated proteins due to the presence of NAPs with differing amino acid sequences [39].

Although sequence coverage of 10–50% is sufficient to differentiate BoNT serotypes, increased sequence coverage assures confident differentiation. In the work previously described in this section [31, 32, 39], a mixture of proteins was digested and analyzed. Analysis of protein mixtures can often result in decreased sequence coverage for any individual protein. In 2011, Moura et al published on the mass spectral identification of BoNT/C and BoNT/D complexes [40]. In this work, they separated the components of the progenitor complexes by gel and then digested each gel band containing an individual protein. Although the same proteins were identified compared to previous work [39], sequence coverage of most proteins reported increased even though lower amounts of toxin were used for this experiment [40]. Additionally, this work also utilized a label-free mass spectrometric quantitation method (MS^E) to report that 31% of the BoNT/C complex consisted of the neurotoxin whereas only 22% of the BoNT/D complex consisted of the neurotoxin [40].

This work also reported on a 3 minute in-solution digestion of the BoNT/C and /D complexes [40]. Shortening of the digestion time from a traditional overnight digestion to a 3 minute process allows for more rapid identification of the serotype of BoNT as the overnight digestion step is the longest step in the process. Many of these same procedures were used by Terilli et al in 2011, reporting on the proteomic analysis of BoNT/G. Sequence coverage were reported as 66% and above for BoNT/G, NTNH, HA-70, and HA-17[41], allowing for easy differentiation of the serotype of toxin complex. The label-free quantitation method (MS^E) reported a molar ratio of BoNT:NTNH:HA-70:HA-17 as 1:1:2:1, indicating that there are two molecules of NTNH and HA-70 for every one molecule of BoNT and HA-17.

BoNT Serotype Detection in Complex Matrices

BoNTs are purified and sold for research purposes either in the purified neurotoxin form or in the purified progenitor complex form. Additionally, the toxin can be found in bacterial cultures, foods, and animal/human clinical samples such as serum and stool. These matrices contain many abundant proteins and in most cases, the level of toxin proteins is far below that of the level of matrix proteins. Therefore, it is of interest to be able to detect and differentiate the serotype of BoNT in the presence of a complex matrix.

Klaubert et al published in 2009 on a technique to identify BoNT/A, /B, /E, and /F (the four serotypes which are associated with human cases of botulism) in the presence of a bacterial culture [42]. Because one role of the NAPs is to protect the toxin from digestive conditions, the bacterial culture was exposed to pepsin in pH 2, and matrix proteins were digested whereas the toxin complex remained intact. Peptides were then separated from proteins, and the proteins alone were digested with trypsin and separated by 2D nano-LC using ion exchange chromatography coupled with reversed phase chromatography. The peptides were then analyzed by nano-ESI MS/MS with data searched against the NCBI database. Although sequence coverage was less than 10%, toxin directly from bacterial cultures of four serotypes could be identified and differentiated [42].

Another approach to identify BoNT in the presence of a complex matrix has been the use of immunoaffinity purification of the toxin from other proteins present in the matrix. Using antibodies to the toxin, the toxin has been isolated from food [43–45] or bacterial culture [44, 46]. Through this process, both BoNT/A [43–45] and BoNT/B [44, 46] were reported to be detected even in the presence of complex matrices. The sequence coverage on the neurotoxin using this process was reported as between 65 and 98%. These high sequence coverage ensured identification and differentiation of these toxins. Although these techniques report the use of serotype-specific antibodies for toxin extraction, it should be noted that an antibody has been reported to bind BoNT/A, /B, /E, and /F, the four serotypes associated with human botulism [47] and that this antibody could be used to isolate any BoNT associated with human cases from its complex matrix.

Differentiation of BoNT at the Subtype/Toxin Variant Level

Differentiation of BoNT at the subtype/toxin variant level can be important for forensic or epidemiological reasons. Although there are many methods available to detect BoNT and differentiate it at the serotype level, few methods exist to differentiate the toxin below the serotype level, and most of these methods rely upon the presence of DNA for that determination. The serotypes of BoNT are 34–64% identical at the amino acid level. Because amino acid identity increases at the subtype level (70–97.5%) and increases even further at the toxin variant level (greater than 97.5%), identification of as much of the amino acid composition of the toxin as possible enables differentiation of the neurotoxin below the serotype level.

The first example of mass spectrometric detection and differentiation of BoNT below the serotype level was in 2005 [43]. In this work, BoNT/A1 and /A2 were spiked into milk and extracted with antibodies to BoNT/A. Both toxins were identified as BoNT/A through their

enzymatic activity upon a peptide substrate, and were then digested with trypsin and analyzed by LC-MS/MS. Upon searching the data against the NCBI database, the toxins were identified as either BoNT/A1 or /A2 with sequence coverages of 65–70% [43]. In 2010, differentiation of BoNT/B1 and /B4 from culture supernatants was reported following extraction of the toxin with immunoaffinity, digestion of the extracted toxin with trypsin, and MS/MS analysis by MALDI-TOF [44]. Similarly, differentiation of BoNT/B1–/B5 extracted from culture supernatants was reported in 2012 [46]. Sequence coverages of 66–77% enabled differentiation of these five different toxins at the subtype level despite similarities as high as 98% in the case of BoNT/B2 and /B3 [46].

In 2012, Wang et al reported on a new technique to improve sequence coverage of BoNT proteins [45]. This work consisted of separation of the protein complex components by SDS-PAGE followed by digestion of each gel band with multiple enzymes and sequential in-gel digestion. Peptides from the gel band containing the neurotoxin were then separated by LC and analyzed by MS/MS followed by database searching. This analysis yielded sequence coverage of 90% and greater for all serotypes of BoNT [45]. Furthermore, this technique was used to identify the toxin present in a toxin-contaminated carrot juice sample associated with a complex botulism outbreak. The toxin was first extracted from the carrot juice with monoclonal antibodies to BoNT/A prior to analysis with this technique. The sequence coverage of the toxin was 98.6%, allowing for a determination of the toxin as BoNT/A1 (GeneBank accession no. ABY56330) [45]. It is important to note that this high level of sequence coverage allowed for determination of the toxin variant in this case as there are two similar toxin variants which are 99.9% and 99.6% identical to the toxin variant identified in this sample.

All of these proteomic methods described above relied upon matching the mass spectral data to sequences of proteins within a database. Forcing the data to fit a protein in the database can lead to inaccurate identifications of proteins if the protein in question has not yet been discovered and sequenced. In 2012, a method was reported to identify novel subtypes or toxin variants of BoNT/B which have not yet been sequenced [46]. This method relied upon the creation of an amino acid substitution database in which each individual amino acid within the sequence of BoNT/B1 was mutated to the other 19 possibilities. The MS/MS data were then searched against this database of possible mutations for a match to a possible mutation. This technique was used to analyze toxin extracted from a culture supernatant of BoNT/B via immunoaffinity purification and resulted in the identification of 5 novel amino acid differences and the identification of a new subtype of BoNT/B, BoNT/B7 [46]. DNA sequencing confirmed the mass spectrometry results, and when the correct amino acid sequence was added to the database, sequence coverage of the toxin was 68% [46].

Conclusions and Future Directions

Mass spectral analysis of botulinum neurotoxins allows for identification of the toxin at the serotype level as well as subtype or toxin variant level provided that the sequence coverage of the toxin is sufficient. These analyses can be performed by ESI or MALDI mass spectrometers and can encompass identification of the neurotoxin-associated proteins in addition to the neurotoxin. BoNTs can be identified and differentiated even in the presence

of a complex matrix such as food or bacterial culture, and it is now possible for de novo identification of novel BoNTs through mass spectrometric techniques. All of this work can be accomplished in the absence of DNA, and is summarized in Table 1.

Identification and differentiation of botulinum neurotoxins is a growing field, and there are many potential new directions. One possibility includes detection of the toxin directly from clinical samples, particularly stool which is the dominant diagnostic matrix for infant botulism. Although there are mass spectrometric methods which detect the presence of BoNT in stool [48], there is no published work describing the proteomics of BoNT present in a stool sample. Obtaining amino acid sequence on BoNT through mass spectrometry typically requires ng levels of toxin, and often, clinical samples contain pg or lower levels of toxin. Therefore, toxin is not often present in clinical samples at levels adequate for subtype or toxin variant differentiation, and methods which could make these determinations in the presence of as little as 10 pg (67 attomole) of toxin could play an important role in timely epidemiologic or forensic investigations.

Increased sequence coverage would allow for more confident differentiation of the subtype or toxin variant and would also assist in epidemiologic or forensic investigations. It is possible that additional amino acid sequence information and hence higher sequence coverage could be obtained through alternate mass spectrometric fragmentation techniques such as electron-transfer dissociation (ETD) or electron-capture dissociation (ECD) or perhaps a combination of those techniques, as both techniques are reported to yield increased fragmentation information on larger peptides [49, 50]. A decrease in the time needed to make these analyses would also be an important improvement. Other possibilities for improvement exist in this growing field to combat the deadly disease of botulism.

The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Centers of Disease Control and Prevention.

Abbreviations used

BoNT	Botulinum neurotoxin
SNAP	synaptosomal-associated protein
NAP	neurotoxin-associated protein
mAb	monoclonal antibodies
MALDI	matrix-assisted laser desorption/ionization
PSD	post-source decay
MS/MS	tandem mass spectrometry
PMF	peptide mass fingerprinting
HA	hemagglutinin
NTNH	nontoxic-nonhemagglutinin
ESI	electrospray ionization

LC	liquid chromatography
QTOF	quadrupole time-of-flight
ETD	electron-transfer dissociation
ECD	electron-capture dissociation

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Table 1

Summary of pertinent information from references cited in this review.

Reference	Method used	Sequence coverage on toxin(s)	Complex matrix?	NAPs?
2002 van Baar	MALDI-PSD/ LC-ESI-MS/MS	35% BoNT/A; 25% BoNT/B	No	No
2004 van Baar	MALDI-PSD/ LC-ESI-MS/MS	11% BoNT/C; 49% BoNT/D, 30% BoNT/E, 53% BoNT/F	No	No
2005 Hines	LC-ESI-MS/MS	18% BoNT/A; 23.5% BoNT/B; 33.3% BoNT/C; 18.6% BoNT/D; 36.5% BoNT/E; 21.9% BoNT/F; 16.1% BoNT/G	No	Yes
2011 Moura	LC-ESI-MS/MS	44% BoNT/C; 18.4% BoNT/DC	No	Yes
2011 Terilli	LC-ESI-MS/MS	66% BoNT/G	No	Yes
2009 Klaubert	LC-ESI-MS/MS	6% BoNT/B, not mentioned for BoNT/A, /E, or /F	Culture supernatant	Yes
2005 Kalb	LC-ESI-MS/MS	65–70% BoNT/A1 and /A2	Milk	No
2010 Küll	MALDI-MS	Not mentioned	Milk, apple juice, orange juice, ham	No
2012 Wang	Gel then LC-ESI-MS/MS	99% BoNT/A; 95% BoNT/B; 90% BoNT/C; 96% BoNT/D; 92% BoNT/E; 97% BoNT/F; 91% BoNT/G	Carrot juice	No
2012 Kalb	LC-ESI-MS/MS	76% BoNT/B1; 76% BoNT/B2; 66% BoNT/B3; 75% BoNT/B4; 74% BoNT/B5	Culture supernatant	No