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Proposed BoNT/A and /B Peptide Substrates Cannot Detect Multiple Subtypes in the Endopep-MS Assay

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Abstract

Botulinum neurotoxins (BoNTs) are a family of protein toxins consisting of seven known serotypes (BoNT/A—BoNT/G) and multiple subtypes within the serotypes, all of which cause the disease botulism; a disease of great public health concern. Accurate detection of BoNTs in human clinical samples is therefore an important public health goal. To achieve this goal, our laboratory developed a mass spectrometry-based assay detecting the presence of BoNT via its enzymatic activity on a peptide substrate. Recently, publications reported the use of new peptide substrates to detect BoNT/A and /B with improved results over other peptide substrates. However, the authors did not provide results of their peptide substrate on multiple subtypes of BoNT. In this work, we describe the results of testing the new substrates with multiple BoNT/A and /B subtypes and find that the substrates cannot detect many subtypes of BoNT/A and /B.

Keywords

Botulinum Neurotoxin; Detection; Botulism; Mass Spectrometry; Peptide Substrate

Introduction

Botulism is a disease characterized by flaccid paralysis and is potentially lethal. Botulism is caused by exposure to botulinum neurotoxins (BoNTs), protein neurotoxins produced by *Clostridium botulinum* and related *Clostridia*. BoNTs cause flaccid paralysis by cleaving proteins needed for acetylcholine release into the neuromuscular junction. This paralysis can affect respiration, lasts from weeks to months, and often requires ventilator support for the patient (1). Due to its toxicity, accurate detection of BoNT in human clinical samples is an important public health goal.

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There are seven confirmed serotypes of BoNT, A-G, which are classified by their antitoxin neutralization ability. Serotypes A, B, E, and F are of greatest public health concern because these serotypes are known to cause botulism in humans. BoNTs are 150 kDa proteins consisting of a heavy chain needed for receptor binding and a light chain which has enzymatic activity. It is the enzymatic activity of the light chain which causes paralysis as BoNTs cleave proteins in the Soluble NSF Attachment Protein Receptor (SNARE) complex. BoNT/A, /C, and /E cleave SNAP (synaptosomal-associated protein)-25 (2–7) and BoNT/B, /D, /F, and /G cleave synaptobrevin-2 (also known as VAMP-2) (8–13). BoNT/C also cleaves syntaxin (14, 15). Cleavage of any of these SNARE complex proteins results in paralysis.

Adding to the diversity of BoNTs, many serotypes also contain amino acid sequence variation in the toxin. These variations, resulting from protein sequence changes, have led to the designation of subtypes of BoNT. Historically, subtypes have been defined based on unique and differing biochemical characteristics (16), enzyme functional differences (17), or differential binding to monoclonal antibodies (mAbs) (18–20). More recently, it was recommended that subtypes be designated based on an amino acid variance of greater than 2.6% (21). Some of the enzyme functional differences between the subtypes can be quite pronounced, as exemplified by the discovery that BoNT/F5 cleaves its substrate in a different location than the other BoNT/F subtypes (8). Whereas only certain subtypes of BoNT are used for commercial purposes, detection of BoNT for public health purposes needs to encompass all subtypes, as all of the subtypes of BoNT/A, /B, /E, and /F can cause human botulism.

Various assays exist to detect BoNT in clinical samples in order for a laboratory to confirm a suspected case of botulism. One assay developed in our laboratory at the CDC, called Endopep-MS, detects the toxin based on its enzymatic activity. The assay uses magnetic beads coated with serotype specific monoclonal antibodies to extract the toxin from a clinical sample. Following extraction and washing of the beads, a reaction buffer and peptide substrate are added. The peptide substrate is an optimized, shortened mimic of SNAP-25 or VAMP-2 which are cleaved by BoNT. Cleavage of the substrate is detected by mass spectrometry, which can detect both cleavage of the substrate as well as the location of cleavage.

The composition of the peptide substrate is a critical factor for success of the Endopep-MS method. Because of this, several publications detail the discovery of modifications to the peptide substrates which improve BoNT detection, either by lowering the limit of detection or by decreasing the possibility of a false positive through creating more rugged substrates that are more stable and resistant to proteases common to clinical matrices such as serum and stool. Most of the publications also report on the use of a new peptide substrate with multiple subtypes of BoNT (22–25). This is crucial for public health detection as it is known that not all peptide substrates can be cleaved by all subtypes of BoNT. One important example details the addition of a five amino acid N-terminal extension for the detection of BoNT/F7 produced by *C. baratii* (26). Prior to this extension, the peptide substrate in use for Endopep-MS could not be used for detection of BoNT/F7, although it worked for detection

of all other subtypes. BoNT/F7 causes human botulism (27–30), therefore, it is imperative that any assay designed for public health purposes can detect this subtype.

The peptide substrates for the detection of BoNT/A and /B used in the validated Endopep-MS assay are Ac-(Nal)(Nal)EKAPARGFNKPKIDAGNGRATR(Nle)LGGR-amide (Aval) and LSELDDRADALQAGASQFESSAAKLKRKYWWKNLK (Bval) (31). Recently, new substrates with the sequences RTRIDEGNQRATR(Nle)LG (Anew) and LSELDDRADALQAGASQFETSAAKLKRK (Bnew) were reported for the detection of BoNT/A and /B (32, 33). The A substrate is significantly shortened at the N-terminus and the B substrate is 7 amino acids shorter on the C-terminus with other mutations in both substrates. The authors reported a 5-fold increase in sensitivity for the detection of BoNT/B with this substrate using the BoNT/B1 subtype(32). However, the authors did not indicate that data was obtained on the use of these new substrates for multiple subtype detection. Because our laboratory uses the validated Endopep-MS assay for BoNT/A and /B detection in human clinical specimens, we were optimistic that the use of these substrates might improve our ability to detect BoNT/A and /B in human clinical samples. This study describes our testing of Anew and Bnew with multiple subtypes of BoNT/A and /B and provides data on the ability of Aval and Bval to detect the known subtypes for BoNT/A and /B.

Experimental

Materials

Botulinum neurotoxin is extremely toxic and therefore necessitates appropriate safety measures. BoNT was handled in a class 2 biosafety cabinet equipped with HEPA filters. BoNT/A1 and /B1 complex toxins were purchased (Metabiologics, Madison, WI, USA). Monoclonal antibodies RAZ1, CR2, 2B18.2 and B12.1 were acquired from Dr. James Marks at the University of California at San Francisco. Dynabeads® (M-280/Strepavidin) were purchased (Invitrogen, Carlsbad, CA, USA). Sulfo-NHS-Biotin was purchased from Thermo Fisher Scientific (Waltham, MA). Human serum and stool were purchased from BioIVT (Westbury, NY). All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) except where indicated.

Production of BoNT culture supernatants

Crude culture supernatants were produced by incubating subcultures of each strain for 5 days at 30−35°C. After centrifugation, supernatants were removed and filtered through 0.22 μm filters. Supernatants were stored at −70°C until needed. Information on the strains used in these studies is listed in Table 1.

Peptide Synthesis

Aval, Anew, Bval and Bnew peptide substrates were prepared in house by a solid phase peptide synthesis method using Fmoc chemistry on a Liberty Blue automated microwave peptide synthesizer (CEM, Matthews, NC, USA). Protected peptides were cleaved and deblocked using a reagent mixture of 92.5% trifluroacetic acid (TFA):2.5% water: 2.5% 3, 6-dioxa-1, 8-octanedithiol:2.5% triisopropylsilane and purified on an semi preparative

reversed-phase HPLC using a water:acetonitrile:0.1% TFA gradient. All peptides were dissolved in deionized water as a 0.5 mM stock solution for Aval and Anew and a 1 mM stock solution for Bval and Bnew and were stored at −70°C until further use. The amino acid sequences of all substrates, their respective cleavage products, and the subsequent m/z of all peptides are listed in Table 2.

Preparation of mAb-coated beads and neurotoxin extraction

Monoclonal antibodies RAZ1 and CR2 bind the heavy chain of BoNT/A and 1B18 and B12.1 bind the heavy chain of BoNT/B and were previously shown to bind multiple subtypes of BoNT/A and /B for use in the Endopep-MS assay [33]. The mAbs were biotinylated in a fresh solution of 300 μM sulfo-NHS-biotin in water. A ratio of 4 μL of the 300 μM biotin was added to 20 μg of total mAb. The mixtures were incubated overnight at room temperature with no mixing. The biotinylated mAbs were bound to streptavidin beads after being rinsed three times with phosphate buffered saline with 0.01% Tween (PBST) buffer. A 20 μg aliquot of biotinylated antibody was used per 1 mL of beads. A standard orbital shaker was used to bind antibody onto the beads for 1 h. Following washing three times with PBST, the mAb-coated beads were stored at −20°C until needed.

For the BoNT extraction assay, an aliquot of 20 μL of antibody-coated beads was mixed for 1 h with either 2 μL of BoNT/A1 or /B1 complex toxin corresponding to 100 mouse LD_{50} (mLD_{50}) diluted in 500 μL of PBST or a solution of 100 μL of each culture supernatant of unknown toxin concentration and 400 μL of PBST buffer in a deep well plate, with the exception of the BoNT/A7 culture supernatant in which 100 mLD_{50} was diluted in 50% human serum and 50% PBST to a volume of 500 μL. The deep well plate was capped and placed on a plate shaker for 1 hr at the minimal speed needed to keep beads in solution. The deep well plate was then uncapped and placed into a KingFisher Flex magnetic particle processor (Thermo Fisher Scientific, Waltham, MA) for automated bead washing, which included two washes with 1 mL each of 2M NaCl followed by two washes with 1 mL each of 1× PBST. The beads were eluted from the KingFisher Flex into 80 μL of water and removed from the KingFisher Flex.

A negative control without toxin consisting of either 500 μL of PBST buffer (comparison to purified toxin or to blank clinical matrix), 400 μL of PBST buffer and 100 μL of culture medium (comparison to culture supernatants) or 500 μL of 50% human serum and 50% PBST (comparison to BoNT/A7) was processed in parallel with all toxin containing samples with the remainder of the protocol as above. For the blank clinical matrix experiments, an aliquot of 20 μL of antibody-coated beads was mixed for 1 h with a mixture of either 500 μL of human serum and 50 μL of 10× PBST buffer or 100 μL of human stool extract, 100 μL of human serum, and 300 μL of PBST buffer in a deep well plate with the remainder of the protocol as above.

Endopep-MS Reaction with MALDI-TOF analysis

The water was removed from the beads by pipetting, and the beads were reconstituted in reaction buffer consisting of 0.05 M Hepes (pH 7.3), 25 mM dithiothreitol, and 20 μM $ZnCl_2$ and peptide substrate Aval, Anew, Bval or Bnew at a final concentration of 50 μM for

Aval and Anew and 100 μM for Bval and Bnew. Samples then were incubated at 37°C for 4 hr with no agitation except to study the effect of bead extraction and reaction buffer upon Anew and Aval as noted. A 2-μL aliquot of each reaction supernatant was mixed with 18 μL of matrix solution consisting of α-cyano-4-hydroxy cinnamic acid (CHCA) at 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA), and 1 mM ammonium citrate. A 0.5-μL aliquot of this mixture was pipeted onto one spot of a 384-spot matrix-assisted laser desorption/ionization (MALDI) plate (Applied Biosystems, Framingham, MA or Bruker, Billerica, MA). Mass spectra of each spot were obtained by scanning from 700 to 4500 *m/z* in MS-positive ion reflector mode on an Applied Biosystems 5800 Proteomics Analyzer (Framingham, MA) or a Bruker ultrafleXtreme (Billerica, MA) for BoNT/A7 analysis.

Results

Figure 1A depicts the mass spectrum generated by the reaction of Anew without toxin. The mass of the intact substrate is present at *m/z* 1857. For comparison, a mass spectrum was generated without toxin using Aval (Figure 1B), with the mass of the intact substrate of this peptide present at *m/z* 3287. Next, both substrates were incubated with BoNT/A1 complex. Cleavage of Anew by BoNT/A1 generated two cleavage products at *m/z* 787 and 1089 as seen in Figure 1C. Unfortunately, small peaks at *m/z* 787 and 1089 corresponding to the cleavage products are visible in the negative control (Figure 1A); however, it is apparent that these peaks dominate the spectrum of the sample containing the toxin and therefore can be considered to be a mark of toxin activity at these high intensities. Cleavage of Aval by BoNT/A1 also generated cleavage products at *m/z* 999 and 2307 (Figure 1D), and these peaks serve as markers of the presence of active BoNT/A in reactions with Aval.

Searching for a reason for the existence of small intensity cleavage products in the negative control with Anew, we repeated the negative controls consisting of just PBST buffer, but eliminated the incubation of substrate with the antibody-coated beads for 4 hours at 37°C. No cleavage products were visible either in the reaction with Anew or Aval (Figure 2). Wanting to understand the effect of clinical matrix on the substrates, we examined negative controls of the reaction of both substrates with human serum and human stool extract, absent of BoNT/A, but including the incubation of the substrate at 37°C for 4 hrs. Cleavage products were not visible with Aval; however significant levels of cleavage products were visible with Anew for both human serum and human stool extract (Figure 2).

Having characterized the negative controls, we focused on evaluation of multiple subtypes of BoNT/A with Anew and Aval. We tested Anew and Aval with BoNT/A2–A8, extracted from culture supernatant using mAbs previously demonstrated to extract these subtypes(22). The presence of peaks at *m/z* 787 and 1089 at levels exceeding those of the negative control indicate cleavage of Anew by these subtypes, and the presence of peaks at *m/z* 999 and 2307 indicate cleavage of Aval. Absence of peaks at those *m/z* indicate no observable cleavage of Anew or Aval by those subtypes. Cleavage products were visible for all subtypes using Aval (Supplementary Figure 1) and cleavage products were visible for subtypes BoNT/A2–A7 using Anew (Supplementary Figure 2). Figure 3 depicts the data generated by the reaction of no toxin, BoNT/A1, and BoNT/A8 with Aval. Cleavage of Aval is evident with incubation with BoNT/A1 and /A8. However, the reaction of BoNT/A8 with Anew (Figure 3B)

generated no increase in cleavage products at *m/z* 787 and 1089 compared to the negative control.

Figure 4A depicts the mass spectrum generated by the reaction of Bnew without toxin. The mass of the intact substrate is present at *m/z* 3021. For comparison, a mass spectrum was generated without toxin using Bval (Figure 4B), with the mass of the intact substrate at *m/z* 4026. Next, both substrates were incubated with the same amount of BoNT/B1 complex. Cleavage of Bnew by BoNT/B1 generated two cleavage products at *m/z* 1279 and 1760, visible in Figure 4C. BoNT/B1 was also added to a reaction with Bval, producing cleavage products at *m/z* 1760 and 2284 (Figure 4D).

We next tested Bnew and Bval with BoNT/B2-/B7, extracted from culture supernatant using mAbs previously demonstrated to extract these subtypes (34). The presence of peaks at *m/z* 1279 and 1760 indicate cleavage of Bnew by these subtypes, and the presence of peaks at *m/z* 1760 and 2284 indicate cleavage of Bval. Absence of peaks at those *m/z* indicate no observable cleavage of Bnew or Bval by those subtypes. Cleavage products were visible for all BoNT/B subtypes using Bval (Supplementary Figure 3) and cleavage products were visible for some subtypes of BoNT/B using Bnew (Supplementary Figure 4). Figure 5A depicts the data generated by the reaction of BoNT/B2 with Bnew. Cleavage products at *m/z* 1279 and 1760 corresponding to the cleavage of Bnew by BoNT/B2 are not present. In contrast, cleavage products are present for the reaction of the same amount of BoNT/B2 with Bval (Figure 5B). Similarly, BoNT/B4 did not produce cleavage of Bnew as seen in the absence of cleavage products in Figure 5A, yet the same amount of BoNT/B4 produces cleavage products with Bval (Figure 5B). Lastly, when Bnew and Bval are used to test the same amount of BoNT/B6, cleavage products are not present in the reaction with Bnew (Figure 5A) yet are visible in the reaction with Bval (Figure 5B).

Discussion

During the incubation of substrate with beads, small amounts of cleavage products appeared in the Anew reaction without BoNT/A (Figure 1A). Because the cleavage products are not present in the reaction supernatant immediately upon mixing (Figure 2), Anew appeared to spontaneously dissociate at the site cleaved by BoNT/A with time and incubation at 37°C, creating small amounts of cleavage products in the absence of the toxin. This phenomenon has been reported before, especially with BoNT/A(22) and was a major consideration in the optimization of the Aval substrate. Instability of a BoNT/A substrate does not necessarily mean that this cannot be used as a substrate for BoNT/A; however, steps must be taken to ensure the absence of a false positive. Some remedies for this situation include a careful characterization of the negative control to select a positive threshold, or incubation at a lower temperature or for a shorter time.

In our experience with the design of substrates for BoNT detection, spontaneous dissociation of the substrate can sometimes increase upon exposure to more complex matrices. To test this, we studied the performance of Anew with the clinical matrices which our laboratory currently uses for laboratory confirmation of human botulism; specifically, human serum and human stool extract. Figure 2 shows that this substrate appears to have an increase in

dissociation at the BoNT cleavage site, particularly with human stool extract. Again, this does not necessarily eliminate this substrate as a possibility for BoNT/A detection; however, great care must be taken to ensure the absence of false positives. Higher blank signal levels also generally affect the sensitivity of the assay because the threshold for a positive result increases.

Upon testing Anew with multiple BoNT/A subtypes, cleavage products were visible in reactions with subtypes A1–A7, and therefore, Anew can be used to detect those subtypes of BoNT/A via their enzymatic activity upon the peptide substrate. However, the absence of cleavage products at levels above that of the negative control in the reaction with BoNT/A8 indicates that this is not a good substrate choice for the detection of BoNT/A8 via its enzymatic activity upon a peptide substrate. The same amount of BoNT/A8 was easily detected using a different substrate for BoNT/A (Aval) with a longer N-terminus and amino acid mutations.

Although BoNT/A8 is not the most divergent of the BoNT/A subtypes, Anew was not cleaved by BoNT/A8 whereas it was cleaved by other, more divergent subtypes. A comparison of the amino acid differences unique to BoNT/A8 show that most of the unique residues and an amino acid insertion are located in the N-terminal portion of the receptor-binding domain, H_{CN}, a region of the toxin not responsible for enzymatic activity. It is possible that alterations in a remote region of the toxin are responsible for differences in substrate recognition. However, there are three unique residues in the light chain portion of BoNT/A8; perhaps one of these amino acids is responsible for the failure of BoNT/A8 to recognize and/or cleave Anew.

Cleavage products were visible in reactions with subtypes B1, B3, B5, and B7, indicating that Bnew could be used to detect those subtypes of BoNT/B via their enzymatic activity upon the peptide substrate. However, the absence of cleavage products in the reactions with BoNT/B2, /B4, and /B6 indicates that this is not a good substrate choice for the detection of those subtypes via their enzymatic activity upon a peptide substrate. Rather, a peptide substrate with the sequence of Bval, encompassing both a C-terminal extension of 7 amino acids as well as a substitution of S to T in the 20^{th} position has been shown to be effective for detection of BoNT/B1–B7 (34, 35), and able to detect all seven BoNT/B subtypes tested in parallel with Bnew using the same levels of toxin for both substrates.

An examination of the amino acid sequence of BoNT/B subtypes (21) shows that the BoNT/B subtype most divergent from BoNT/B1 is BoNT/B4, which is 6.8% different. Subtypes BoNT/B2 and /B6 are 4.4% and 3.9% divergent, respectively. As the amino acid sequence of a protein changes, this can affect its interactions with other proteins, and in this case, although subtypes BoNT/B2, /B4, and /B6 all cleave VAMP-2 and the peptide LSELDDRADALQAGASQFESSAAKLKRKYWWKNLK (Bval), those subtypes do not cleave the peptide LSELDDRADALQAGASQFETSAAKLKRK (Bnew). It is often difficult to predict how two proteins will interact with amino acid changes in one of the proteins; Bnew may be used to detect the presence of BoNT/B3 which is 4.0% divergent from BoNT/B1, but may not be used to detect the presence of BoNT/B6 which is 3.9% divergent.

The location of amino acid change is often more important than the amount of amino acid change when examining protein-protein interaction.

An additional consideration in the design of an optimized peptide substrate for the detection of BoNT is the amount of cleavage of the substrate by each of the BoNT subtypes. Comparing the ratios of the substrate ions to the cleavage product ions for each subtype (Supplementary Figures 1 and 2) for Aval and Anew clearly show superior cleavage of Aval over Anew for each of the subtypes, especially A2, A6, and A7. These experiments were done with equal aliquots of toxin supernatants, on the same day at the same time. A similar comparison for BoNT/B cleavage of Bval and Bnew (Supplementary Figures 3 and 4) show a higher ratio of cleavage products to substrate for all subtypes of BoNT B especially B3. Again, these experiments were performed with equal aliquots of toxin and on the same day at the same time. This may partially be due to the ionization efficiencies of the two substrate peptides but careful examination of all the spectra in Supplementary Figures 1 – 4 show cleaner spectra and more easily detected cleavage products for BoNT/A cleavage of Aval over Anew and for BoNT/B cleavage of Bval over Bnew.

Conclusion

In summary, peptide RTRIDEGNQRATR(Nle)LG cannot be used to detect BoNT/A8 and peptide LSELDDRADALQAGASQFETSAAKLKRK cannot be used to detect BoNT/B2, /B4, or /B6, although all of these subtypes have been involved in botulism outbreaks. Furthermore, the validated peptides (Aval and Bval) in the Endopep-MS assay, in this study, gave superior performance to the recently proposed peptides (Anew and Bnew) for all toxin subtypes. Due to the inability to detect multiple subtypes of BoNT/A and /B, these peptides are therefore not a good choice for botulism investigations, where the subtype of toxin is not known. When designing a peptide substrate to be used for detection of BoNT when the subtype is not known, it is necessary to test the proposed peptide substrate against all available subtypes, to prevent false negative findings in botulism outbreaks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BoNT	Botulinum Neurotoxin
SNARE	Soluble NSF Attachment Protein Receptor
SNAP	Synaptosomal-associated protein

VAMP	Vesicle-associated membrane protein
mAbs	monoclonal antibodies
PBST	phosphate buffered saline with 0.01% tween
TFA	trifluroacetic acid

References:

1. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, et al. Botulinum toxin as a biological weapon: medical and public health management. Jama. 2001;285(8):1059–70. [PubMed: 11209178]
2. Binz T, Blasi J, Yamasaki S, Baumeister A, Link E, Sudhof TC, et al. Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J Biol Chem. 1994;269(3):1617–20. [PubMed: 8294407]
3. Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, et al. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature. 1993;365(6442):160–3. [PubMed: 8103915]
4. Foran P, Lawrence GW, Shone CC, Foster KA, Dolly JO. Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. Biochemistry. 1996;35(8):2630–6. [PubMed: 8611567]
5. Schiavo G, Rossetto O, Catsicas S, Polverino de Laureto P, DasGupta BR, Benfenati F, et al. Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. J Biol Chem. 1993;268(32):23784–7. [PubMed: 8226912]
6. Schiavo G, Santucci A, Dasgupta BR, Mehta PP, Jontes J, Benfenati F, et al. Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. FEBS letters. 1993;335(1):99–103. [PubMed: 8243676]
7. Williamson LC, Halpern JL, Montecucco C, Brown JE, Neale EA. Clostridial neurotoxins and substrate proteolysis in intact neurons: botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. J Biol Chem. 1996;271(13):7694–9. [PubMed: 8631808]
8. Kalb SR, Baudys J, Webb RP, Wright P, Smith TJ, Smith LA, et al. Discovery of a novel enzymatic cleavage site for botulinum neurotoxin F5. FEBS letters. 2012;586(2):109–15. doi: 10.1016/j.febslet.2011.11.033. [PubMed: 22172278]
9. Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR, et al. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature. 1992;359(6398):832–5. [PubMed: 1331807]
10. Schiavo G, Malizio C, Trimble WS, Polverino de Laureto P, Milan G, Sugiyama H, et al. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J Biol Chem. 1994;269(32):20213–6. [PubMed: 8051110]
11. Schiavo G, Shone CC, Rossetto O, Alexander FC, Montecucco C. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J Biol Chem. 1993;268(16):11516–9. [PubMed: 8505288]
12. Yamasaki S, Baumeister A, Binz T, Blasi J, Link E, Cornille F, et al. Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. J Biol Chem. 1994;269(17):12764–72. [PubMed: 8175689]
13. Yamasaki S, Binz T, Hayashi T, Szabo E, Yamasaki N, Eklund M, et al. Botulinum neurotoxin type G proteolyses the Ala81-Ala82 bond of rat synaptobrevin 2. Biochemical and biophysical research communications. 1994;200(2):829–35. [PubMed: 7910017]
14. Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. The EMBO journal. 1993;12(12):4821–8. [PubMed: 7901002]
15. Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C. Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. J Biol Chem. 1995;270(18):10566–70. [PubMed: 7737992]

16. Wagner E, Meyer KF, Dozier CC. Studies on the Metabolism of B. Botulinus in Various Media. Xxvi. Journal of bacteriology. 1925;10(4):321–412. [PubMed: 16559144]
17. Kozaki S, Kamata Y, Nishiki T, Kakinuma H, Maruyama H, Takahashi H, et al. Characterization of Clostridium botulinum type B neurotoxin associated with infant botulism in japan. Infection and immunity. 1998;66(10):4811–6. [PubMed: 9746583]
18. Gibson AM, Modi NK, Roberts TA, Hambleton P, Melling J. 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. The Journal of applied bacteriology. 1988;64(4):285–91. [PubMed: 3049499]
19. Kozaki S, Nakaue S, Kamata Y. Immunological characterization of the neurotoxin produced by Clostridium botulinum type A associated with infant botulism in Japan. Microbiology and immunology. 1995;39(10):767–74. [PubMed: 8577267]
20. Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, Laporte SL, et al. Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infection and immunity. 2005;73(9):5450–7. [PubMed: 16113261]
21. Peck MW, Smith TJ, Anniballi F, Austin JW, Bano L, Bradshaw M, et al. Historical Perspectives and Guidelines for Botulinum Neurotoxin Subtype Nomenclature. Toxins (Basel). 2017;9(1). doi: 10.3390/toxins9010038.
22. Wang D, Baudys J, Hoyt KM, Barr JR, Kalb SR. Further optimization of peptide substrate enhanced assay performance for BoNT/A detection by MALDI-TOF mass spectrometry. Analytical and bioanalytical chemistry. 2017;409(20):4779–86. doi: 10.1007/s00216-017-0421-8. [PubMed: 28573317]
23. Wang D, Baudys J, Ye Y, Rees JC, Barr JR, Pirkle JL, et al. Improved detection of botulinum neurotoxin serotype A by Endopep-MS through peptide substrate modification. Analytical biochemistry. 2013;432(2):115–23. doi: 10.1016/j.ab.2012.09.021. [PubMed: 23017875]
24. Wang D, Krilich J, Baudys J, Barr JR, Kalb SR. Enhanced detection of type C botulinum neurotoxin by the Endopep-MS assay through optimization of peptide substrates. Bioorg Med Chem. 2015;23(13):3667–73. doi: 10.1016/j.bmc.2015.04.012. [PubMed: 25913863]
25. Wang D, Krilich J, Baudys J, Barr JR, Kalb SR. Optimization of peptide substrates for botulinum neurotoxin E improves detection sensitivity in the Endopep-MS assay. Analytical biochemistry. 2015;468:15–21. doi: 10.1016/j.ab.2014.08.026. [PubMed: 25232998]
26. Kalb SR, Baudys J, Egan C, Smith TJ, Smith LA, Pirkle JL, et al. Different substrate recognition requirements for cleavage of synaptobrevin-2 by Clostridium baratii and Clostridium botulinum type F neurotoxins. Applied and environmental microbiology. 2011;77(4):1301–8. [PubMed: 21169446]
27. Castor C, Mazuet C, Saint-Leger M, Vygen S, Coutureau J, Durand M, et al. Cluster of two cases of botulism due to Clostridium baratii type F in France, November 2014. Euro Surveill. 2015;20(6).
28. Hannett GE, Schaffzin JK, Davis SW, Fage MP, Schoonmaker-Bopp D, Dumas NB, et al. Two cases of adult botulism caused by botulinum neurotoxin producing Clostridium baratii. Anaerobe. 2014;30C:178–80. doi: 10.1016/j.anaerobe.2014.10.005.
29. Moodley A, Quinlisk P, Garvey A, Kalas N, Barash JR, Khouri JM, et al. Notes from the field: infant botulism caused by Clostridium baratii type F - Iowa, 2013. MMWR Morb Mortal Wkly Rep. 2015;64(14):400. [PubMed: 25879901]
30. Trehard H, Poujol I, Mazuet C, Blanc Q, Gillet Y, Rossignol F, et al. A cluster of three cases of botulism due to Clostridium baratii type F, France, August 2015. Euro Surveill. 2016;21(4). doi: 10.2807/1560-7917.ES.2016.21.4.30117.
31. Kalb SR, Baudys J, Wang D, Barr JR. Recommended mass spectrometry-based strategies to identify botulinum neurotoxin-containing samples. Toxins (Basel). 2015;7(5):1765–78. doi: 10.3390/toxins7051765. [PubMed: 25996606]
32. Rosen O, Feldberg L, Gura S, Zichel R. A new peptide substrate for enhanced botulinum neurotoxin type B detection by endopeptidase-liquid chromatography-tandem mass spectrometry/multiple reaction monitoring assay. Analytical biochemistry. 2015;473:7–10. doi: 10.1016/j.ab.2014.09.016. [PubMed: 25277815]

33. Rosen O, Feldberg L, Yamin TS, Dor E, Barnea A, Weissberg A, et al. Development of a multiplex Endopep-MS assay for simultaneous detection of botulinum toxins A, B and E. Sci Rep. 2017;7(1):14859. doi: 10.1038/s41598-017-14911-x. [PubMed: 29093524]

34. Kalb SR, Santana WI, Geren IN, Garcia-Rodriguez C, Lou J, Smith TJ, et al. 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 biochemistry. 2011;12:58. doi: 10.1186/1471-2091-12-58. [PubMed: 22085466]

35. Kalb SR, Baudys J, Rees JC, Smith TJ, Smith LA, Helma CH, et al. De novo subtype and strain identification of botulinum neurotoxin type B through toxin proteomics. Analytical and bioanalytical chemistry. 2012;403(1):215–26. doi: 10.1007/s00216-012-5767-3. [PubMed: 22395449]

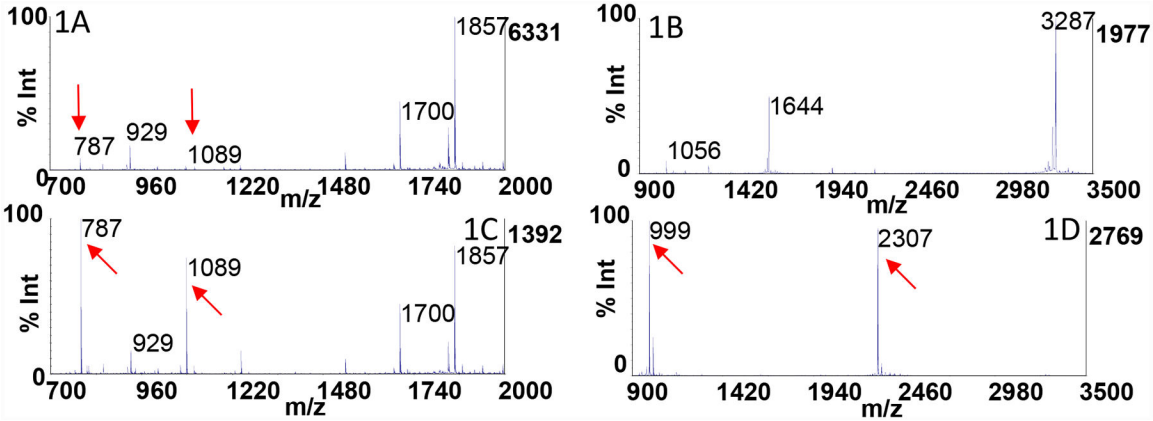

Figure 1.

Mass spectra from the reaction of no BoNT/A with Anew (1A), no BoNT/A with Aval (1B), BoNT/A1 with Anew (1C), and BoNT/A1 with Aval (1D). Cleavage products indicating the presence of BoNT/A are present at *m/z* 787 and 1089 for Anew and at 999 and 2307 for Aval. Substrate ions are at *m/z* 1857 and the doubly charged ion at *m/z* 929 for Anew in 1A and 1C and *m/z* 3287 and the doubly charged ion at *m/z* 1644 for Aval in 1B. No substrate ion was visible after cleavage reaction for Aval in spectrum 1D.

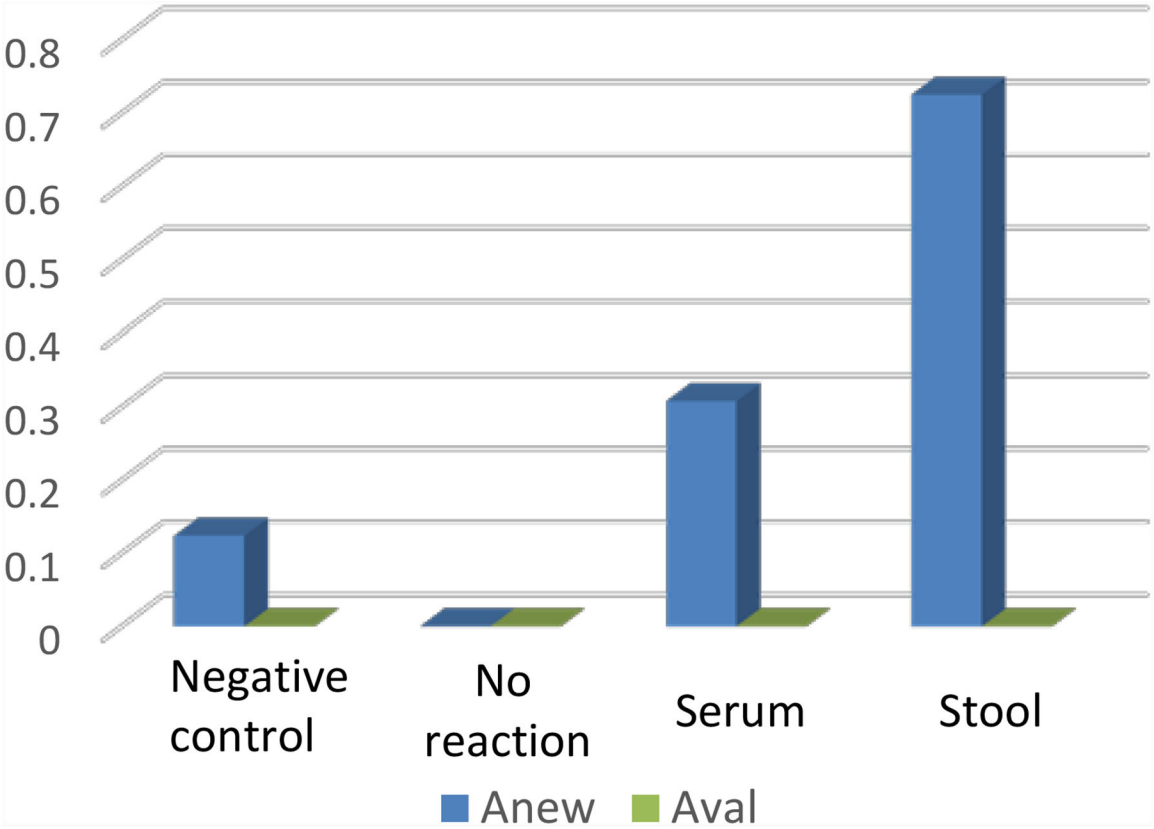

Figure 2.

Comparison of Anew and Aval after extraction from PBST following incubation for 4 hrs at 37°C (negative control), after extraction from PBST with no reaction (No reaction), after extraction from serum (Serum) and after extraction from stool extract (Stool). Values are calculated from the ratio of the signal/noise of the sum of the cleavage products to the signal/noise of the intact substrate.

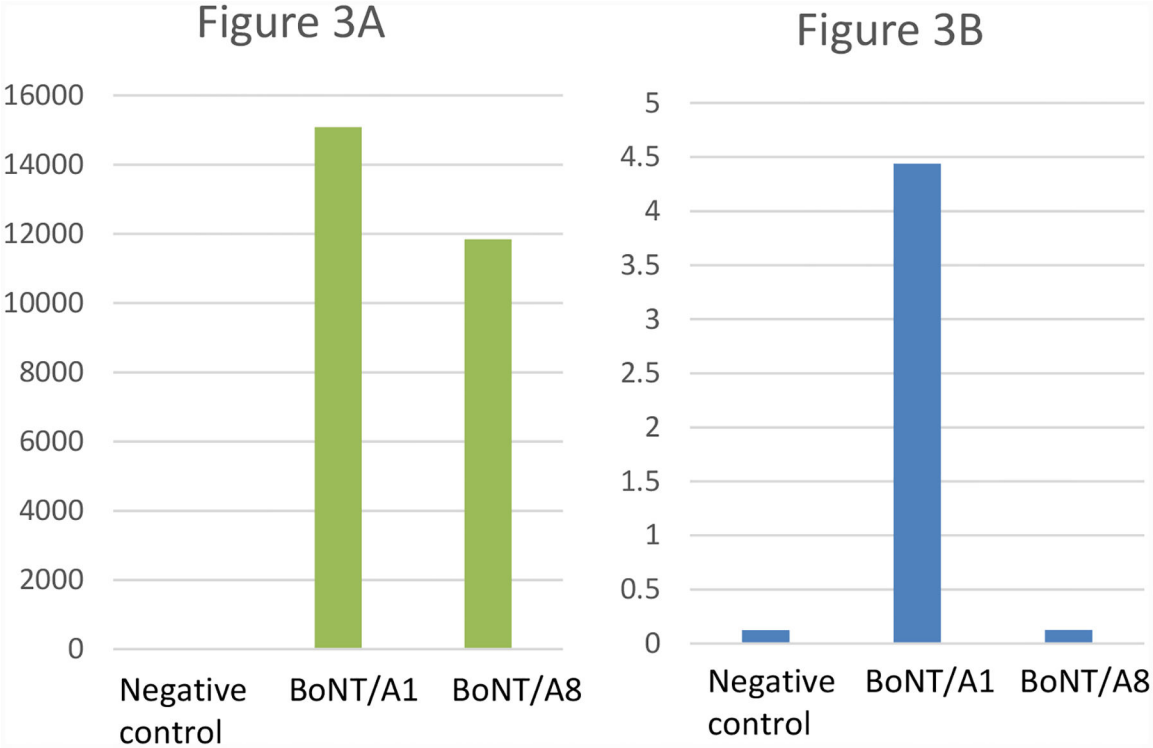

Figure 3.

Comparison of Aval (3A) and Anew (3B) to the negative control, BoNT/A1, and BoNT/A8. Values are calculated from the ratio of the signal/noise of the sum of the cleavage products to the signal/noise of the intact substrate.

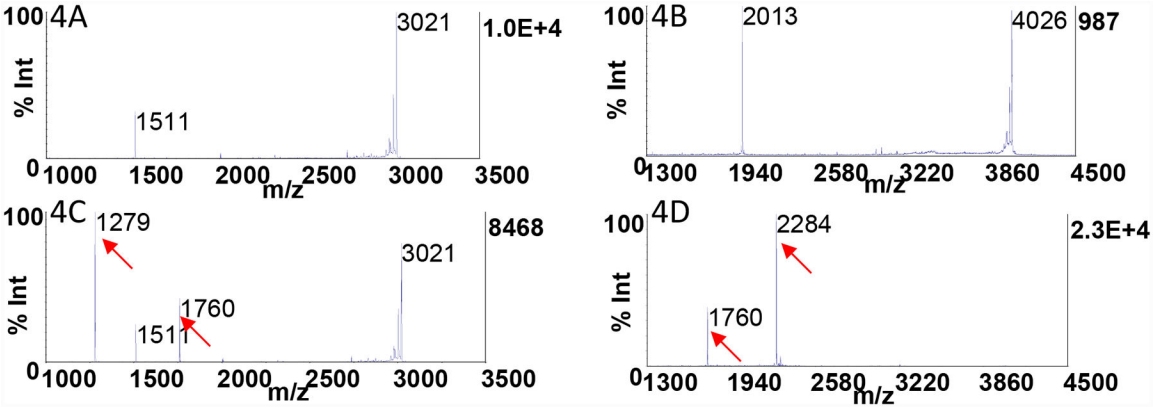

Figure 4.

Mass spectra from the reaction of no BoNT/B with Bnew (4A), no BoNT/B with Bval (4B), BoNT/B1 with Bnew (4C), and BoNT/B1 with Bval (4D). Cleavage products indicating the presence of BoNT/B are present at *m/z* 1279 and 1760 for Bnew and at 1760 and 2284 for Bval. Intact substrate ions are at *m/z* 3021 and doubly charged at *m/z* 1511 for Bnew in 4A and 4C and at *m/z* 4026 and the doubly charged ion at *m/z* 2013 for Bval in 4B. No substrate ions were visible after cleavage reaction for Bval in spectrum 4D.

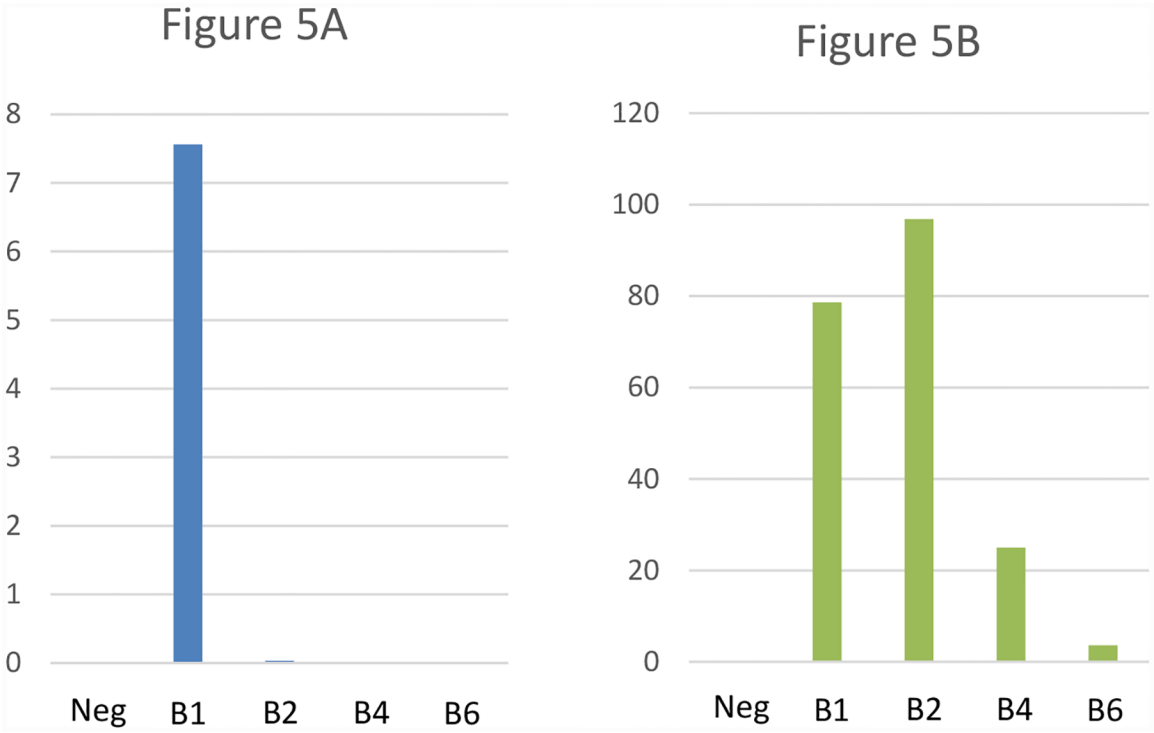

Figure 5.

Comparison of Bnew (5A) and Bval (5B) to the negative control, BoNT/B1, BoNT/B2, BoNT/B4, and BoNT/B6. Values are calculated from the ratio of the signal/noise of the sum of the cleavage products to the signal/noise of the intact substrate.

Table 1.

Strain information on culture supernatants used for this study.

Sample	Strain	NCBI accession #
BoNT/A2	CDC2171	ADB85243
BoNT/A3	CDC 40234	AFM77661
BoNT/A4 and /B5	CDC 657	ABA29018/ABY56323
BoNT/A5	A661222	ADJ68240
BoNT/A6	CDC 41370	ACW83608
BoNT/A7	2008–148	AFV13854
BoNT/A8	Chemnitz	AJA05787
BoNT/B2	CDC 1828	ABM73988
BoNT/B3	CDC795	ABM73977
BoNT/B4	Eklund 17B	ABM73987
BoNT/B6	CDC 6291	ABM73978
BoNT/B7	NCTC3807	AEN25581

Table 2.

Information on peptide substrates used for this study where NT is the N-terminal cleavage product and CT is the C-terminal cleavage product.

Peptide	Amino acid sequence	*m/z* observed
Aval	Ac-(Nal)(Nal)EKAPARGFNKPKIDAGNGRATR(Nle)LGGR-amide	3287
NT Aval	Ac-(Nal)(Nal)EKAPARGFNKPKIDAGNG	2307
CT Aval	RATR(Nle)LGGR-amide	999
Anew	RTRIDEGNQRATR(Nle)LG	1857
NT Anew	RTRIDEGNQ	1089
CT Anew	RATR(Nle)LG	787
Bval	LSELDDRADALQAGASQFESSAAKLKRKYWWKNLK	4026
NT Bval	LSELDDRADALQAGASQ	1760
CT Bval	FESSAAKLKRKYWWKNLK	2284
Bnew	LSELDDRADALQAGASQFETSAAKLKRK	3021
NT Bnew	LSELDDRADALQAGASQ	1760
CT Bnew	FETSAAKLKRK	1279