Journal of Infectious Diseases Advance Access published June 10, 2015

A Novel Botulinum Toxin, Previously Reported as Serotype H, has a Hybrid Structure of Known Serotypes A and F that is Neutralized with Serotype A Antitoxin

Susan E. Maslanka[1,*], Carolina Lúquez[1], Janet K. Dykes[1], William H. Tepp[2], Christina L. Pier[2], Sabine Pellett[2], Brian H. Raphael[1], Suzanne R. Kalb[1], John R. Barr[1], Agam Rao[1], Eric A. Johnson[2]

[1]Centers for Disease Control and Prevention, Atlanta, Georgia 30329

[2]Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53706

[*]Corresponding author: Susan E. Maslanka, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30329. Telephone: (404) 639-0895. Fax: (404) 639-4290. Email: SMaslanka@cdc.gov

ABSTRACT

Botulism is a potentially fatal paralytic disease caused by the action of botulinum neurotoxin (BoNT) on nerve cells. There are 7 known serotypes (A through G) of BoNT and up to 40 genetic variants. *Clostridium botulinum* strain IBCA10-7060 was recently reported to produce BoNT serotype B (BoNT/B) and a novel BoNT, designated by the authors as BoNT/H. The botulinum neurotoxin gene (*bont*) sequence of BoNT/H was analyzed against known related *bont* sequences. Genetic analysis suggested that BoNT/H has a hybrid-like structure containing regions of similarity to BoNT/A1 and /F5. This novel BoNT was serologically characterized by the mouse neutralization assay and a neuronal cell-based assay. The toxic effects of this hybrid-like BoNT were completely eliminated by existing serotype A antitoxin including those contained in multivalent therapeutic antitoxin products that are the mainstay for human botulism treatment.

INTRODUCTION

Botulism is a rare, potentially lethal, neuroparalytic disease which manifests naturally in humans in three primary forms: 1) Foodborne, 2) Intestinal Colonization (Infant botulism and rarely in adults), and 3) Wound [1]. The reported occurrence of each botulism form varies worldwide. Botulism is a nationally reportable disease in the United States; the Centers for Disease Control and Prevention (CDC) is responsible for compiling reports of laboratory confirmed cases through its National Botulism Surveillance System. Approximately 120 human cases are reported annually in the United States; roughly 70% of these are Infant botulism cases.

Botulinum antitoxin is the only specific pharmacological treatment for botulism and is the cornerstone of clinical management. In the United States, non-infant botulism is treated with BAT™, an equine-based heptavalent product that treats all known serotypes of botulinum toxin (BoNT/A through G)[2, 3]. Infant botulism is typically treated by Botulism Immune Globulin Intravenous (Human) (BabyBIG®) which is licensed for BoNT/A and B only [4]. Improvements in medical management practices since the 1950's have reduced overall botulism mortality from ~ 60% to only 5 to 10% [5]. Therapeutic antitoxin provides a protective benefit in botulism patients by reducing both mortality and the long term consequences of this paralytic disease [6]. A retrospective review of foodborne botulism cases showed that patients that received antitoxin were more likely (46% versus 10%) to survive. Patients receiving antitoxin early in the course of their illness appeared to recover more quickly; 10 hospital days versus 41 to 56 days for late or no antitoxin, respectively. A double blind study of BabyBIG® in Infant botulism demonstrated that antitoxin administration decreases hospital stay and improves clinical outcome [4].

The seven recognized serotypes of BoNT were originally defined by neutralization of toxicity by specific polyclonal antibodies. However, limited information is available about the relationship

between the structure and function of the BoNT molecule [7]. On its most basic level, BoNT is a 150kDa protein which when activated is converted to a dichain (Light Chain, LC and Heavy Chain, HC) with 3 domains weakly held together by a peptide belt, a disulfide bond, and surface charges. The LC domain is responsible for the toxin's enzymatic activity which results in the paralytic symptoms of botulism. The HC_N (translocation domain) facilitates the translocation of the LC into the neuronal cytosol. The HC_C (binding domain) is responsible for docking the toxin to the exterior of the neuronal cell membrane. However, molecular characterization of the gene contained within diverse strains has expanded the knowledge of BoNT diversity beyond what could be achieved through classical microbiological methods alone. At least 40 unique BoNTs, often called subtypes, have been identified by DNA sequencing; some have an impact on BoNT function [7]. For instance, molecular studies have provided evidence for cross-reactive serological observations of a single BoNT containing structural components of both BoNT serotypes C and D [8,9,10,11]. Sequences of BoNT/F were found to be particularly variable [12]. BoNT/F functional diversity was demonstrated when it was discovered that one variant (BoNT/F5) cleaved VAMP- 2 (a synaptic vesicle membrane protein involved in acetylcholine release) at L^{54} while all other BoNT/F variants (F1-F4, F6-F7) cleaved VAMP-2 at Q^{58} [13]. Currently there is not sufficient experimental evidence to correlate all observed variances in the botulinum neurotoxin gene (*bont)* with functional changes in the toxin [7]. However, it is clear that availability of these genetic data facilitates our understanding of BoNT diversity and assists in the interpretation of functional differences observed through serological methods.

Recently, researchers from the California Department of Public Health (CDPH) described the identification of a novel bivalent (produces 2 BoNTs) strain (IBCA10-7060) of *C. botulinum*, isolated from a naturally occurring case of Infant botulism [14]. While rare, other bivalent producing *C. botulinum* strains have been reported which produce combinations of BoNT A, B, and F. Many of these strains produce one of the toxins in excess of the other and denoted in accordance to relative BoNT expression

level (e.g. Af). CDPH researchers reported that IBCA10-7060 produced BoNT /B in excess (24:1) of the novel BoNT. The novel toxin was described by the CDPH researchers as a newly defined serotype H which could not be neutralized by existing antibody products [14]. However, the designation of BoNT/H has been questioned and additional studies were recommended to confirm its identity [7,15].

Both the traditional mouse neutralization assay (MNA) and an *in vitro* neuronal cell-based (NCB) assay were used to independently evaluate the first new BoNT serotype reported in >40 years. Serological results were analyzed in the context of the newly released DNA sequence posted in GenBank (acessession number: JSCF01000000) [16]. Our serological data is consistent with the genetic evidence that the novel BoNT produced by IBCA10-7060 has a hybrid-like structure of BoNT/A1 and BoNT/F5.

METHODS AND MATERIALS

Gene analyses. Neurotoxin gene sequences were retrieved from GenBank, aligned using ClustalW, and compared using SimPlot [17]. Predicted amino acid sequences were aligned pairwise using EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Toxin Preparation and Estimate of Toxin Ratio. Cultures were prepared and toxin produced at two different institutions, CDC and University of Wisconsin-Madison (UW-Madison). Toxicity levels (LD_{50} per ml) were determined in both laboratories by mouse bioassay endpoint analysis [18,19].

At CDC, strain IBCA10-7060 was streaked for isolation on egg yolk agar and incubated anaerobically at 35 ^{o}C for 2 days. A single colony was selected, assigned the designation of CDC69016 (per CDC laboratory policy), and inoculated into Cooked Meat Glucose Starch (CMGS) medium (Remel, Lenexa, KS) for overnight growth at 35 ^{o}C [18,20]. This culture (300 µl) was inoculated into 150 ml of Trypticase Peptone Glucose Yeast Extract (TPGY) medium (Remel, Lenexa, KS) with 15 ml of 1% sterile trypsin (added to ensure complete BoNT activation), and incubated anaerobically 5 days at 30 ^{o}C. After the incubation, the toxin was acid precipitated [21]. The precipitated toxin was concentrated using an

Amicon Ultra-15 Centrifugal Filter Unit with a Ultracel-50kDa membrane (EMD Millipore, Billerica, MA) and referred to in these studies as "CDC Toxin" (282,800 LD_{50}/ml).

At UW-Madison , strain CDC69016 (derived from IBCA10-7060 at CDC) was grown for 5 days at 37°C in toxin production medium (TPM) (2 % NZ Case TT, 1 % yeast extract, 0.5 % glucose) [21]. The culture was centrifuged at 12,000 × *g* for 10 min. The culture supernatant was adjusted to pH 6.2 and incubated with 5 µg/ml TPCK-treated trypsin (Worthington, Lakewood NJ) at 37 °C for 60 min to ensure complete BoNT activation. Soybean trypsin inhibitor (Sigma-Aldrich, St. Louis MO), final concentration of 10 µg/ml, was added to the culture supernatant. The trypsinized culture supernatant, referred to in these studies as "UW Toxin" (22,400 LD_{50}/ml), was diluted 1:10 in GelPhos buffer (30 mM sodium phosphate, 0.2% gelatin [pH 6.3]) for storage.

The ratio of the 2 toxins was determined in both laboratories by mouse bioassay endpoint titration with and without serotype B antitoxin. Additionally, the ratio was estimated based on Endopep-MS as previously described [22]. Culture supernatant from FDA115, which expresses BoNT/B2, was used as a control for the estimate of VAMP-2 cleavage product produced by both the known BoNT/B and the novel toxin in CDC69016.

Mouse Neutralization Assay (MNA). CDC Toxin was diluted to either 100 or 2000 LD_{50}/ml in Gelatin Buffered Saline (GBS)[18]. Antitoxins were diluted in GBS and 0.25 ml of each dilution was mixed with 1 ml of the respective toxin dilution. The toxin-antitoxin mixtures were incubated at ambient temperature for 30 min and then mice were exposed by intraperitoneal (IP) injection (0.5 ml/mouse). UW Toxin was diluted to 200 or 2,000 LD_{50}/ml with GelPhos buffer. Antitoxins were combined with 0.45 ml diluted UW Toxin and incubated at ambient temperature for 1 hr then injected IP into mice (0.5 ml/mouse). Mice were observed for signs of botulism for at least the standard MNA endpoint of 4 days [18]. All animal studies were conducted according to protocols approved by either the CDC or UW-Madison Institutional Animal Care and Use Committee.

CDC diagnostic antitoxin types A, B, and F, and trivalent antitoxin types A, B, and E had potency values from 2 to 10 IU/ml. Equine monovalent research antitoxins (Auburn University, Auburn AL) had the following potency: type A, 2,623 IU/ml; type B, 691 IU/ml; type C, 370 IU/ml; type D, ≥200 IU/ml; type E, 2,378 IU/ml; type F, 996 IU/ml; type G, 196 IU/ml. Rabbit polyclonal antitoxins were raised in Dr. Johnson's laboratory, UW-Madison, against BoNT/A1 or /B1 toxoid, respectively. Both antibody stocks were estimated to contain 100 IU/ml. Additionally 2 commercially produced therapeutic products were used: (1)Botulism Antitoxin bivalent (Equine) Types A and B (bivalentAB) (Sanofi Pasteur Limited, Canada) with stated antitoxin titers of ≥600 IU/ml for each of serotype A and B; (2)Botulism Antitoxin Heptavalent (A,B,C.D,E,F,G)-(Equine) (BAT™) (EmergentBioSolutions, Rockville MD) with stated antitoxin titers of ≥300 IU/ml for each serotype A through G. The AB product (discontinued in 2010) was held in the CDC laboratory at 4°C ± 2°. BAT™ (current therapeutic) was stored under pharmaceutical product conditions by the CDC Drug Services Office prior to use.

Neuronal Cell-based (NCB) assay. The cell-based assay using hiPSC derived neurons (Cellular Dynamics Inc) was performed as previously described [23]. A second UW Toxin (UW Toxin2) was prepared as described above; the toxicity of UW Toxin2 was 6 x 10^5 LD_{50}/ml. UW Toxin2 was combined with BAT™ in 100 µl of culture media and incubated for 1 hr at 37°C. The toxin-antitoxin mixtures were then added to cells (100 µl/well) and incubated for 24 hr at 37°C, 5 % CO_2. Cell lysates were analyzed by Western blot for VAMP2 cleavage as previously described [24,25]. Images were obtained using PhosphaGlo reagent (KPL) and a Foto/Analyst FX imaging system.

RESULTS

Genetic analysis of the novel *bont* gene. The novel toxin gene contains regions of similarity to both *bont* /A1and *bont* /F5 (Figure 1a). Comparative analysis of the nucleotide gene sequence demonstrates that the region corresponding to the HC_C domain is nearly identical (>90%) to *bont/* A1 while the region

corresponding to the LC is similar to *bont/*F5 (Figure 1b). This hybrid-like structure is similar to that described for certain serotype C and D strains (Figure 1c and Figure 1d). However, in contrast to the C/D hybrids, the HC_N domain of this hybrid is less similar (ie < 80%) to the HC_N of either *bont/*A1 or *bont*/F5. Independent verification of the novel BoNT gene sequence inCDC69016 showed 100% alignment with the GenBank sequence of IBCA10-7060 (data not shown).

MNA. The toxic effect of either the CDC Toxin or UW Toxin in mice was eliminated, even up to 21 days, only when both monovalent antitoxins A and B were added to the test sample (Table 1). The results were equivalent even though different toxin preparations and different antitoxins were used. No other single or combination of antitoxins reduced the effects of the toxin. With one exception, BAT™ provided complete neutralization with CDC Toxin at 2,000 LD_{50}/ml; partial protection was observed when the product was diluted (Table 2). The cause of death in the one exception was uncertain since symptoms were not observed in this animal prior to death. Complete neutralization of 2,000 LD_{50}/ml was also observed with the bivalent AB product. BAT™ protected animals at lower test dose at both CDC and UW-Madison. CDC Diagnostic Trivalent ABE also provided complete neutralization of CDC Toxin at 100 LD_{50}/ml; partial protection was observed at 2,000 LD_{50}/ml (Table 2).

NCB Assay. A reduction in VAMP2 was observed when UW Toxin was added to the cells without BAT™ (control), indicating VAMP2 cleavage. The addition of BAT™ protected against VAMP2 cleavage, indicating the presence of neutralizing antibodies (Figure 2).

Ratio of BoNT/B to Novel BoNT and highest effective dilution of type A antitoxin. The ratio of BoNT/B to the novel toxin in the UW Toxin was estimated to be~ 1:1 by the observation that the endpoint titer in the presence of serotype B antitoxin was ½ the titer in the absence of antitoxin (Table 3). Mice receiving toxin plus excess serotype B antitoxin but not serotype A antitoxin exhibited symptoms consistent with botulism establishing that the novel toxin in IBCA10-7060 can cause botulism. Equivalent MNA results were obtained with the CDC Toxin (data not shown). A ratio of 4:1 was obtained

with the CDC Toxin by the Endopep-MS quantitative assay (data not shown). Dilutions ≤ 1:400 (≥7 IU/ml) of the type A monovalent research antitoxin A antitoxin still provided complete neutralization against the toxic effects of an estimated level of 1,000 LD_{50}/ml of the novel toxin while ≤3.5 IU /ml did not (Table 4).

DISCUSSION

Our studies show that strain CDC69016 (derived from CDPH strain IBCA10-7060) produces 2 toxins (BoNT/B and a novel BoNT), in approximately equal proportions, and the toxicity in animals can be completely neutralized using a combination of serotypes B and A antitoxins. These results were demonstrated in 2 independent laboratories using different antitoxins. No other combination of antitoxins protected animals, indicating that the apparent neutralization with serotypes A and B antitoxins was specific for the novel BoNT and BoNT/B, respectively. Additionally, BAT™ (containing antitoxins for all 7 known BoNT serotypes) eliminated the toxic effects of both BoNTs as demonstrated in both the traditional MNA and an *in vitro* neuronal cell-based assay indicating that current therapeutic treatment products would likely be effective in individuals exposed to this hybrid toxin.

Serotype identification is critical to the laboratory confirmation of human botulism; however, DNA sequencing provides critical evidence aiding in the interpretations of serological observations of neutralization and cross-reactivite strain variations. Historically, investigators were forced to speculate on the causes of differences in serological observations which sometimes led to misinterpretations [26]. For example, the BoNT from one strain of *C. botulinum* was initially considered to be an atypical variant of serotype B because a 1,000-fold excess of antitoxin was required to neutralize its effects [27]. However, this interpretation later was determined to be incorrect when this strain (657) was shown to produce both serotype B and A (ie. *C. botulinum* Ba) [28]. DNA sequencing may have prevented the initial misidentification of this dual-toxin producing strain. More recently, sequence data provided

structural evidence of hybrid-like structures which explained the observed cross-reactivity between some *C. botulinum* serotype C and D strains [10,11]. The molecular study of the novel toxin of IBCA10-7060 showed that the *bont* gene contained areas of similarity with known toxin serotypes A and F (specifically F5)[29]. Our DNA analysis is in agreement with the previous study. However, we conclude that the DNA sequence of the novel gene appears to represent a hybrid structure between known *bont* subtypes A1 and F5 similar to those described between BoNT serotypes C and D. The CDPH authors designated the novel toxin as serotype H because of their serological observation that single or combinations of monovalent diagnostic antitoxins could not neutralize the effects of the novel BoNT [14]. However, our studies show that this novel toxin can be neutralized by existing serotype A antitoxin. Our DNA analysis shows that the binding domain (HC_C) of the novel toxin gene is nearly identical with *bont*/A1. The protective capacity of antibody directed toward the HC (neuronal cell binding domain) of BoNT is well established through work with monoclonal antibodies and the HC is currently being pursued for a next generation vaccine [30,31,32]. Therefore our observed neutralization of the novel toxin with serotype A antitoxin was not surprising.

The neutralization assay has been used for decades to establish and identify BoNT. In 1919, the first 2 BoNT serotypes (A and B) were defined through serological analysis; subsequent BoNT serotypes were similarly discovered through production of BoNT -specific antibody [33]. While standardized reagents were not necessarily required for BoNT identification, there was a need for reference antitoxins to facilitate inter-laboratory and lot-to-lot potency comparisons as investigators began developing therapeutic products (antitoxin and toxin) . As a result, the WHO International antitoxin reference standards were established [34,35]. The WHO standards were produced with BoNT from specific strains and, somewhat arbitrarily, assigned potency (International Units, IU) based on a designated toxicity level (I IU neutralized 10,000 LD_{50} for BoNT serotypes A-D, and F; 1 IU neutralized 1,000 LD_{50} for BoNT serotype E) of fully characterized BoNT derived from these identical strains. These

reference antitoxins provided a standard approach for describing neutralization capacity of therapeutic antitoxin products. The neutralization capacity of research and diagnostic antitoxin products are similarly assigned potency but with far less stringency then required for therapeutic products. As more strains were studied following outbreak investigations, variations were observed in neutralization capacity of research/diagnostic antitoxins towards BoNT derived from non-reference strains of the identical serotype; these were described as intratypic serological variants (27,36,37). Similarly, distinct antigenic properties have been described for BoNT/A1, /A2, and /A3 subtypes (named as a result of DNA sequencing) using panels of monoclonal antibodies [38,39]. Although it was suggested that an upper limit be imposed on the amount of antitoxin required to neutralize a particular BoNT from a particular strain compared to a reference toxin to help identify new serotypes, none were ever defined [36]. So the stated potency of the WHO reference standards, therapeutic antitoxin products, and diagnostic reagents only applies to the neutralization capacity of the antitoxin towards a very specific BoNT preparation; similar capacity towards non-reference BoNT (e.g. a different BoNT subtype within a serotype) cannot be assumed. Based on our DNA analyses indicating only ~1/3 of the novel toxin in IBCA10-7060 having similarity to serotype A, our studies, not unexpectedly, showed that a higher level of serotype A antitoxin was required to neutralize the effects of the novel toxin compared to BoNT/A1. Since full protection from the effects of the novel toxin was achieved using serotype A antitoxin alone, our serological observations would be consistent, based on historical precedent, with the designation of an atypical serological BoNT/A variant. The DNA evidence shows this serological variant has a hybrid structure. Recently this BoNT hybrid was confirmed to have the same VAMP-2 cleavage site as F5 [40].

Actual potency (IU per ml) of an antitoxin towards an individual BoNT can only be quantified under highly specific experimental conditions using both predefined reference standards for both toxin and antitoxin [34,41]. Purified and characterized BoNT is required for both the reference and test toxins. Additionally, changes in assay conditions, such as reference toxin, toxin test dose, buffers,

number of animals, or even reference antitoxin, affect final laboratory-specific potency results [42]. The stringent conditions required to define antitoxin potency to a specific toxin cannot be found in a typical research or clinical laboratory. Additionally, values assigned to available distributed antitoxin products (other than recognized standards such as WHO), including CDC diagnostic reagents, must be assumed to be approximate since these were never designed to be quantitative primary reference standards [20]. So, assessment of antitoxin potency towards the novel BoNT in the absence of a validated test using fully qualified materials is speculative. However, we did observe differences in antitoxin neutralization capacity towards the novel BoNT compared to what would be expected with reference toxins. At least 2 IU of monovalent serotype A research antitoxin was required to neutralize an estimated 1,000 LD_{50} of the novel toxin suggesting that an ~20-fold increase in antitoxin requirement compared to the expected neutralization of reference BoNT/A1. An even higher amount (~200-fold) of the CDC diagnostic reagent was required (only the ~50 LD_{50} per ml of the novel toxin was neutralized by ~1 IU) then expected compared to BoNT/A1. Additionally, ≥500-fold increase in BAT™ was required compared to BoNT/A1 (data not shown). So clearly this novel toxin is distinct from reference BoNT/A1. However, even these relative serological observations will need to be confirmed when purified BoNT F/A becomes available.

There are no published studies on the protective benefit of BAT™. However, the level of available type A and B antitoxin in BAT™ is nearly equivalent to the previous licensed bivalent AB product (~7500 IU) so the protective benefit is likely similar. This level of antitoxin can neutralize 25 X 10^3 LD_{50} per ml of BoNT/A1 circulating in an adult (3L plasma volume). While there are isolated reports of higher values, CDC reported in 1984 that the highest level of BoNT detected in any patient was 32 LD_{50} per ml [43]. So the available antitoxin in a single vial is ≥800 times more than needed for the treatment of most botulism cases. While our study data does not allow us to predict with accuracy the absolute potency of BAT™ against the novel toxin in IBCA10-7060, our observed protection in both the

mouse neutralization assay and the neuronal cell-based assay suggests that this therapeutic product would effectively neutralize this toxin in exposed individuals.

In summary, our studies on CDC69016, derived from IBCA10-7060, confirm the presence of 2 toxins: serotype B and a novel BoNT. The novel BoNT is a serotype A variant consisting of a hybrid structure between *bont* A1 and F5, which can be neutralized with existing serotype A antitoxin. Further studies, using purified toxin, are necessary to assign the appropriate nomenclature to this novel BoNT and to further characterize its risks.

ACKNOWLEDGEMENT

IBCA10-7060 was provided to CDC by Dr. Paul Kimsey and Dr. James Watt, California Department of Public Health. Transfer of IBCA10-7060 to CDC was facilitated through the assistance of: Ms. Angela Sanchez, Technology Transfer Office, CDC; Mr. Joseph Foster, Office of General Counsel, CDC; Dr. Michael Kurilla, Director, Office of Biodefense, Research Resources, and Translational Research, NIAID. Authors do not have a commercial or other association that might pose a conflict of interest. This publication partially was supported by funds made available from the Centers for Disease Control and Prevention, Office of Public Health Preparedness and Response (all CDC authors). Additionally, a portion was funded by the National Institute of Allergy and Infectious Diseases, R01AI095274 (all U Wisconsin authors). The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

REFERENCES

1. Yu PA, Maslanka SE, St Louis ME, Swerdlow DL. Botulism. In: Abrutyn E, Brachman PS, eds. Bacterial Infections in Humans. New York: Springer, **2009**:159-76.
2. CDC. Investigational Heptavalent botulinum antitoxin (HBAT) to replace licensed botulinum antitoxin AB and investigational Botulinum antitoxin E. MMWR **2010**; 59:299.
3. FDA News Release. FDA approves first Botulism Antitoxin for use in neutralizing all seven known botulinum nerve toxin serotypes. March 22, 2013. http://www.fda.gov/newsEvents/Newsroom/Press/Announcements/ucm345128.htm
4. Arnon SS, Schechter R, Maslanka SE, Jewell NP, Hatheway CL. Human botulism immune globulin for the treatment of infant botulism. New Eng J Med **2005**; 354:462-71.
5. Shapiro RL, Hatheway C, Swerdlow DL. Botulism in the United States: A clinical and epidemiologic review. Annals of Internal Med **1998**; 123:221-8.
6. Tacket CO, Shandera WX, Mann JM, Hargrett NT, Blake PA. Equine use and other factors that predict outcome in type A foodborne botulism. Am J Med **1984**; 76:794-8.
7. Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: genetic, structural and mechanistic insights. Nature **2014**; 12:535-49.
8. Arndt JW, Jacobson MJ, Abola EE, et al. A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1-A4. J Mol Biol **2006**; 362:733-42.
9. Hill KK, Smith TJ, Helma CH, et al. Genetic diversity among botulinum neurotoxin-producing Clostridial strains. **2007**; 189:818-32.
10. Moriishi K, Koura M, Abe Norio, et al. Mosaic structures of neurotoxins produced from *Clostridium botulinum* types C and D organisms. Biochimica et Biophysica Acta **1996**; 1307: 123-6.

11. Nakamura K, Kohda T, Umeda K, Yamamoto H, Mukamoto M, Kosaki S. Characterization of the D/C mosaic neurotoxin produced by *Clostridium botulinum* associated with bovine botulism in Japan. Vet Microbiol **2010**; 140:147-54.
12. Raphael BH, Choudoir MJ, Lúquez C, Fernández, Maslanka SE. Sequence diversity of genes encoding botulinum neurotoxin type F. Appl Environ Microbiol **2010**; 76:4805-12.
13. Kalb SR, Baudys J, Smith TJ, Smith LA, Barr JR. Three enzymatically active neurotoxins of *Clostridium botulinum* strain Af84;BoNT/A2, /F4, and /F5. Anal Chem **2014**; 86:3254-62.
14. Barash JR, Arnon SS. A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins. J Infect Dis **2013**; 209:183-91.
15. Johnson EA. Validity of botulinum neurotoxin serotype H. JID **2014**; http://jid.oxfordjournals.org Accessed on April 21, 2014.
16. Gonzalez-Escalona N, Thirunavukkarasu N, Singh A, et al. Draft genome sequence of bivalent *Clostridum botulinum* strain IBCA10-7060, encoding botulinum neurotoxin B and a new FA mosaic type. Genome Announcements **2014**; 2(6):e01275-14.
17. Lole KS, Bollinger RC, Paranjape RS, et al. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 1999; 73:152-60.
18. Maslanka SE, Solomon HM, Sharma S, and Johnson EA. Clostridium botulinum and Its Toxins. In: Tortorello ML, Downes FP, Doores S, Ito K, Salfinger Y, eds. Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association. **2013**: eBook ISBN: 978-0-87553-022-2
19. Reed LJ, Muench H. A simple method for estimating fifty percent end points. Am J Hyg **1938**; 27:493-7.

20. Hatheway CL. Botulism. In: Balows A, Hausler Jr. WJ, Ohashi M, Turano A, eds. Laboratory Diagnosis of Infectious Diseases: Principles and Practice, Vol. 1. Berlin:Springer-Verlag, **1988**:111-133.

21. Malizio CJ, Goodnough MC, Johnson EA. Purification of Clostridium botulinum Type A Neurotoxin. In: O. Holst, ed. Bacterial Toxins: Methods and Protocols , Methods in Molecular Biology. Totowa, NJ: Humana Press. **2000**; 145:27-39.

22. Wang D, Baudys J, Krilich JC, Smith TJ, Barr JR, Kalb SR. A two-stage multiplex method for quantitative analysis of botulinum neurotoxin type A, B, E, and F by MALDI-TOF mass spectrometry. Anal Chem **2014;** 86:10847-54.

23. Whitemarsh RC, Strathman MJ, Chase LG, Stankewicz C, Tepp WH, Johnson EA, Pellett S. Novel application of human neurons derived from induced pluripotent stem cells for highly sensitive botulinum neurotoxin detection, Toxicological sciences : an official journal of the Society of Toxicology **2012**; 126:426-35.

24. Pellett S, Tepp WH, Clancy CM, Borodic GE, Johnson EA. A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. FEBS letters **2007**; 581: 4803-4808.

25. Pellett S, Tepp WH, Toth SI, Johnson EA. Comparison of the primary rat spinal cord cell (RSC) assay and the mouse bioassay for botulinum neurotoxin type A potency determination. Journal of Pharmacological and Toxicological Methods **2010**; 61: 304-310.

26. Smith TJ, Hill KK, Raphael BH. Historical and current perspectives on *Clostridium botulinum* diversity. Res Microbiol **2014**; In Press.

27. Hatheway CL, McCroskey LM, Lombard GL, Dowell Jr VR. Atypical toxin variant of *Clostridium botulinum* type B associated with infant botulism. J Clin Microbiol **1981**; 14:607-11.

28. Giménez DF. *Clostridium botulinum* subtype Ba. Zbl Bakt Hyg A **1984**; 257:68-72.

29. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis **2013**; 209:192-202

30. Levy R, Forsyth CM, LaPorte SL, Geren IN, Smith LA, Marks JD. Fine and domain-level epitope mapping of botulinum neurotoxin type A neutralizing antibodies by yeast surface display. J Mol Biol **2007**; 365: 196-210.

31. Webb RP, Smith LA. What next for botulism vaccine development? Expert Rev Vaccines **2013**; 12:481-92.

32. Przedpelski A, Tepp WH, Kroken AR, Fu Z, Kim JP, Johnson EA, Barbieri JT. Enhancing the protective immune response against botulism. Infect Immun **2013**; 81:2638-2644.

33. Burke GS. Notes on *Bacillus botulinus*. J Bacteriol **1919**; 4:555-71.

34. Bowmer EJ. Preparation and assay of the International standards for *Clostridium botulinum* types A, B, C, D, and E antitoxins. Bull Wld Hlth Org **1963**; 29:701-9.

35. Jones RGA, Corbel MJ, Sesardic D. A review of WHO International standards for botulinum antitoxins. Biologicals **2006**; 34:223-6.

36. Giménez DF, Ciccarelli AS. Variaciones antigenicas en toxinas botulinicas del tipo F. Ensayo de definiciones para la tipificacion serologica y clasificacion de *Clostridium botulinum*. Medicina (Bs Aires) **1972**; 32:596-606

37. Giménez DF, Giménez JA. Serological subtypes of botulinal neurotoxins. In: DasGupta BR, ed. Botulinum and Tetanus Neurotoxins. New York: Plenus Press, **1993**:421-430.

38. Smith TJ, Lou J, Geren IN, et al. Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun **2005;** 73:5450-7.

39. Kalb SR, Lou J, Garcia-Rodriguez C, et al. Extraction and inhibition of enzymatic activity of botulinum neurotoxins/A1, /A2, and /A3 by a panel of monoclonal anti-BoNT/A antibodies. PLoS One **2009**; 4:e5355. doi: 10.1371/journal.pone.0005355. Epub 2009 Apr 28.

40. Kalb, SR, Baudys J, Raphael BH, Dykes JK, Lúquez C, Maslanka SE, Barr JR. Functional characterization of botulinum neurotoxin serotype H as a hybrid of known serotypes F and A (BoNT F/A. Anal Chem 2015; doi:10.1021/ac504716v. Epub 2015 March 3.
41. Bengtson IA. Standardization of botulism antitoxins. Amer J Pub Health **1921**; 11:352-357.
42. Sesardic D, Leung T, Gaines Das R. Role of standards in assays of botulinum toxins: international collaborative study of three preparations of botulinum type A toxin. Biologicals **2003**; 31: 265-76.
43. Hatheway CH, Snyder JD, Seals JE, Edell TA, Lewis GE. Antitoxin levels in botulism patients treated with trivalent equine botulism antitoxin to toxin types A, B, and E. JID **1984**; 150:407-12.

TABLES

Table 1. Mouse Neutralization Assay using research antitoxins [a]

Toxin	Control[b]	A	B	B+A	B+C	B+D	B+E	B+F	B+G
CDC Toxin[c]	0/6	0/6	0/6	6/6[d]	0/6	0/6	0/6	0/6	0/6
UW Toxin[e]	0/5	0/5	0/5	5/5	NT[f]	NT	NT	NT	NT

[a]Data expressed as number of animals survived at 4 days/number of animals tested

[b]Toxin only control; no antitoxin added.

[c]Centers for Disease Control and Prevention, CDC. Toxin tested at 2000 LD_{50} per ml. Monovalent antitoxins: A (2,623 IU/ml), B (691 IU/ml), C (370 IU/ml), D (units not available), E (2,378 IU/ml;), F (996 IU/ml), G(196 IU/ml). Results are from 3 independent experiments.

[d]Animals observed for 21 days; no botulism symptoms developed.

[e]University of Wisconsin-Madison, UW Toxin tested at 2000 LD_{50} per ml. UW rabbit types A and B antitoxin: 100 IU per ml. Equivalent results obtained when tested at 200 LD_{50} per ml.

[f]Not Tested, NT

Shaded areas represent antitoxin combinations which provided protection from toxic effects.

Table 2. Mouse Neutralization Assay using non-research antitoxins[a]

Toxin	Control[b]	Therapeutic		CDC Diagnostic	
		hBAT[c]	BAT AB[d]	Trivalent[e]	AB[f]
CDC Toxin[g]	0/10	7/8[h]	2/2	NT[i]	NT
CDC Toxin[j]	0/18	10/10[k]	NT	4/4	0/4[l]
UW Toxin[m]	0/4	4/4	NT	NT	NT

[a]Data expressed as number of animals survived at 4 days/number of animals tested.

[b]Toxin only control; no antitoxin added.

[c]Botulism Antitoxin Heptavalent (BAT™), EmergentBioSolutions, Rockville MD (different unexpired lots used at CDC and UW-Madison).

[d]Botulism Antitoxin Bivalent, Types A&B, Sanofi Pasteur, Canada.

[e]CDC Diagnostic Antitoxin Trivalent (A, B, E), CDC

[f]CDC Diagnostic Antitoxins A plus B, CDC

[g]Centers for Disease Control Toxin test dose: 2,000 LD_{50} per ml.

[h]One animal asymptomatic until 4th day. Partial protection (symptom onset delayed for 3 days) achieved even when BAT™ diluted 1:32.

[i]Not Tested, NT

[j]CDC Toxin test dose: 100 LD_{50} per ml.

[k]Partial protection (symptom onset delayed for 4 days) achieved even when BAT™ diluted 1:160.

[l]Partial protection (symptom onset delayed for 2 days).

[m]University of Wisconsin-Madison, UW Toxin test dose: 2000 LD_{50} per mL

Shaded areas represent antitoxin combinations which provided protection from toxic effects.

Table 3. Estimation of ratio of BoNT/ B to Novel BoNT in UW Toxin[a]

Antitoxin	Culture Dilutions Tested[b]					
Treatment	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000
None[c]	0/4	0/4	0/4	0/4	4/4	4/4
B[d]	0/4	0/4	0/4	4/4	4/4	
B + A[e]	4/4					

[a]Toxin produced at University of Wisconsin (UW Toxin)

[b]Results expressed as number of animals survived at 4 days/total animals tested.

[c]Serial dilution of culture alone. Toxicity level of 22,400 LD_{50} per ml.

[d]3IU type B antitoxin added to each dilution. Both toxins present at equivalent level of ~ 11,200 LD_{50} per ml.

[e]Addition of 10 IU of type A antitoxin provided complete neutralization demonstrating that the addition of 3IU of type B antitoxin eliminated the toxic effects of the BoNT B in all dilutions. No symptoms of botulism observed in any animals.

Table 4. Neutralization capacity of monovalent type A antitoxin for Novel BoNT[a]

Toxin	Dilution of antitoxin type A[b]				
	1:100	1:200	1:400	1:800	1:1600
CDC Toxin[c]	4/4	4/4	4/4[d]	0/4	0/4

[a]Data expressed as number of animals survived at 4 days/number of animals tested

[b]Research monovalent type A antitoxin (2,623 IU per ml).

[c] Centers for Disease Control and Prevention, CDC Toxin. Based on 1:1 ratio of B to novel BoNT, the estimated test dose of 1,000 LD_{50} per ml of novel BoNT was used in the presence of 8 IU/ml of type B antitoxin.

[d]Addition of monovalent type F antitoxin did not alter the minimum dilution of type A antitoxin that protected animals.

FIGURE LEGENDS

Figure 1. Nucleotide similarity plots (derived from SimPlot [7]) are shown in Panels A and C for the novel *bont/* FA and previously recognized *bont/* CD, respectively. The percent similarity shown on the Y-axis was generated using a 200-bp window and a 20-bp step. In Panel A, *bont/A1* (shown in green) and *bont/F5* (shown in red) are compared with *bont/FA* [individual nucleotide data was obtained from Genbank accession numbers: *bont/A1* (AM412317), *bont/F5* (GU213212), *bont/FA* (JSCF00000000)]. In Panel C, *bont/D* (shown in green) and *bont/C* (shown in red) are compared with *bont/CD* [individual nucleotide data was obtained from Genbank accession numbers: *bont/D* (JENR01000128), *bont/C* (AB200358), *bont/CD* (AB200360)]. The gene regions encoding the 3 domains (light chain; LC, N-terminal heavy chain; HC_N and C-terminal heavy chain; HC_C) are indicated by dotted lines. The predicted amino acid identity of BoNT LC, HC_N, and HC_C domains are shown in Panels B and D for the hybrid toxins BoNT F/A and BoNT C/D, respectively. Domains sharing ≥ 80% amino acid identity in pairwise alignments between the associated hybrid toxins and the comparison toxins are shaded. The % amino acid identity of the most similar domains is also indicated. The novel toxin (Panels A and B) contained in strain IBCA10-7060 (BoNT F/A), has significant similarity to the LC domain of BoNT/F% and the HC_C domain of BoNT/A1, respectively. For comparison, the structure of another hybrid toxin (BoNT C/D) is shown in Panels C and D.

Figure 2. Neutralization of UW Toxin2 with BAT™ in NCB assay. The indicated amounts of extract were incubated with no (top) or 2 µl (bottom) of hBAT in 100 µl of culture media, and incubated for 1 hr at 37°C. The toxin/BAT™ mixtures were then added to hiPSC derived neurons (100 µl/well), and incubated for 24 hr at 37°C, 5 % CO2. Cell lysates were prepared in 50 µl of lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) and analyzed by Western blot for levels of VAMP2, syntaxin, and SNAP-25 as

previously described [24,25]. VAMP-2 remains intact when the culture supernatant is pretreated with HBAT demonstrating a protective capacity.