A Novel Botulinum Neurotoxin, Previously Reported as Serotype H, Has a Hybrid-Like Structure With Regions of Similarity to the Structures of Serotypes A and F and Is Neutralized With Serotype A Antitoxin

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Botulism is a potentially fatal paralytic disease caused by the action of botulinum neurotoxin (BoNT) on nerve cells. There are 7 known serotypes (A–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 as BoNT/H. The BoNT gene (*bont*) sequence of BoNT/H was compared to known *bont* sequences. Genetic analysis suggested that BoNT/H has a hybrid-like structure containing regions of similarity to the structures of BoNT/A1 and BoNT/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 antitoxins, including those contained in multivalent therapeutic antitoxin products that are the mainstay of human botulism treatment.

Keywords. botulinum toxin; *Clostridium botulinum*; serotype; botulism; neutralization; neuronal cell-based assay.

Botulism is a rare, potentially lethal, neuroparalytic disease that manifests naturally in humans in 3 primary forms: foodborne botulism, intestinal colonization botulism (infant botulism and, rarely, adult intestinal colonization botulism), and wound botulism [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 Heptavalent Botulism Antitoxin (BAT), an equine-based heptavalent product that treats all known serotypes of botulinum toxin (BoNT/A–G) [2, 3]. Infant botulism is typically treated by Botulism Immune Globulin Intravenous (human), marketed as BabyBIG (Baxter Biosciences, Thousand Oaks, California), which is licensed for BoNT/A and BoNT/B only [4]. Improvements in medical management practices since the 1950s have reduced overall botulism mortality from approximately 60% to only 5%–10% [5]. Therapeutic antitoxin provides a protective benefit in patients with botulism by reducing both mortality due to and the long-term consequences of this paralytic disease [6]. A retrospective review of foodborne botulism cases showed that patients who received antitoxin were more likely (46% vs 10%) to survive. Patients receiving antitoxin early in the course of their illness appeared to recover more quickly, with a hospitalization

duration of 10 days, compared with 41 and 56 days for late and no receipt of antitoxin, respectively. A double-blind study of BabyBIG in infant botulism demonstrated that antitoxin administration decreases hospital stay and improves clinical outcome [4].

The 7 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 150-kDa protein that, when activated, is converted to a dichain (composed of a 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 BoNT serotypes C and D [8–11]. 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 and F6-F7) cleaved VAMP-2 at Q^{58} [13]. Currently, there is not sufficient experimental evidence to correlate all observed variances in the BoNT 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 strain (ie, a strain that produces 2 BoNTs), *C. botulinum* IBCA10-7060, which was isolated from a naturally occurring case of infant botulism [14]. While rare, other bivalent *C. botulinum* strains have been reported that produce combinations of BoNT A, B, and F. Many of these strains produce one of the toxins in excess of the other, and this characteristic is denoted by specifying the BoNT subtype with the greatest level of expression first (eg, "Af" denotes a bivalent strain in which BoNT/A is produced in greater quantity than BoNT/F). CDPH researchers reported that strain 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 toxin that 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 (accession number, JSCF01000000) [16]. Our serological data are consistent with the genetic evidence that the novel BoNT produced by strain IBCA10-7060 has a hybrid-like structure of BoNT/A1 and BoNT/F5.

MATERIALS AND METHODS

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

Preparation of Toxin and Estimation of Toxin Ratio
Cultures were prepared and toxin produced at 2 different institutions, the CDC and the University of Wisconsin–Madison (UW-Madison). Toxicity levels (measured as the dose per milliliter that is required to kill 50% of recipients [LD_{50}]) were determined in both laboratories by mouse bioassay end point analysis [18, 19].

At the CDC, strain IBCA10-7060 was streaked for isolation on egg yolk agar and incubated anaerobically at 35°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 medium (Remel, Lenexa, KS) for overnight growth at 35°C [18, 20]. This culture (volume, 300 µL) was inoculated into 150 mL of trypticase peptone glucose yeast extract medium (Remel, Lenexa, Kansas) with 15 mL of 1% sterile trypsin (added to ensure complete BoNT activation) and incubated anaerobically for 5 days at 30°C. After incubation, the toxin underwent acid precipitation [21]. The precipitated toxin was concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with a Ultracel 50-kDa membrane (EMD Millipore, Billerica, Massachusetts) and is referred to hereafter as the "CDC toxin" (282 800 LD_{50}/mL).

At the UW-Madison, strain CDC69016 (derived from strain IBCA10-7060 at the CDC) was grown for 5 days at 37°C in toxin production medium (2% NZ Case TT, 1% yeast extract, and 0.5% glucose) [21]. The culture was centrifuged at 12 000*g* for 10 minutes. The culture supernatant was adjusted to pH 6.2 and incubated with 5 µg/mL of TPCK-treated trypsin (Worthington, Lakewood, New Jersey) at 37°C for 60 minutes
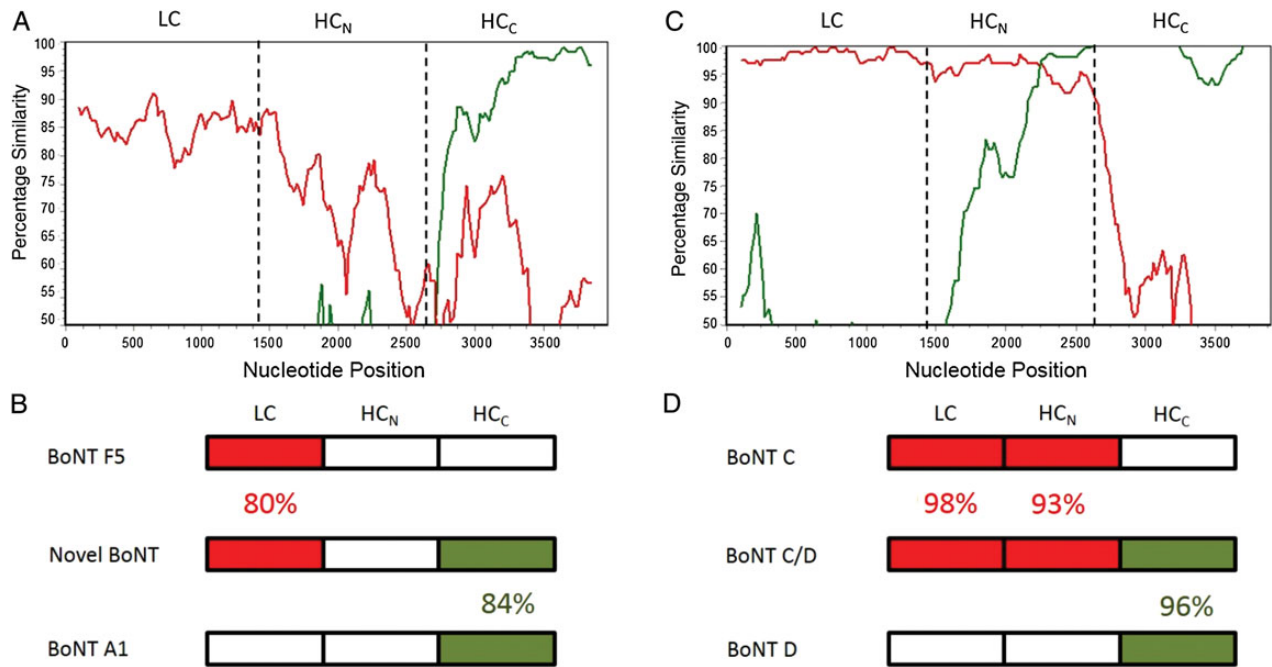
Figure 1. *A* and *C*, Nucleotide similarity plots (derived from SimPlot [7]) are shown for the novel *bont/FA* (*A*) and the previously recognized *bont/CD* (*C*). The percentage similarity was generated using a 200-bp window and a 20-bp step. *A*, *bont/A1* (green) and *bont/F5* (red) are shown with *bont/FA* (individual nucleotide data were obtained from GenBank accession numbers AM412317 [for *bont/A1*], GU213212 [for *bont/F5*], and JSCF00000000 [for *bont/FA*]). *B*, *bont/D* (green) and *bont/C* (red) are shown with *bont/CD* (individual nucleotide data were obtained from GenBank accession numbers JENR01000128 [for *bont/D*], AB200358 [for *bont/C*], and AB200360 [for *bont/CD*]). 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. *B* and *D*, The predicted amino acid identity of botulinum neurotoxin (BoNT) LC, HC_N, and HC_C domains are for the hybrid toxins BoNT F/A (*B*) and BoNT C/D (*D*). Domains sharing ≥80% amino acid identity in pairwise alignments between the associated hybrid toxins and the comparison toxins are shaded. The percentage amino acid identity of the most similar domains is also indicated. The structure of the novel toxin contained in strain IBCA10-7060 (BoNT F/A) has significant similarity to the LC domain of BoNT/F5 (*A*) and the HC_C domain of BoNT/A1 (*B*). For comparison, the structure of another hybrid toxin (BoNT C/D) is also shown (*C* and *D*).

to ensure complete BoNT activation. Soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, Missouri) was added to the culture supernatant to yield a final concentration of 10 μg/mL. The trypsinized culture supernatant, referred to hereafter as "UW toxin" (22 400 LD$_{50}$/mL), was diluted 1:10 in GelPhos buffer (30 mM sodium phosphate and 0.2% gelatin [pH 6.3]) for storage.

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

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 minutes and then mice were exposed by intraperitoneal (IP) injection (0.5 mL/mouse). UW toxin was

diluted to 200 or 2000 LD$_{50}$/mL with GelPhos buffer. Antitoxins were combined with 0.45 mL diluted UW toxin and incubated at ambient temperature for 1 hour 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 international units (IU)/mL. Equine monovalent research antitoxins (Auburn University, Auburn, Alabama) had the following potency: type A, 2623 IU/mL; type B, 691 IU/mL; type C, 370 IU/mL; type D, ≥200 IU/mL; type E, 2378 IU/mL; type F, 996 IU/mL; and type G, 196 IU/mL. Rabbit polyclonal antitoxins were raised in the laboratory of one of the authors (E. A. J.; UW-Madison) against BoNT/A1 or BoNT/B1 toxoid. Both antibody stocks were estimated to contain 100 IU/mL. Additionally, 2 commercially produced therapeutic products were used: (1) Bivalent Botulism Antitoxin against types A and B (Equine; bivalent-AB) (Sanofi Pasteur, Canada), with stated antitoxin titers of ≥600 IU/mL for both serotypes; and (2) Heptavalent Botulism

Table 1. Mouse Neutralization Assay, Using Research Antitoxins

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

Data denote the no. of animals alive at day 4/no. tested.

Abbreviations: CDC, Centers for Disease Control and Prevention; IU, international units; LD_{50}, dose required to kill 50% of recipients; NT, not tested; UW, University of Wisconsin–Madison.

[a] Toxin-only control; no antitoxin was added.

[b] Tested at 2000 LD_{50}/mL. Monovalent antitoxin potency: A, 2623 IU/mL; B, 691 IU/mL; C, 370 IU/mL; D, not available; E, 2378 IU/mL; F, 996 IU/mL; and G, 196 IU/mL. Results are from 3 independent experiments.

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

[d] Tested at 2000 LD_{50}/mL. A and B antitoxin potency: 100 IU/mL. Equivalent results were obtained when tested at 200 LD_{50}/mL.

Antitoxin against types A, B, C, D, E, F, and G (Equine; BAT; Emergent BioSolutions, Rockville, Maryland), with stated antitoxin titers of ≥300 IU/mL for each serotype. The AB product (discontinued in 2010) was held in the CDC laboratory at 4°C ± 2°. BAT (the current therapeutic) was stored under pharmaceutical product conditions by the CDC Drug Services Office before use.

NCB Assay

The cell-based assay using hiPSC-derived neurons (Cellular Dynamics) was performed as previously described [23]. A second UW toxin (UW toxin 2) was prepared as described above; the toxicity of UW toxin 2 was 6×10^5 LD_{50}/mL. UW toxin 2 was combined with BAT in 100 µL of culture medium and incubated for 1 hour at 37°C. The toxin-antitoxin mixtures were then added to cells (100 µL/well) and incubated for 24 hours at 37°C in 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*/A1 and *bont*/F5 (Figure 1A). Comparative analysis of the nucleotide gene sequence demonstrated that the region corresponding to the HC_C domain was nearly identical (>90%) to *bont*/A1, whereas the region corresponding to the LC was 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 1D). However, in contrast to the C/D hybrids, the HC_N domain of this hybrid is less similar (ie, <80% similarity) to the HC_N of either *bont*/A1 or *bont*/F5. Independent verification of the novel BoNT gene sequence in strain CDC69016 showed 100% alignment with the GenBank sequence of strain IBCA10-7060 (data not shown).

MNA Findings

The toxic effect of either the CDC toxin or UW toxin in mice was eliminated for up to 21 days when monovalent antitoxin A

and antitoxin B were added together to the test sample (Table 1). The results were equivalent, even though different toxin preparations and different antitoxins were used. No other single antitoxin or combination of antitoxins reduced the effects of the toxin. With one exception, BAT provided complete neutralization of the CDC toxin at 2000 LD_{50}/mL; partial protection was observed when the product was diluted (Table 2). The cause of death in the one exception was uncertain because symptoms were not observed in this animal before it died. Complete neutralization at 2000 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

Table 2. Mouse Neutralization Assay, Using Nonresearch Antitoxins

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

Data denote the no. of animals alive at day 4/no. tested.

Abbreviations: CDC, Centers for Disease Control and Prevention; LD_{50}, dose required to kill 50% of recipients; NT, not tested; UW, University of Wisconsin–Madison.

[a] Toxin-only control; no antitoxin was added.

[b] Heptavalent botulism antitoxin (BAT), was produced by Emergent BioSolutions (Rockville, Maryland). Different unexpired lots were used at the CDC and UW-Madison.

[c] Bivalent botulism antitoxin against types A and B (Bivalent AB) was produced by Sanofi Pasteur, Canada.

[d] CDC diagnostic antitoxin, trivalent (A, B, and E).

[e] CDC diagnostic antitoxins A and B.

[f] Tested at 2000 LD_{50}/mL.

[g] One animal was asymptomatic until day 4. Partial protection (ie, delay of symptom onset for 3 days) was achieved even when BAT was diluted 1:32.

[h] Tested at 100 LD_{50}/mL.

[i] Partial protection (ie, delay of symptom onset for 4 days) was achieved even when BAT was diluted 1:160.

[j] Partial protection (ie, delay of symptom onset for 2 days) was achieved.
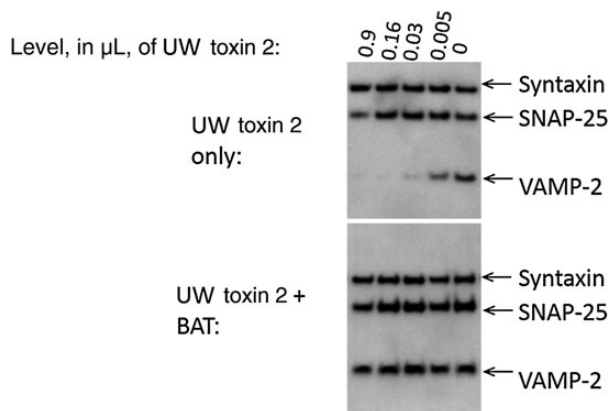
[k] Tested at 2000 LD_{50}/mL.

Figure 2. Neutralization of University of Wisconsin–Madison (UW) toxin 2 with heptavalent botulism antitoxin (BAT) in a neuronal cell–based assay. The indicated amounts of extract were incubated without (top) or with 2 μL (bottom) of heptavalent botulism antitoxin, in 100 μL of culture medium and incubated for 1 hour at 37°C. The toxin/BAT mixtures were then added to hiPSC-derived neurons (100 μL/well) and incubated for 24 hours at 37°C in 5% CO_2. Cell lysates were prepared in 50 μL of lithium dodecyl sulfate 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 BAT, demonstrating a protective capacity.

provided complete neutralization of CDC toxin at 100 LD_{50}/mL; partial protection was observed at 2000 LD_{50}/mL (Table 2).

NCB Assay Findings

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).

Table 3. Estimation of Ratio of Botulinum Neurotoxin Subtype B (BoNT/B) to Novel BoNT in the University of Wisconsin–Madison (UW) Toxin

Antitoxin Treatment	Culture Dilution					
	1:1000	1:2000	1:4000	1:8000	1:16 000	1:32 000
None[a]	0/4	0/4	0/4	0/4	4/4	4/4
B[b]	0/4	0/4	0/4	4/4	4/4	. . .
B + A[c]	4/4	. . .	. . .	. . .	. . .	. . .

Data denote the no. of animals alive at day 4/no. tested. No symptoms of botulism were observed in any animals.

Abbreviations: IU, international units; LD_{50}, dose required to kill 50% of recipients.

[a] Serial dilution of culture alone. The toxicity level was 22 400 LD_{50}/mL.

[b] A total of 3 IU of type B antitoxin was added to each dilution. Both toxins present at equivalent level of approximately 11 200 LD_{50}/mL.

[c] The addition of 10 IU of type A antitoxin provided complete neutralization, demonstrating that the addition of 3 IU of type B antitoxin eliminated the toxic effects of BoNT/B in all dilutions.

Table 4. Neutralization Capacity of Monovalent Type A Antitoxin for Novel Botulinum Neurotoxin (BoNT)

Toxin	Dilution of Antitoxin Type A[a]				
	1:100	1:200	1:400	1:800	1:1600
CDC toxin[b]	4/4	4/4	4/4[c]	0/4	0/4

Data denote the no. of animals alive at day 4/no. tested.

Abbreviations: CDC, Centers for Disease Control and Prevention; LD_{50}, dose required to kill 50% of recipients.

[a] Research monovalent type A antitoxin (2623 IU per mL).

[b] On the basis of a 1:1 ratio of B to novel BoNT, the estimated test dose of 1000 LD_{50}/mL of novel BoNT was used in the presence of 8 IU/mL of type B antitoxin.

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

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 approximately 1:1 by the observation that the end point titer in the presence of serotype B antitoxin was one half 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 CDC toxin (data not shown). A ratio of 4:1 was obtained with CDC toxin by the Endopep mass spectrometry quantitative assay (data not shown). Dilutions of ≤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 1000 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 that the toxicity in animals can be completely neutralized using a combination of serotype 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 NCB 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-reactive 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 1000-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 strain IBCA10-7060 showed that *bont* 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-like 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 antitoxins. Our DNA analysis shows that the binding domain (HC_C) of the novel toxin gene is nearly identical with that of *bont*/A1. The protective capacity of antibody directed toward the HC (the 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 [30–32]. 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 known 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 interlaboratory and lot-to-lot potency comparisons as investigators began developing therapeutic products (antitoxin and toxin). As a result, the World Health Organization (WHO) international antitoxin reference standards were established [34, 35]. The WHO standards were produced with BoNT from specific strains and, somewhat arbitrarily, were assigned a potency (expressed in IU) on the basis of a designated toxicity level (1 IU neutralized $10\,000$ LD_{50} for BoNT serotypes A–D and F, and 1 IU neutralized 1000 LD_{50} for BoNT serotype E) of fully characterized BoNTs derived from these identical strains. These reference antitoxins provided a standard approach for describing the neutralization capacity of therapeutic antitoxin products. The neutralization capacity of research and diagnostic antitoxin products are similarly assigned a potency but with far less stringency then required for therapeutic products. As more strains were studied following outbreak investigations, variations were observed in the neutralization capacity of research and diagnostic antitoxins toward BoNT derived from nonreference 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, BoNT/A2, and BoNT/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 with the amount of 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 toward a very specific BoNT preparation; similar capacity toward nonreference BoNT (eg, a different BoNT subtype within a serotype) cannot be assumed. On the basis of our DNA analyses indicating that the novel toxin in IBCA10-7060 was only approximately 33% similar 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 with 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 that 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/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 the potency of antitoxin 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 those of the WHO), including CDC diagnostic reagents, must be assumed to be approximate because these were never designed to be quantitative primary reference standards [20]. So, assessment of antitoxin potency toward the novel BoNT in the absence of a validated test that uses fully qualified materials is speculative. However, we did observe

differences in antitoxin neutralization capacity toward the novel BoNT, compared with what would be expected with reference toxins. At least 2 IU of monovalent serotype A research antitoxin was required to neutralize an estimated 1000 LD_{50} of the novel toxin, suggesting an approximately 20-fold increase in antitoxin requirement, compared with the amount expected to neutralize reference BoNT/A1. An even higher amount (approximately 200-fold) of the CDC diagnostic reagent was required (only the approximately 50 LD_{50}/mL of the novel toxin was neutralized by approximately 1 IU) than expected, compared with BoNT/A1. Additionally, a ≥500-fold increase in BAT was required, compared with BoNT/A1 (data not shown). So, it is clear that 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 that in the previous licensed bivalent AB product (approximately 7500 IU), so the protective benefit is likely similar. This level of antitoxin can neutralize 25×10^3 LD_{50}/mL of BoNT/A1 circulating in an adult (plasma volume, 3 L). While there are isolated reports of higher values, the CDC reported in 1984 that the highest level of BoNT detected in any patient was 32 LD_{50}/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 do not allow us to predict with accuracy the absolute potency of BAT against the novel toxin in strain IBCA10-7060, the protection we observed in both the MNA and the NCB assay suggests that this therapeutic product would effectively neutralize this toxin in exposed individuals.

In summary, our studies on strain CDC69016, derived from strain IBCA10-7060, confirm the presence of 2 toxins: serotype B and a novel toxin, BoNT. BoNT is a serotype A variant consisting of a hybrid-like 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.

Notes

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