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A 10 Year Analysis of the Use of Multiplex Real-Time PCR Screening for Botulinum Neurotoxin-Producing Clostridium Species

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Abstract

Prompt testing of specimens suspected of containingbotulinum neurotoxins produced by *Clostridium botulinum*, *C. butyricum*, and *C. baratiiisis* essential due to the potential of these toxins to produce rapid morbidity and mortality in humans. The Standard Mouse Bioassay (SMB) is the gold standard for *C. botulinum* neurotoxin (BoNT) testing but has several limitations including a long, labor intensive testing process and difficult result interpretation. We have developed and evaluated a sensitive screening tool for the detection of *Clostridium spp*. neurotoxin genes, BoNT types A, B, E, F and *C. baratii* F, by real-time PCR using an automated DNA extraction. Clinical specimens and environmental samples were analyzed over a 10-year period by real-time PCR, SMB, and culture. A total of 61 cases, clinically compatible with foodborne or infant botulism were submitted to our laboratory between 2003-2013. PCR was positive for 31 cases, 81% of these were confirmed by culture and 45% were confirmed by SMB. We also found that screening specimens by PCR provides an early indication of botulism in approximately 4 hours on all specimen types. Comparatively, SMB testing requires at least four days, is not appropriate for all specimen types, and requires sufficient quantities of specimen for analysis which precluded its use in 28 cases in our study. This rapid, high-confidence, cost-effective assay that detects the major BoNT types is a great addition to the investigation of suspect cases of botulism.

Keywords: *Clostridium botulinum*; Botulinum toxin; Botulism; Real-time PCR; Multiplex PCR

Abbreviations

ATCC: American Tissue Type Culture Collection; BoNT: *C. botulinum* Neurotoxin; CBI: *C. botulinum* Isolation Agar; CFU: Colony Forming Units; LOD: Limit Of Detection; NYSDOH: New York State Department of Health; spp: Species; PCR; Polymerase chain reaction; SMB: Standard Mouse Bioassay; TPGY: Trypticase-Peptone-Glucose-Yeast-Extract

Introduction

Clostridium botulinum, *C. baratii*, and *C. butyricum* are gram positive, anaerobic bacteria which may produce potent neurotoxins. Rapid clinical testing is critical because intoxication from BoNT types A, B, E, F, and *C. baratii* F can result in fatal illness in humans [1]. The CDC case definitions of botulism as of 2011 for foodborne botulism and infant botulism have been described [2]. A confirmed case of foodborne botulism is a clinically compatible case that is laboratory confirmed or that occurs among a person who ate the same food as persons who have laboratory-confirmed botulism. The laboratory criteria for diagnosis include the detection of botulinum toxin in serum, stool or patient's food or the isolation of *C. botulinum* from stool. There is a similar definition for infant botulism however the case must be in a child less than 1 year of age and detection in food is not relevant.

The standard mouse bioassay (SMB), which detects active toxin, is the "gold standard" for BoNT testing [3]. This method has several limitations including being very labor intensive, expensive, ethically challenging due to the use of live mice, and having the potential of producing false positive results [4]. Even though the use of the SMB has been much debated, particularly for comparison of toxin preparations in clinical use, this assay utilizing mouse lethality remains the standard for detection and quantitation of *in-vivo* neurotoxicity [5,6,7]. Other methods of identification of toxicity such as those determining immunological reactivity or catalytic activity need to be more extensively compared to the mouse bioassay before they are adopted for general use [8].

Polymerase Chain Reaction (PCR) assays have been developed and utilized in BoNT testing previously [9,10]. The few reports that have been published have evaluated foods and fecal material using PCR based methods but none used real-time testing directly on clinical specimens. Also, the published assays have not included all of the most relevant BoNT types detected in North America (including *C. baratii* toxin type F) that cause the majority of botulism cases in a multiplex format nor did they utilize laboratory automation that is beneficial for large outbreaks or response to potential bio-threat events.

Real-time PCR testing is also valuable where there is insufficient

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volume of clinical specimen for direct mouse bioassay testing. In these situations, culture and isolation of the toxigenic bacteria must be performed before any indication of botulism is detected or confirmed in the laboratory thus delaying the identification of botulism which can lead to a serious impact to patient treatment and care. Culture and isolation of bacterial colonies from primary specimens is time consuming and often results in having to test many suspect colonies that have similar morphology to *C. botulinum*. In addition, there may be *Clostridium spp*. present in stool that do not produce toxin. When testing for infant botulism, specimens are frequently received in the laboratory that does not meet the minimum volume criteria for SMB testing. PCR testing is extremely useful in these situations as very small volumes can be successfully testing using this method. Botulism can cause paralysis of the GI tract and thus infants cannot produce the necessary specimen quantity needed for the SMB. Often, diagnosis by molecular methods is the only mechanism by which a highly reliable and sensitive result is obtained within a time frame relevant for providing meaningful results to physicians and public health epidemiologists.

We developed a real-time PCR screening assay using automated nucleic acid extraction from specimens which specifically detects DNA from *C. botulinum* types A, B, E, F, and *C. baratii* F (BoNT real-time PCR). The real-time PCR can be utilized directly on stool, rectal swabs, and enema specimens and results can be obtained in <5 hrs. This new assay was evaluated with SMB and culture on 61 clinical specimens received at the Wadsworth Center, New York State Department of Health (NYSDOH) over a 10-year period. This study was designed to determine if this new multiplex real-time PCR assay is an acceptable screening tool that can be used by public health and/or clinical laboratories for rapid detection of botulism cases.

Materials and Methods

Bacterial strains

All strains utilized in the testing including *C. botulinum* and *C. baratii* strains were obtained from the culture collection at the Wadsworth Center, New York State DOH or the American Tissue Type Culture Collection (ATCC). *C. botulinum* and *C. baratii* were grown at 37°C overnight on sheep blood agar plates or CDC anaerobe agar plates. An anaerobic environment was created using Gas Pack jars utilizing AnaeroGen (Oxiod) or an anaerobic chamber (Coy Labs).

C. botulinum and *C. baratii* culture from stool specimens

Stool samples were cultured by inoculation to *C. botulinum* Isolation agar (CBI) [11]. Plates were incubated for at least 48 h at 37°C. For isolation of *C. botulinum*, lipase positive colonies were picked and identified using conventional biochemical reactions. *C. baratii* was isolated by picking colonies that showed a characteristic narrow zone of lecithinase on CBI. Toxin production of isolates was tested by cultivation of the organism in Trypticase-Peptone-Glucose-Yeast-extract (TPGY) broth [12] for 48h. The broth was filtered by passing through a 0.45 micron filter. SMB was performed on filtrates in the same manner as stool samples. Selected stool specimens were enriched by inoculation of stool in TPGY. Broths were incubated in an anaerobic atmosphere for 48h at 37°C.

Extraction of clinical specimens

Clinical stool specimens were obtained from the NYSDOH Bacteriology Laboratory. Pretreatment to remove solid materials from stool specimens was required before performing the DNA extraction as per the manufacturer. Stool specimens and environmental samples were diluted in Tris EDTA buffer to obtain 200ul of liquid content, mixed thoroughly and heat treated at 95°C for 30min. The specimen was then centrifuged for 10s at 14,000 RPM and the supernatant was recovered. The supernatant was utilized for nucleic acid extraction by either manual or automated methods. Manual extraction was performed using the Master Pure complete DNA and RNA Purification Kit (Epicentre Technologies) with the modification of a 30min lysis step followed by filtering in a spin module column (Bio 101, Inc.) to remove un-lysed spores.

Automated DNA extraction of specimens was performed utilizing the MagNA Pure LC and Total Nucleic Acid Extraction Kit I. (Roche Life Sciences). Kit I contains wash buffers for removing PCR inhibitors, salts and proteins, lyses and binding solution, proteinase K, magnetic beads for binding of DNA and an elution buffer. Post extraction filtering in a spin module column was also performed as stated above.

BoNT real-time PCR primer and probes

The BoNT real-time PCR assay targets the toxin genes of *C. botulinum* toxin types A, B, E, F, and *C. baratii* F. Primers and probes were designed using Primer Express v.3.0 (Life Technologies). All primer and probes were manufactured by Integrated DNA Technologies, Inc. (Table 1). GenBank accession numbers for the original sequences used to generate these primer and probe sets in Primer Express are: X73423-toxin type A; AF295926-toxin type B; AB039264-toxin type E; X81714-toxin type F; and X68262-baratii toxin type F.

BoNT real-time PCR

Both of the multiplex reactions consisted of 25-µl reaction volumes and utilized LightCycler FastStart DNA Master Hybridization Probes kit (Roche Life Sciences). Each reaction mixture had the final concentration of the following reagents: 1×LightCycler FastStart DNA Master Hybridization Probes mix, 4mM MgCl2, 450nM forward and reverse primers, 125nM probes, and 5µl of sample volume.

All real-time PCR reactions were performed using the ABI 7000 or 7500 Fast Sequence Detection System with SDS software version 1.4.0 (Thermo Fisher). The real-time PCR reactions were performed using standard cycling conditions that included a 95°C denaturation for 10min, followed by 45 cycles of 95°C for 15s and 60°C for 60s, with no passive reference dye utilized. Each run was analyzed separately for each probe dye utilized.

Sensitivity testing

The analytical sensitivity of each primer and probe set within the multiplex reactions was determined for both cultured isolates and clinical stool matrix. Culture and matrix sensitivity sample preparation, purification and real-time PCR analysis was completed in triplicate for each target primer and probe set. A heavy suspension was made for each toxin type from organism grown overnight at 37°C on sheep blood agar or CDC anaerobe agar plates under anaerobic conditions. The suspension was then serially diluted and 200ul of

Table 1: Primers and Probes of BoNT real-time PCR.

Target	Oligo	Length (bp)	Sequence
A	Forward	24	TTT GGTTTTGAGGAGTCACTT GAA
A	Reverse	26	CATGTGCTAATGTTACTGCTGGATCT
A	Probe	32	6FAM TTGATACAAATCCTCTTTTAGGTGCAGGCAAA BHQ-1
B	Forward	30	AACACAAACATTGCTAGTGTAACTGTTAAT
B	Reverse	19	AAACTGGCCCAGGTCCAAA
B	Probe	34	JOE AATTAATCAGTAATCCAGGAGAAGTGGAGCGAAA BHQ-2
E	Forward	23	TTATTTTTTGTGGCTTCCGAGAA
E	Reverse	26	AAGTCCAGGTGCTGATTCACTATTAA
E	Probe	32	6FAM ACTCCTAAAGAAATTGACGATACAGTAACTTC BHQ-1
F	Forward	23	CAGGTGGAGTTTATGACCCAAGT
F	Reverse	32	TGCAATAAATGATTCTGTACTACTGTTATACC
F	Probe	36	JOE TTTTGGATCAATTAATATCGTGACATTTTCACCTGA BHQ-2
C. baratii F	Forward	25	TGG TTT ATA CGG AGC TAA AGG TGT T
C. baratii F	Reverse	30	GAC CTC CAA AGG TTA TAA ACT CTT CTA TTT
C. baratii F	Probe	28	ROX NHS Ester AGA AGT AGA TCA GGG TGC CCT CAT GGC A Iowa Black RQ

Table 2: Limit of Detection Studies for *C. botulinum* and *C. baratii*for heat lysed, and nucleic acid extracted using manual and automated methods.

Clostridium toxin type DNA	Heat-Lysed Bacteria (CFUs)	Manual Extraction from bacteria(CFUs)	Manual Extraction from Stool (CFUs)	Automated Extraction from Stool(CFUs)
A	11	12	31	58.5
B	4	20	2	104
E	1	73	10	32
F	19	53	295	40
C. baratii F	56	22	150	123

Table 3: Human Clinical botulism test from 2003-2013.

Year	Cases	Clinical and Food Samples	Positive samples
2003	2	2	2
2004	5	9	1
2005	1	4	0
2006	1	2	2
2007	10	40	8
2008	5	5	2
2009	4	5	1
2010	1	2	2
2011	6	8	4
2012	15	57	17
2013	11	32	7
Total	61	166	46

each suspension was utilized for analytical sensitivity studies or added to approximately 400ul of a stool specimen for clinical sensitivity studies. For bacterial heat kill studies, the suspensions were subjected to heat treatment at 115°C for 30min. For clinical sensitivity studies, either a manual or automated extraction was performed as described above. To calculate colony forming units (cfu's) in each dilution and PCR reaction, 100ul of each dilution was placed on sheep blood agar plates and incubated at 37°C overnight. Spread plating and colony counts prepared for each day of testing from each organism. All of the final nucleic acid preparations in the real-time PCR reactions were analyzed in triplicate. The lowest dilution concentration that consistently generated positive amplification crossing the Cycle Threshold (Ct) in 100% of the replicates was considered the sensitivity limit for that target.

Specificity testing

The specificity of the multiplex assays was determined by testing eight known strains of *C. botulinum* (Type A, Type B, Type E, Type F) and *C. baratii* and other non-*botulinum* toxin producing Clostridia such as *C. perfringens*, *C. histolyticum*, *C.sporogenes*, *C.sordellii*, and *C. tetani.* Additionally, 32 other species and 4 viral isolates that were either genetically related, or cause similar gastrointestinal illness, or are normal flora were also tested including: *Bacteroiodesfragilis*, *Bordetella pertussis*, *Borreliaburgdorferi*, *Corynebacterium diphtheriae*, *Escherichiacoli* O157 H7, *Escherichia coli* TN11, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Eggerthellalenta*, *Haemophilusinfluenzae*, *Lactobacillus acidophilus*, *Legionella pneumophila*, *Leptospirainterrogans*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Varicella Zoster Virus*, Herpes Simplex Virus-1, and Herpes Simplex Virus. Also included for specificity testing since this assay could be used for bio-threat agent testing were other select agent organisms such as *Bacillus anthracis*, *Brucella melitensis*, *Francisellatularensis*, *Yersinia pestis*, and Vaccinia virus.

Table 4: Summary of clinical cases received for botulinum neurotoxin-producing *C. botulinum* and *C. baratii*testing.

	Date	Sample Type	PCR Result	Bioassay Result	Isolate
1	09/17/03	Infant Stool	B	Insufficient material	*C.botulinum* B
2	09/23/03	Infant Stool	B	B	Cancelled[a]
3	02/09/04	Stool	Neg.	Neg.	Cancelled
4	07/14/04	Infant Stool	B	B	*C. botulinum* B
5	09/27/04	Stool	Neg.	Insufficient material	Culture Neg.
7	12/23/04	Stool	Neg.	Neg.	Culture Neg.
8	03/03/05	Infant Stool	Neg.	Insufficient material	Culture Neg.
9	03/03/06	Infant Stool	B	Insufficient material	*C. botulinum* B
10	05/02/07	Infant Stool	B	B	*C. botulinum* B
11	05/14/07	Adult Stool	*C. baratii* F	F	*C. baratii* F
12	05/25/07	Adult Stool	*C. baratii* F	F	*C. baratii* F
13	06/06/07	Infant Stool	B	B	Culture Neg.
14	07/24/07	Infant Stool	B	B	*C.botulinum* B
15	08/29/07	Adult Stool	Neg.	Insufficient material	Culture Neg.
16	10/02/07	Infant Stool	B	Insufficient material	Culture Neg.
17	10/05/07	Adult Stool	Neg.	Neg.	Culture Neg.
18	10/12/07	Infant Swab	B	Insufficient material	*C.botulinum* B
19	11/30/07	Adult Stool	Neg.	Neg.	Culture Neg.
20	4/30/08	Stool	Neg.	Neg.	Culture Neg.
21	7/11/08	Infant stool	Neg.	Insufficient material	Culture Neg.
22	7/23/08	Infant Stool	B	Insufficient material	Culture Neg.
23	8/9/08	Rectal Swab	Neg.	Insufficient material	Culture Neg.
24	8/29/08	Infant stool	B	B[b]	*C. botulinum* B
25	3/03/09	Infant stool	A	A	*C. botulinum* A
26	4/20/09	Child stool	Neg.	Neg.	Culture Neg.
27	9/20/09	Infant stool	Neg.	Neg.	Culture Neg.
28	12/21/09	Stool	Neg.	Neg.	Culture Neg.
29	4/20/10	Infant swab	B	B	*C. botulinum* B
30	4/22/11	Infant stool	B	B	*C. botulinum* B
31	4/22/11	Stool	Neg.	Neg.	Culture Neg.
32	4/28/11	Enema wash	Neg.	Neg.	Culture Neg.
33	4/29/11	Stool	A + B[c]	A	*C.botulinum* A(b)[d]
34	8/9/11	Stool	Neg.	Insufficient material	Culture Neg.
35	9/3/11	Infant stool	B	Neg.	*C. botulinum* B
36	3/23/12	Stool	B	B	*C. botulinum* B
37	4/3/12	Stool	A	Insufficient material	Culture Neg.
38	4/12/12	Rectal swab	A + B	Insufficient material	*C.botulinum* A(b)
39	5/16/12	Infant stool	B	Insufficient material	Culture Neg.
40	5/30/12	Stool	Neg.	Neg.	Culture Neg.
41	5/30/12	Infant stool	A + B	A	*C.botulinum* A(b)
42	5/31/12	Infant stool	Neg.	Insufficient material	Culture Neg.
43	6/15/12	Infant stool	B	Insufficient material	*C. botulinum* B
44	6/26/12	Enema wash	A + B	Cancelled	*C.botulinum* A(b)
45	6/26/12	Infant stool	B	Insufficient material	*C. botulinum* B

46	8/17/12	Child stool	Neg.	Cancelled	Culture Neg.
47	9-14-12	Infant stool	Neg.	Cancelled	Culture Neg.
48	10/23/12	Infant stool	B	B	*C. botulinum* B
49	11/14/12	Infant stool	Neg.	Neg.	Culture Neg.
50	11/29/12	Adult stool	Neg.	Insufficient material	Culture Neg.
51	01/08/13	Rectal swab	Neg.	Insufficient material	Culture Neg.
52	01/18/13	Infant swab	B	Insufficient material	*C. botulinum* B
53	2/13/13	Infant stool	B	Insufficient material	*C. botulinum* B
54	4/9/13	Infant stool	Neg.	Neg.	Culture Neg.
55	6/6/13	Child stool	Neg.	Neg.	Culture Neg.
56	6/25/13	Infant stool	Neg.	Insufficient material	Culture Neg.
57	7/10/13	Infant stool	Neg.	Insufficient material	Culture Neg.
58	10/22/13	Infant stool	Neg.	Insufficient material	Culture Neg.
59	11/20/13	Infant stool	Neg.	Insufficient material	Culture Neg.
60	12/18/13	Adult stool	*C.baratii* F	Insufficient material	*C. baratii* F
61	12/21/13	Infant stool	B	Insufficient material	*C. botulinum* B

[a]Cancelled testing resulting from duplicate specimens received or negative PCR result.
[b]Specimen received from the Connecticut Department of Health for real-time PCR testing. SMB testing performed by CDC.
[c]A + B DNA from BoNT types A and B are detected.
[d]A(b) Isolate contains both A and B genes but only expresses BoNT type A toxin.

Approximately 106 genome copies of DNA purified from these organisms were extracted using the Epicentre Master Pure Total Nucleic Acid Extraction Kit and tested in both multiplex reactions.

Standard Mouse Bioassay

SMB was performed according to the procedure of Dowell [13]. Briefly, an equal volume of gelatin diluent (w/v) was added to stool. The stool was thoroughly mixed, and refrigerated overnight at 5oC. The stool extract was centrifuged (12,350 x g, 5°C, 20min) and the supernatant tested for BoNT. The supernatant was split, and an aliquot was neutralized with type specific antitoxin. (Antitoxin was provided by the CDC, Atlanta, GA). Intraperitoneal injection of Swiss Webster mice was performed using either an un-neutralized or neutralized extract. Mice were injected in pairs and observed for at least 4 days. Specimens were positive for the presence of BoNT if the mice that received the neutralized extract survived, and mice that received the un-neutralized extract either developed signs of botulism intoxication or expired.

Evaluation of real-time PCR Compared to SMB and culture for clinical specimens. Sixty-one clinical cases of suspect foodborne and infant botulism were received at the NYSDOH over the ten-year period from 2003-2013. Each specimen was tested, if sufficient quantity present for each method, by BoNT real-time PCR, SMB, and culture.

Results

Sensitivity and specificity

The Limit Of Detection (LOD) of the BoNT real-time PCR assay targets were determined utilizing a dilution series for each target of heat-killed bacteria as well as purified genomic *C. botulinum* DNA that utilized a manual extraction of nucleic acid. Positive results were required in 100% of samples for a determination of the LOD. Table 2 liststhe LOD for each real-time PCR assay target in cfu's. The analytical sensitivity for this assay was determined to be <100 gene copies for all targets. Linearity analysis was performed for all targets and the efficiency for all targets was determined to be greater than 90% (data not shown).

Sensitivity of the assay and the extraction methods were determined through spiking stool specimens with a dilution series of *C. botulinum* and *C. baratii* organisms. The results show that the BoNT real-time PCR targets are sensitive and detect <300 organisms for all 5 targets. The automated extraction method utilized proved to have comparablerecovery (or in some cases superior recovery) to that of the manual extraction performed for each *C. botulinum* and *C. baratii* toxin type (Table 2).

Specificity

In order to determine the specificity of the assay, an extensive number of bacteria and viruses were tested using the multiplex real-time PCR assays. Thirty-seven strains of bacteria and 4 viruses were extracted and tested in duplicate in the multiplex real-time PCR assays. The organisms and viruses tested include pathogens that could be present in stool, cause similar symptomlogy, as well as near neighbors such as closely related *Clostridium spp*. All organisms and viruses tested negative (including the closely related Clostridial strains such as *C. histolyticum*, *C. sporogenes*, *C. sordellii*, and *C. tetani*). Strains of *C. botulinum* and *C. baratii* tested negative for all other targets other than the appropriate toxin gene expected including the other bio-threat agents tested. There was no cross reactivity found between *C. botulinum* toxin type F and *C. baratiitoxin* type F.

Botulinum toxin testing in the Public Health Laboratory

Table 3 depicts the number of specimens and samples per year that our public health laboratory has received from 2003-2013. The number of clinical cases varied during this time period from a

Table 5: Summary comparing BoNT real-time PCR, SMB and culture results.

	Final Identification	BoNTreal-time PCR Result	SMB Result (% confirmed)	Culture Result (% confirmed)
C. botulinum B	22	22 (B)	9 (41%)	17 (77%)
C. botulinum A	6	6[a] (A; A + B)	3 (50%)	5 (83%)
C. baratii F	3	3 (F)	2 (67%)	3 (100%)
Positive		31	14	25
Neg	30	30	15	34
Total	61	61	29[c]	59[b]

[a]To date all specimens PCR positive for A and B toxin genes have exhibited neurotoxin A activity.
[b]Two cultures were cancelled.
[c]28 cases had insufficient material to test; 1 specimen was sent to CDC; three tests were cancelled.

low of 1 case per year to a high of 11 cases in 2012. A total of 61 samples were received during this time. In addition to the clinical specimens received in the laboratory, there were numerous food and environmental samples associated with these investigations that were implicated as potential causes of illness. A total of 46 of those samples including clinical specimens as well as food and environmental samples were positive for C. botulinum/*C. baratii* toxin or toxin DNA. Validation testing was also performed on numerous food and environmental samples that included different pre-processing steps for food samples to determine the applicability of these assays for various sample matrices (data not shown).

Evaluation of real-time PCR Compared to SMB and culture for clinical specimens. Between 2003 and 2013, our laboratory received a total of 61 cases of suspect botulism with an appropriate specimen submission. Of these, 31 (51%) were determined to be positive by at least one method (Table 4). BoNT real-time PCR was positive for 100% of positive cases. These included 22 *C. botulinum* type B, 6 *C. botulinum* type A and A+B, and 3 *C. baratii* type F results. To date, all strains received that have been identified as BoNT real-time PCR positive for types A and B have only expressed type A toxin. All clinical specimens received during this 10-year period were negative for type E DNA. Testing of environmental samples associated with an ongoing Great Lake botulism outbreak in wildlife identified Type E toxin DNA in these samples [14] however, there have been no associated clinical specimens during this time period. Of note, 30 cases were received with insufficient quantities to test by SMB.

Table 5 summarizes a comparison of the real-time PCR assays, SMB, and culture results for the 10-year period. Overall, the real-time PCR multiplex assays had a detection rate of 100% in comparison to 81% culture confirmations of the specimens. The SMB had a much lower positivity rate of 45% for all toxin types combined. In addition, the SMB was only able to confirm a negative result for 50% of the specimens tested.

Discussion

The BoNT multiplex real-time PCR assays are highly specific and sensitive assays for identifying *C. botulinum* types A, B, E, F, and *C. baratii* F as shown by the limit of detection, sensitivity and specificity studies provided. These assays are extremely useful when specimen volume is limited (e.g., infant stool). In addition, the automated extraction methods developed for this assay improved laboratory workflow, decreased extraction time, removed potential inhibitors that were present in some of the original specimens, as well as provide additional benefit for laboratorians. In cases where multiple specimens and associated samples are sent to the laboratory, automated extraction improves laboratory efficiency and allows for expedited results to be communicated back to the physicians and public health officials.

The ability to test specimens and food samples directly by real-time PCR provides results in a much faster manner than relying on only the SMB and culture. In addition to the 4-5 days needed once mice are injected with specimens and anti-toxin, processing of stools for SMB analysis involves lengthy (>8hr) incubation and there are often other bacteria present in stools that can result in death of the mice and confound assay results. When using the SMB, processed specimen is bound with the various antitoxins resulting in mice surviving from one anti-toxin type indicated the type of toxin present in the specimen. Mice injected with processed and bound specimen must be observed for at least 2 days for final results, however, specimens that contain low concentrations of toxin or other bacteria can often cause delays in the SMB (2-5 days) or result in repeat testing. Testing results using the multiplex real-time PCR assays can take as little as 4.5 hours from processing to result with less hands-on time for analysts. Culturing *Clostrial* organisms from specimens and food samples can be very time consuming and difficult as there may be many bacterial organisms present in stool and food samples that can out-compete the toxin producing organism of interest. In addition, often there are other non-toxin producing Clostridium that results in additional identification testing that must be performed such as biochemical and SMB. Real-time PCR provides a rapid method to ensure the presence of the toxin gene in a sample or from an organism that is isolated from a sample.

There are a limited number of rapid immunoassays available but the tests can take up to 1½ days to complete, and they are not able to successfully differentiate *C. baratii* toxin type F from *C. botulinum* toxin type F. In addition, the assays can cross react with other substances that can be found in stool or food matrices making this technology difficult to utilize for a rapid screening method for patient testing.

Utilizing real-time PCR for rapid testing of suspect botulinum specimens presents an essential supplement to testing for toxin via the mouse neutralization assay and in many cases may be the only test to obtain results. Performing the SMB for testing of stool specimens can take 2 to 5 days for results and in many cases is inconclusive due to other organisms present in the stool. In many cases of infant botulism, there is often not enough stool for toxin testing as the

patient becomes constipated due to botulinum intoxication. In 30 of 61 cases overall in this extensive study, there was insufficient material for toxin testing via the mouse bioassay (Table 4). In 2 cases of infant botulism, the specimens submitted were very small specimens on a rectal swab, and in both of these cases a positive PCR result was obtained. These findings provide data demonstrating the utility of this method as an effective screen for the detection of BoNT DNA in clinical specimens.

In the human cases identified in our laboratory, since 2003, there have been 22 positive type B specimens, 6 positive type A specimens, 3 positive *C. baratii* type F specimens identified using the real-time PCR multiplex assays, and no E, or C. botulinum type F specimens. These results support the use of these multiplex assays as a screening tool for rapid detection. It should be noted that serum specimens do not provide an appropriate specimen for extraction of bacterial DNA and testing by real-time PCR in botulism cases. Organisms are not present in serum and in limited studies in our laboratory; we have never detected *C. botulinum* DNA in sera (data not shown). The most appropriate specimen type for DNA testing by PCR is the stool specimen.

For the first 4 years of testing from 2003-2007, the multiplex assay that was utilized for initial testing consisted of targets for *C. botulinum* types A,B, E, and F. In 2007, a limited volume specimen was submitted to our laboratory limiting our SMB testing to the trivalent toxins (types A, B, and E antitoxin). The SMB resulted in a delayed positive after several days which also required repeat testing with additional specimen. The real-time PCR for *C. botulinum* types A, B, E, and F was negative. Testing with a supplemental PCR test for *C. baratii* type F revealed a positive for *C. baratii* type F DNA. The SMB cannot distinguish between *C. botulinum* type F and *C. baratii* type F due to the high degree of homology between these toxins. There is also enough homology between type E toxin, type F toxin, and *C. baratii* type F toxin which can result in cross reactivity. A*C. baratii* type F toxin will results in a positive SMB test with type E antitoxin, a component of the trivalent antitoxin which is the cause of the observed delayed positive result in the SMB.

Of the botulism cases reported to the CDC from 1981 to 2002, only 1% was type F. In 69 % of those cases a *C. baratii* was identified [15]. The real-time PCR assays that we developed for detection of *C. botulinum* toxin types E and F DNA do not detect *C. baratii* type F. In 2007, after identifying the 2nd case of *C. baratii* in New York State, this target was subsequently incorporated into the first line multiplex PCR assays and has been included in the multiplex *C. botulinum* panel for the last 8 years. *C. baratii* is also considered a select agent since it produces a *C. botulinum*-like toxin, therefore rapid identification of this agent and toxin is also of importance to public health and security officials.

These multiplex assays have been extremely useful in the rapid identification of botulinum toxin. In many of the cases identified, patients had typical symptomology indicative of botulism and thus the presence of BoNT DNA was not unexpected. We have tested specimens with patients that have exhibited atypical presentations of botulism in which testing is necessary before botulism treatment has been made available to the patient. In these cases, the rapid screening multiplex assays have been extremely important for patient treatment. We have utilized these assays to decrease the number of mice needed based on the identification of specific toxin gene present in the specimen. In addition, rapid identification of particular toxin types is very important and provides critical information for public health officials for epidemiological investigations to determine the source of illness.

While these assays have been very useful public health epidemiological tools, *C. botulinum* and *C. baratii* produce potent neurotoxins that are considered select agents and have been thought to be one of the most likely biological agents used in a bio-threat attack. Rapid detection and the use of automation allows for the testing of large numbers of specimens and samples that could be generated in a potential bio-threat event. As shown in Table 3, botulism cases can generate large number of associated samples that need testing. We have been able to rapidly perform direct testing on these specimens and clinical samples, similar to what could be expected in a bio-threat event. This is the first report of the use of automated nucleic acid extraction combined with the use of multiplex real-time PCR assays for the detection of *C. botulinum* and *C. baratii* toxin DNA. The development of these rapid, high-confidence, cost-effective assay for the major *C. botulinum* neurotoxin types will greatly improve response to clinical intoxication and potential outbreaks and biothreat events.

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