A Quadraplexed Real-Time PCR Assay for Rapid Detection and Differentiation of the *Clostridium botulinum* Toxin Genes A, B, E, and F in Pure Culture and Environmental Samples

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ABSTRACT

Clostridium botulinum is the etiologic agent of botulism, a disease marked by flaccid paralysis that can progress to asphyxiation and death. This species of bacteria produces the most potent toxins known with an LD_{50} in primates of 1-10 ng kg^{-1} of body weight. Because of their potency, these toxins have the potential to be used as biological weapons. Therefore, *C. botulinum* has been classified as a select agent by the United States Centers for Disease Control and is considered to be equally dangerous by other governments. There are four related but antigenically distinct botulinum toxins that cause disease in humans (A, B, E, and F) and these can enter the body via three different routes: inhalation, ingestion, and absorption from wound infections. Ingestion of *C. botulinum* spores by infants has been associated with sudden infant death syndrome. The mouse bioassay is the current gold standard by which toxin type is confirmed. However, this method is expensive, slow, and very labor intensive, taking up to four days to complete. In addition, this assay carries ethical concerns due to the need to sacrifice mice. Commercial biochemical tests have failed in identifying various toxin-producing strains of *C. botulinum*. PCR-based assays have been used extensively for the detection of botulinum toxin-producing bacteria in food, animals, and fecal samples, and recently, to help diagnose disease in humans. Most of these are traditional PCR methods, though assays have been published in recent years that use real-time PCR to target one or more botulinum toxin genes. However, no assay has been published that involves real-time PCR detection of the four human disease-causing toxin genes A, B, E, and F in a single-tube, multiplex reaction. This report describes the development of a real-time PCR single-tube assay that uniquely identifies these four botulinum toxin types responsible for human disease. A total of 83 *C. botulinum* isolates were evaluated in this study, as well as numerous near-neighbors and other bacterial species. Included were isolates which had genes for each of the toxins A, B, E, and F with some natural isolates containing genes for more than one toxin. *C. botulinum* isolates producing the toxins C and D, which do not cause disease in humans, were also included as controls. Results showed that this quadraplexed assay was capable of detecting any of the four toxin genes in a given sample at a sensitivity of about 130-840 fg of genomic DNA. Furthermore, it was able to detect the presence of two, three, or all four toxin genes in a given sample, indicating the lack of type-to-type interference. The test was also functional in the presence of extraneous organic matter commonly found in various environmental samples. This assay could prove to be a useful tool in the rapid identification of a specific type of disease, or the potential toxic threat of a substance to human health.

METHODS

Bacterial Isolates and DNA Extraction

Bacterial isolates used in this study were acquired from American Type Culture Collection (ATCC), the CDC, and the Utah State Department of Health. Isolates were grown anaerobically on Reinforced Clostridial Agar at 37 °C for 3-5 days prior to DNA extraction. DNA was extracted using a Roche MagNA Pure LC system (Roche Diagnostics). DNA concentrations were measured using a TBS-380 (Turner Biosystems) and a PicoGreen Quant-it kit (Invitrogen).

Optimizing and Quadraplexing the Real-time PCR Assay

Each single reaction was optimized for temperature and cycling conditions followed by an optimization of the four assays together in the quadraplexed format. GE Healthcare illustra Hot Start Mix RTG Master Mix (GE Healthcare) beads were used for all reactions and the sample volume was always 25 µL per reaction. For the quadraplex assay 1 or 2 master mix beads were added to a mixture of 250 nM of each primer and probe for toxin A and 500 nM of each primer and probe for toxins B, E, and F. 1-10 nM of target DNA was used, and PCR-grade H_20 was then added to 25 µL. Thermal cycling conditions were an initial denaturization at 95°C for 120 sec followed by 40 cycles of 95°C for 12 sec, then 62°C for 20 sec, and 72°C for 15 sec. A positive signal was determined by the crossing of a fluorescence threshold of 15 before cycle 40.

Environmental Samples

To ascertain the effectiveness of this assay on environmental samples as well as pure culture samples, 400 mg each of soil, sausage, and vegetable matter (Beech Nut® Mixed Vegetables Baby Food) were inoculated individually with 100 µL of concentrated (about 10^8 CFUs mL^{-1}) cultures of *C. botulinum* isolates known to contain the A, B, E, and F toxin genes. For negative controls, a concentrated culture of an isolate containing the C toxin gene and 100 µL of TE buffer were used. After the inoculation, a bead beating protocol was used to homogenize each sample, followed by DNA extraction with the Roche MagNA Pure LC.



RESULTS

Table 1. Summary of PCR data

Species	Toxin A	Toxin B	Toxin E	Toxin F	Toxins A/B	Purported C isolates	Purported D isolates	Unknown (likely C or D)	Total
C. botulinum	37	13	5	4	6	9	3	6	83
C. argentinense	0	0	0	0	-	-	-	-	2
C. beijerinckii	0	0	0	0	-	-	-	-	1
C. haemolyticum	0	0	0	0	-	-	-	-	1
C. perfringens	0	0	0	0	-	-	-	-	1
C. subterminale	0	0	0	0	-	-	-	-	1
C. tetani	0	0	0	0	-	-	-	-	1

Specificity Testing

The quadraplexed assay correctly identified 65 isolates containing the toxin types A, B, E, and F, while showing no amplification of other *C. botulinum* toxin types or near neighbors (Table 1). The assay could detect each toxin separately as well as in every combination of two, three, or all four toxin types simultaneously (Fig. 1).

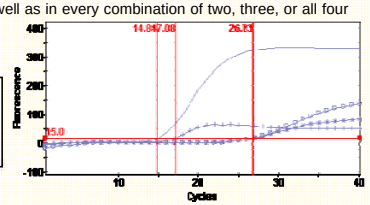

Fig. 1 Simultaneous Detection of Toxins A, B, E, and F.

The quadraplexed assay detected all four toxins concurrently. (——) toxin A, (+–+–+) toxin B, (–•–•–) toxin E, (–••–••–) toxin F.

Sensitivity Testing

Isolates representing each of the toxins A, B, E, and F were used to test the sensitivity of the singleplex and quadraplexed assays. For each isolate, 10-fold serial dilutions were made of the purified genomic DNAs. For the singleplex assays, the threshhold sensitivities for each toxin type were: type A 130 fg, type B 70 fg, type E 8.4 fg, and type F 8.4 fg (Fig. 2). For the quadraplexed assay, the threshold sensitivities for each toxin type were: type A 130 fg, type B 700 fg, type E 840 fg, and type F 840 fg.

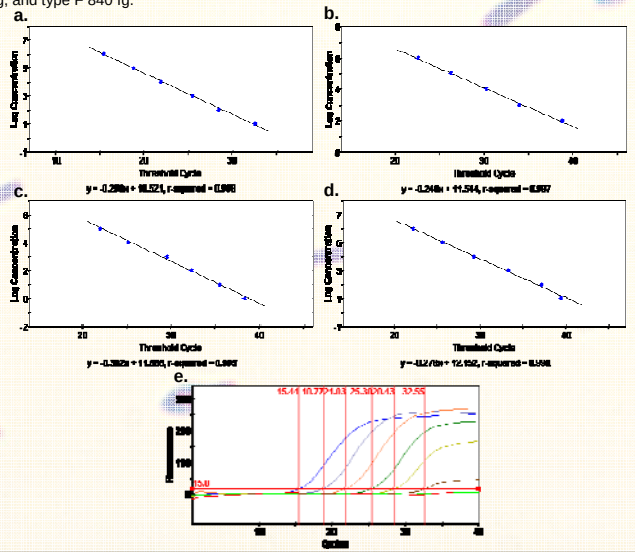

Fig. 2. Sensitivity Testing

Standard curves derived from 10-fold dilutions of purified genomic DNAs for *C. botulinum* isolates containing the toxin genes for toxin A (a), toxin B (b), toxin E (c), and toxin F (d). An example of the sensitivity test that produced the standard curve for toxin A (e).

Environmental Samples

DNA extracted from each environmental sample was interrogated with the quadraplexed assay. It was found that the assay could differentiate between toxin types in the vegetable matter and the sausage with the uninoculated controls showing no signal. Soil contains a high concentration of natural PCR inhibitors. In the soil samples, toxin type A and B were correctly detected, and slight amplification of toxin types E and F were often seen, but they did not cross threshold (Fig. 3). Environmental samples interrogated with the singleplex reactions were able to detect the respective toxin types in all samples, including those containing soil.

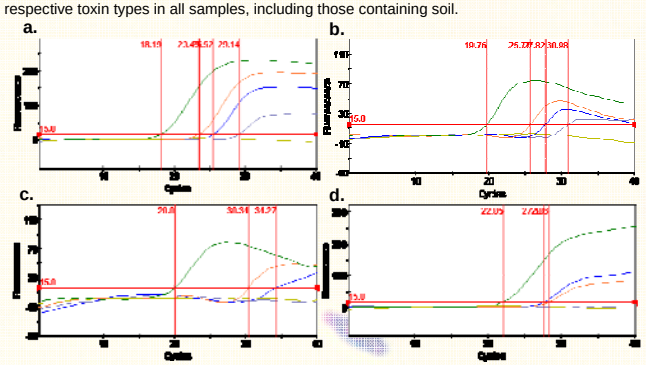

Fig. 3. Detection of the Presence of Toxin Genes in Environmental Samples

The detection of toxin A (a), toxin B (b), toxin E (c), and toxin F (d) after DNA was extracted from environmental samples inoculated with *C. botulinum*. Samples tested included genomic DNA control (green), sausage (orange), vegetable matter (blue), soil (gray), and a no template control (tan).

Toxin Type Variance from ATCC and the CDC

Of the isolates tested using the described assay, those obtained from the ATCC and the CDC consisted of a wide diversity with multiple isolates purportedly representing each of the A, B, C, D, E, and F toxin types. In most cases the purported toxin type matched that detected by the described assay; there were only six discrepancies (Table 2). Other PCR tests were used to verify that the quadraplex assay detected the toxin genes correctly.

Table 2. *Toxin Type Variance from ATCC and the CDC*

Isolate Number	Purported Toxin Type	Detected Toxin Type
ATCC 438	C	A
ATCC 17786	E	A
CDC 10305 T-5	A	A & B
CDC 10306 A-2	A	A & B
ATCC 43757	B & F	B
ATCC 43758	B & F	B

CONCLUSIONS

- **This assay was able to detect and accurately differentiate the genes for botulinum toxin types A, B, E, and F.**
- **Detection sensitivities were in the range of 1-100 genome equivalents.**
- **Toxin genes could be detected in environmental samples, even those known to contain large amounts of nucleic acids and natural PCR inhibitors.**
- **This assay correctly identified misreported toxin types in six *C. botulinum* isolates, while confirming many others.**
- **This assay may be a cheaper, faster, and more ethical alternative to the mouse bioassay for these toxin types.**