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Detection of *Clostridium botulinum* Type C Cells in the Gastrointestinal Tracts of Mozambique Tilapia (*Oreochromis mossambicus*) by Polymerase Chain Reaction

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ABSTRACT: We established a method of directly detecting *Clostridium botulinum* type C cells, while minimizing spore detection, in the intestinal contents of Mozambique tilapia (*Oreochromis mossambicus*). This technique involved extraction of predominantly cellular DNA from tilapia intestinal tracts and used a polymerase chain reaction assay to detect presence of type C_1 toxin gene. We consistently detected *C. botulinum* type C cells in tilapia gastrointestinal contents at a level of 7.5×10^4 cells per 0.25 g material or 1.9×10^3 cells. This technique is useful for determining prevalence of the potentially active organisms within a given population of fish and may be adapted to other types of *C. botulinum* and vertebrate populations as well.

Key words: *Clostridium botulinum* type C, diagnostic technique, polymerase chain reaction, tilapia.

In the last decade, type C avian botulism was documented as the cause of outbreaks involving fish-eating birds at the Salton Sea in southern California (USA) (Rocke et al., 2004). Fish are suspected of harboring the bacteria in their gastrointestinal tracts, where toxin is subsequently formed, perhaps under certain stressful conditions. In the past, detection of *Clostridium botulinum* within an animal has involved inoculation of growth media with tissue, intestinal contents, or fecal material, followed by DNA extraction of the media and finally polymerase chain reaction (Szabo et al., 1994; Hielm et al., 1996, 1998; Kimura et al., 2001; Fach et al., 2002). This method of sample preparation does not distinguish between spores, the resting phase, and cells, the active phase of the organism. When investigating cases of suspected botulism, it is important to identify cells, rather than spores, as evidence of a primary toxin source because cells alone produce toxin and spores are ubiquitous and readily detected. This holds true for wildlife epizootics, and these techniques could be adapted to human and livestock cases as well.

In this study, we established a method for detecting cells of *C. botulinum* type C in Salton Sea Mozambique tilapia (*Oreochromis mossambicus*) intestines for the purpose of population surveys. We used a DNA extraction and polymerase chain reaction (PCR) (Williamson et al., 1999) combination that targets the C_1 toxin gene.

We collected intestines from Mozambique tilapia taken by gill net from the Salton Sea. Intestinal samples were shipped on dry ice to the US Geological Survey, National Wildlife Health Center (Madison, Wisconsin, USA) for further processing. We thawed the samples and aseptically expressed each individual's gut contents into sterile 30-ml Corex tubes (Fisher Scientific, Suwanne, Georgia, USA) in a biologic safety cabinet. The samples were then covered with parafilm and incubated at 4 C overnight for toxin extraction for a related study. The following day, the samples were centrifuged at 4 C and 12,000 × G for 10 min in an IEC MP4R centrifuge (International Equipment Company, Needham Heights, Massachusetts, USA) (Ausubel et al., 1992). We removed the resulting supernatants. We extracted cellular DNA from 0.25 g gut sediment using the UltraClean™ Soil DNA Isolation Kit as per manufacturer's instructions (MoBio Laboratories Inc., Solana Beach, California, USA). Additional purification of the DNA was performed by cetyltrimethylammonium bromide (CTAB)

extraction as described in Ausubel et al. (1992). The samples were then precipitated with ethanol, resuspended to 100 μl in deionized water, and stored at −20 C.

The DNA samples derived from tilapia gastrointestinal sediment were thawed and analyzed for the presence of vegetative *C. botulinum* type C using a seminested PCR modified from the protocol described in Williamson et al. (1999). This PCR targets a portion of the gene that encodes the light chain of type C toxin and is specific to the type C_1 gene. Polymerase chain reaction was performed using a DNA thermal cycler (model 480, Perkin-Elmer, Boston, Massachusetts, USA) and utilized the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Reactions (100 μl) were performed in 0.5-μl thin-walled, polypropylene PCR tubes (PGC Scientifics, Gaithersburg, Maryland, USA) under a 75-μl layer of light mineral oil (Sigma, St. Louis, Missouri, USA). In the initial amplification (30 cycles), forward primer ToxC-625 ($5'^{632}$CTAGACAAGGTAACAACTGGGTTA$^{655}3'$) and reverse primer ToxC-1049R ($5'^{1057}$AATAAGGTCTATAGTTGGACCTCC$^{1033}3'$) were used (Genbank accession number X53751ver.X53751.1) (University of Wisconsin Biotechnology Center, Madison, Wisconsin, USA). Each reaction contained 1× Expand High Fidelity PCR buffer, 3.75 mmol/l MgCl, 0.2 mmol/l of each deoxynucleoside triphosphate (dNTP) (Roche Molecular Biochemicals), 0.6 μmol/l of each primer, 0.875 U expand polymerase, 1.5% polyvinylpyrrolidone-40 (PVP-40), 0.0005% bovine serum albumin (BSA), and 2.5 μl sample DNA. Seminested amplification reactions (15 cycles) were performed using a forward primer ToxC-625 and reverse primer ToxC-850R ($5'^{857}$GAAAATCTACCCTCTCCTACATCA$^{834}3'$) (Genbank accession number X53751ver.X53751.1), and 5.0 μl of the initial amplification reaction mixture was used. Each reaction was heated to 80 C for 5 min prior to addition of dNTP's, the second primer, and the expand polymerase. An amplification profile of 95 C for 1 min, 55 C for 1 min, and 72 C for 2 min followed by a final extension of 72 C for 10 min was used for both the initial and the seminested reactions. A positive and a negative, or no template, control were included in every experiment. For the positive-control reaction, 150 pg of DNA from a *C. botulinum* type C strain, isolated from wetland sediment taken from northern California (96-SAC), was used in the initial reaction, and 0.25μl of the initial reaction mixture was used in the seminested PCR. In addition, a set of initial sample reactions spiked with 150 pg of 96-SAC was run to show that there was no inhibition with the added control DNA. Ten microliters of the initial spiked amplification reactions and the seminested amplification reactions were size fractionated through 2% agarose gels (Invitrogen, Life Technologies Corporation, Carlsbad, California, USA) in 1× TAE buffer (40 mM Tris acetate, 1 mM ethylenediaminetetraacetic acid). Gels were stained in 0.01% Vistra Green (Amersham Biosciences, Sunnyvale, California, USA) for 15 min and products were then visualized using the Fluorimager System (Molecular Dynamics, Sunnyvale, California, USA).

We tested the sensitivity of this method by seeding tilapia gastrointestinal sediment with known numbers of *C. botulinum* type C vegetative cells isolated from a Salton Sea tilapia (SS36). We also seeded gastrointestinal sediment with *C. botulinum* type C spores (96-SAC) in order to confirm that our method minimized spore lysis. The SS36 cell suspensions and 96-SAC bacterial spore suspensions were prepared as described in Williamson et al. (1999). The concentrations of cell and spore suspensions (cells or spores/μl) were determined by microscopic counting using a Petroff-Hausser chamber (AO Scientific Instruments, Buffalo, New York, USA). We aliquoted 0.25 g tilapia gastrointestinal sediment, having previously tested negative for the C_1 gene, into microcentrifuge tubes containing dry garnet beads (MoBio Lab-

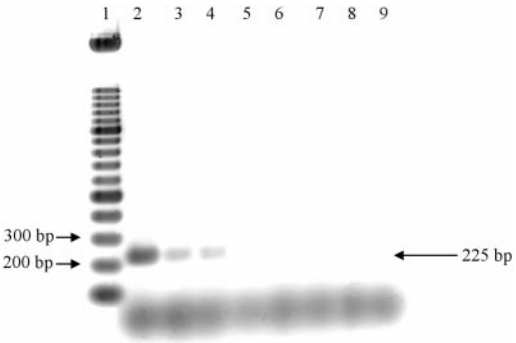

FIGURE 1. Agarose gel depicting amplification of type C toxin gene from DNA extracted from tilapia gastrointestinal contents seeded with multiple dilutions of *Clostridium botulinum* type C cells (SS36). Lanes: 1, 100-bp DNA ladder (Roche); 2, 96-SAC positive control; 3, 1.0×10^5 cells; 4, 7.5×10^4 cells; 5, 5.0×10^4 cells; 6, 2.5×10^4 cells; 7, 1.5×10^4 cells; 8, 1.0×10^4 cells; 9, no template control.

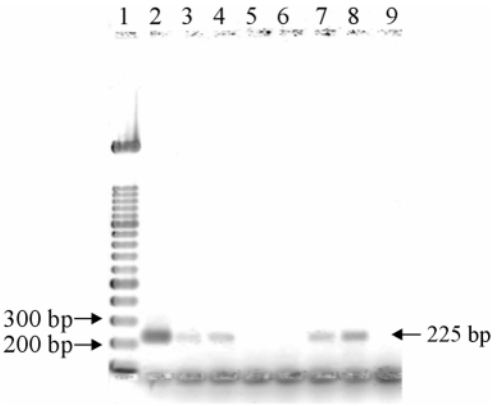

FIGURE 2. Agarose gel depicting amplification of type C toxin gene from DNA extracted from tilapia gastrointestinal contents seeded with multiple dilutions of *Clostridium botulinum* type C cells (SS36). Lanes: 1, 100-bp DNA ladder (Roche); 2, 96-SAC positive control; 3, 1.0×10^5 cells; 4, 7.5×10^4 cells; 5, 5.0×10^4 cells; 6, 2.5×10^4 cells; 7, 1.5×10^4 cells; 8, 1.0×10^4 cells; 9, no template control.

oratories, Inc.). Six aliquots were seeded with decreasing numbers of cells: 1×10^5, 7.5×10^4, 5×10^4, 2.5×10^4, 1.5×10^4, or 1.0×10^4 cells. Ten aliquots were seeded with decreasing numbers of spores, 1×10^6, 9×10^5, 8×10^5, 7×10^5, 6×10^5, 5×10^5, 4×10^5, 3×10^5, 2×10^5, or 1×10^5 spores. Cell or spore stock was added directly to the gastrointestinal sediments, which were then mixed by hand. The samples were centrifuged at 12,000 × G for 10 min (Eppendorf 5417C, Brinkmann, Westbury, New York, USA), the supernatants were removed, and the sediments underwent immediate DNA extraction and purification as described above. The DNA was again resuspended in deionized water to a final volume of 100 μl. Polymerase chain reaction was then performed as described above.

We consistently detected *C. botulinum* type C in 0.25-g gastrointestinal sediment samples seeded with 7.5×10^4 cells when 2.5 μl of a total volume of 100 μl sample DNA was used (Fig. 1). In four consecutive PCRs, all five levels of cell seeding were occasionally detectable, even for the lowest number of cells, 1×10^4 (Fig. 2). Spore DNA could not be detected by our method at any of our seeding levels (Fig. 3).

The methods described in this article were used to investigate the prevalence of *C. botulinum* type C cells within the tilapia population at Salton Sea (Nol et al., 2004), and may be used for other fish populations as well. In addition to fish, this technique could potentially be adapted to a variety of

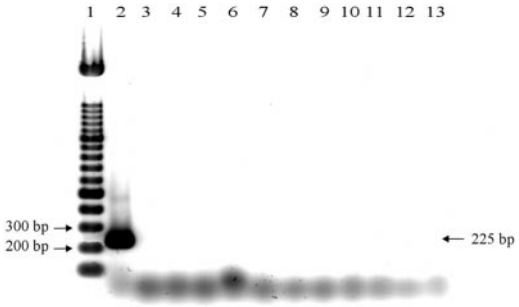

FIGURE 3. Agarose gel demonstrating lack of amplification of type C toxin gene from DNA extracted from tilapia gastrointestinal contents seeded with multiple dilutions of *Clostridium botulinum* type C spores (96-SAC). Lanes: 1, 100-bp DNA ladder (Roche); 2, 96-SAC positive control; 3, 1.0×10^6 spores; 4, 9.0×10^5 spores; 5, 8.0×10^5 spores; 6, 7×10^5 spores; 7, 6.0×10^5 spores; 8, 5×10^5 spores; 9, 4×10^5 spores; 10, 3×10^5 spores; 11, 2×10^5 spores; 12, 1×10^5 spores; 13, no template control.

other species. When dealing with both type C and E avian botulism outbreaks in fish-eating birds, it is useful to know which prey groups harbor *C. botulinum* cells and at what prevalence. Such data could help researchers determine sources of toxin for botulism-affected birds. Furthermore, prevalence of *C. botulinum* cells in fish could also be used to investigate temporal or spatial trends in outbreaks in both fish and fish-eating birds from year to year or in various locations.

Our laboratory achieved reliable detection of 100–500 cells in lake sediment samples seeded with *C. botulinum* type C (Williamson, unpubl. data). In testing gastrointestinal sediment of fish, we ultimately achieved reliable detection of 1.9×10^3 cells. The sensitivity of this method may also be expressed as able to detect 3×10^5 cells/g gastrointestinal sediment. Due to the nature of fish gastrointestinal content samples, we suspect that DNAases and, to a lesser extent, PCR inhibitors lower the sensitivity of the assay. In order to reduce noticeable inhibition, we applied the CTAB procedure to our extraction protocol and used PVP-40 and BSA in our PCR (Ausubel et al., 1992; John, 1992; Koonjul et al., 1999). The purification by CTAB extraction was effective in eliminating the sources of inhibition that were initially preventing consistent detection of the target gene in our samples spiked with 96-SAC DNA. The tradeoff was a reduction in final DNA yield and thus overall detection of the type C_1 toxin gene. In the future, we plan to improve assay sensitivity by experimenting with various reagents or additives that would preserve the integrity of the DNA during and after extraction and also reduce inhibition in the PCR, in order to eliminate the need for additional extraction steps such as CTAB.

Currently, our method for C_1 toxin gene detection in intestinal samples could be used in surveys to aid in epizootiologic investigations. With improvements in sensitivity, a modified assay, without any need for additional enhancement through culture, may be used for diagnostic purposes in individual botulism cases to verify the presence of and possible harborage of toxin-producing *C. botulinum.*

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