

## In Situ Detection of the *Clostridium botulinum* Type C<sub>1</sub> Toxin Gene in Wetland Sediments with a Nested PCR Assay

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**A nested PCR was developed for detection of the *Clostridium botulinum* type C<sub>1</sub> toxin gene in sediments collected from wetlands where avian botulism outbreaks had or had not occurred. The C<sub>1</sub> toxin gene was detected in 16 of 18 sites, demonstrating both the ubiquitous distribution of *C. botulinum* type C in wetland sediments and the sensitivity of the detection assay.**

*Clostridium botulinum* type C is an anaerobic, spore-forming bacterium found naturally in the sediments of lakes and marshes. It is one of seven types of *C. botulinum* (types A to G), which produce serologically distinct neurotoxins that invoke flaccid paralysis and death in humans and other animals (15). Type C<sub>1</sub> botulinum neurotoxin (BoNT/C<sub>1</sub>) is the primary cause of botulism outbreaks in wild waterfowl, and in the last few years, type C botulism has killed an estimated 4 million waterbirds in North America (11).

Limited research has been conducted on *C. botulinum* type C in its natural environment. Investigators previously measured the prevalence of botulinum spores in wetland sediments by incubating samples in enrichment media and performing mouse bioassays to detect botulinum toxin-producing organisms (12, 19). Other investigators have used PCR to detect *C. botulinum* (types A, B, C, E, and F) in sediments (3, 5), but again the bacterial populations were enhanced in media prior to DNA extraction and PCR. Since culture enhancement of sediment samples can result in competition between microbial populations that may inhibit the growth of the target organism (13), it precludes quantitative analysis of naturally occurring populations. In this paper, we describe a procedure for detection of the BoNT/C<sub>1</sub> gene in wetland sediments without prior enrichment.

Sediment samples were collected during avian botulism outbreaks at three wetland complexes: Klamath National Wildlife Refuge (NWR) (Willows, Calif.), Sutter NWR (Willows, Calif.), and Kulm Wetland Management District (WMD) (Kulm, N.D.). The wetlands sampled were classified as outbreak (confirmed bird mortality due to type C botulism) or nonoutbreak (no evident bird mortality) wetlands. Approximately 100 g from the top 10 cm of bottom sediment was collected and then frozen at –20°C within 4 to 6 h of collection. Total DNA was isolated from 0.25 to 0.5 g of sediment by the extraction procedure described by Tebbe and Vahjen (17) with some modifications. In our study, a Mini-Bead Beater (Bio-Spec Products, Bartlesville, Okla.) was used, and sediment DNAs, treated with RNase A (20 µg/ml; Sigma, St. Louis, Mo.), were further purified by 1% cetyltrimethylammonium bromide

(CTAB) extraction (2) and resuspended in 100 µl of double-distilled water.

PCRs (50-µl reaction mixtures) were performed with a DNA thermal cycler (model 480; Perkin-Elmer, Norwalk, Conn.). The amplification reaction mixtures contained 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 3.75 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleoside triphosphate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 1.0 µM each primer, 1.25 U of *Taq* DNA polymerase (Promega, Madison, Wis.), and 5.0 µl of DNA template in a 0.65-ml thin-walled, polypropylene PCR tube (PGC Scientifics, Frederick, Md.) under a layer of light mineral oil (Sigma). Samples were heated to 80°C for 5 min prior to addition of the deoxynucleoside triphosphates, second primer, and *Taq* DNA polymerase. An amplification profile of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min was performed for 30 cycles, followed by one cycle of 72°C for 10 min. Nested amplification reactions (15 cycles) were performed with 1 µl of the initial amplification reaction mixture as the template. A no-template control (NTC) was included in every experiment. Two combinations of primers (University of Wisconsin Biotechnology Center, Madison, Wis.; Gibco BRL, Grand Island, N.Y.) located within the light-chain region of the toxin gene were used to detect the BoNT/C<sub>1</sub> gene (Table 1). In the initial amplification step, a ToxC-384 and ToxC-850R (ToxC-384/850R) or a ToxC-625 and ToxC-1049R (ToxC-625/1049R) primer combination was used. In the nested amplification reactions, the ToxC-625 or ToxC-850R primer was used to give the following primer combinations: ToxC-384/850R/625 and ToxC-625/1049R/850R. Ten-microliter portions of the resulting amplification reaction mixtures were size fractionated through 1.5% agarose gels (Life Technologies Inc., Grand Island, N.Y.) in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA) containing 0.5 µg of ethidium bromide per ml. The amplification products were visualized and photographed with a UV transilluminator (Foto/Phoresis I UV documentation station; Fotodyne, Hartland, Wis.).

A total of 18 sediment samples were analyzed for the BoNT/C<sub>1</sub> gene. Nested PCR analysis produced an appropriately sized DNA fragment in 12 of 13 sediment samples collected from outbreak wetlands and in 4 of 5 sediment samples collected from nonoutbreak wetlands (Fig. 1; Table 2). Nested amplifications with two different primer combinations (ToxC-384/850R/625 and ToxC-625/1049R/850R) appeared to work equally efficiently with the limited number of samples tested. DNA sequence analysis confirmed that the amplification prod-

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TABLE 1. Oligonucleotide primers for PCR analysis of the *C. botulinum* C<sub>1</sub> toxin gene

Direction	Primer <sup>a</sup>	Sequence
Sense	ToxC-384	5' <sup>384</sup> AAACCTCCTCGAGTTACAAGCCC <sup>406</sup> 3'
	ToxC-625	5' <sup>625</sup> CTAGACAAGGTAACAACACTGGGTTA <sup>648</sup> 3'
Antisense	ToxC-850R	5' <sup>850</sup> GAAAATCTACCCTCTCCTACATCA <sup>827</sup> 3'
	ToxC-1049R	5' <sup>1049</sup> AATAAGGTCTATAGTTGGACCTCC <sup>1026</sup> 3'

<sup>a</sup> The locations of the PCR primers are indicated by the primer names. Primers were numbered according to the published strain 468C C<sub>1</sub> toxin gene DNA sequence (3a) (GenBank accession no. X53751).

ucts generated from the sediment samples contained the BoNT/C<sub>1</sub> gene sequence (data not shown).

To determine if our extraction procedure could isolate DNAs from both cells and spores, a sediment sample (K7A-2; 0.5 g), previously shown to test negative for the BoNT/C<sub>1</sub> gene, was inoculated with type C botulinum (strain 96-SAC) cells or spores. Cell suspensions were prepared by incubation in an-

aerobic cooked-meat medium (1.5% Casein Peptone, 0.5% potassium phosphate [dibasic], 0.5% yeast extract, 0.05% L-cysteine-HCl, 0.3% glucose, 0.1% resazurin, 1.25 g of cooked meat/10 ml, 100 µl of vitamin K-hemin/10 ml) at 30°C for 21 h, after which the cells were diluted 80-fold, stained with 0.4% trypan blue, and counted on a Neubauer hemacytometer (AO Scientific Instruments, Buffalo, N.Y.). Aliquots (85 µl of cells

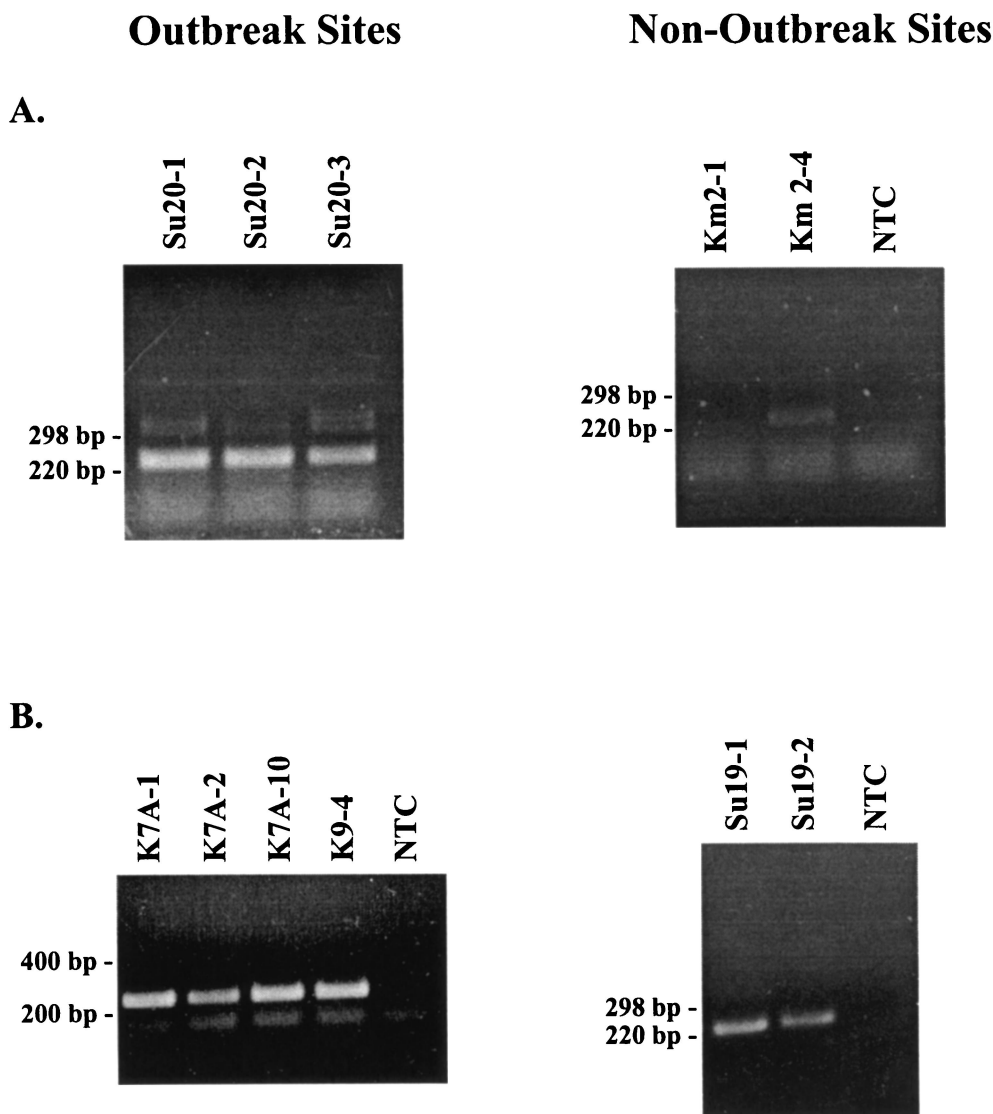


FIG. 1. Detection of the C<sub>1</sub> toxin gene amplification products in DNA samples isolated from sediments collected at botulism outbreak and nonoutbreak sites. (A) ToxC-384/850R/625 primer combination; (B) ToxC-625/1049R/850R primer combination. Images were generated with Photoshop version 5.02 and Freehand version 7.02 software. Letters and numbers above the lanes are sample designations. NTC, no-template control.

TABLE 2. Detection of the C<sub>1</sub> toxin gene in sediments collected from outbreak and nonoutbreak wetlands

Status	Wetland complex	Wetland sample	No. of samples in which product was detected/total no. of samples
Outbreak	Kulm WMD	Km1	2/2 <sup>a</sup>
	Sutter NWR	Su20	3/3 <sup>a</sup>
	Klamath NWR	K4C	1/1 <sup>b</sup>
		K7A	5/6 <sup>b</sup>
		K9	1/1 <sup>b</sup>
Total		12/13	
Nonoutbreak	Kulm WMD	Km2	1/2 <sup>a</sup>
	Sutter NWR	Su19	3/3 <sup>a</sup>
Total		4/5	

<sup>a</sup> Primer combination ToxC-384/850R/625.

<sup>b</sup> Primer combination ToxC-625/1049R/850R.

plus 15  $\mu$ l of glycerol) were prepared and stored at  $-80^{\circ}\text{C}$ . Spore suspensions were prepared by inoculating them into anaerobic fortified egg meat medium (14) and incubating the suspensions at  $35^{\circ}\text{C}$ . Malachite green spore staining (4) was performed daily to determine the optimal time for spore harvesting, at which time the suspension was treated with 50% ethyl alcohol to rupture any remaining cells and the cells were washed four times in sterile double-distilled water, diluted to  $0.9 \times 10^8$  spores/ml, and stored at  $4^{\circ}\text{C}$ . Dilutions of cell or spore suspensions ( $\sim 25$ ,  $3 \times 10^3$ , or  $3 \times 10^5$ ) were added to the sediment sample and placed on ice for 15 min, and the DNAs were then isolated by the extraction and purification procedures described above. PCR yielded the expected amplification product (225 bp) in sediment inoculated with 25 or more bacterial cells and in sediment inoculated with 25 or more bacterial spores. No product was detected in the control sample (Fig. 2).

In the majority of the wetlands we sampled in our study (both outbreak and nonoutbreak wetlands), the BoNT/C<sub>1</sub> gene was detected by PCR analysis. This high prevalence was not unexpected considering the widespread distribution of type C botulinum spores in wetlands (12) as demonstrated by traditional microbiological methods. Also, our DNA extraction method utilized a bead mill homogenizer that has previously been shown to rupture both bacterial spores and cells (9). Our findings confirmed that our procedure could detect low numbers (25 organisms) of either botulinum cells or washed spores in sediment seeded with the organism. In our study, we also found that further purification of the sediment DNA by CTAB extraction was critical for successful amplification of the BoNT/C<sub>1</sub> gene by PCR. Without this purification step, organic materials which copurified with the DNA during the extraction process inhibited the enzymatic activity of the *Taq* DNA polymerase, preventing amplification (1, 7, 18).

Detection of organisms in environmental samples by PCR has become more common as the need to monitor specific pathogens (1, 6) or genetically modified organisms released into the environment (8, 16) arises. Because many indigenous bacteria present in soils or sediments are difficult to culture on laboratory media, including *C. botulinum* (10), PCR analyses, such as ours, provide new opportunities for studying these organisms in their natural environments. Our study demonstrates the utility of PCR for detection of the BoNT/C<sub>1</sub> gene

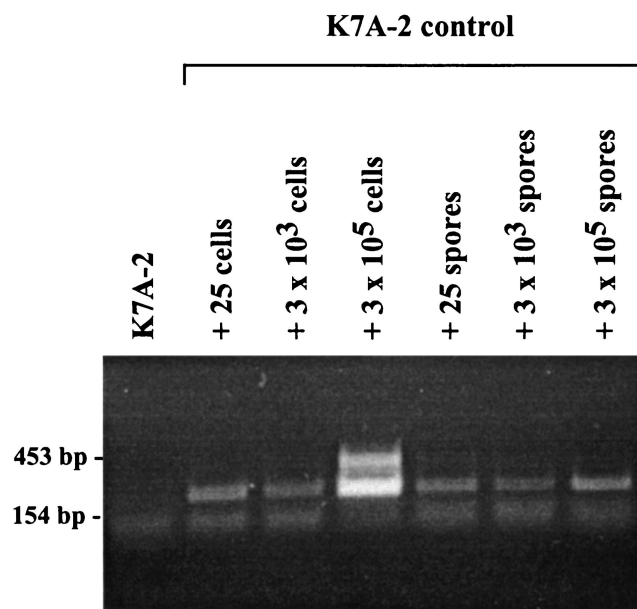


FIG. 2. Detection of the C<sub>1</sub> toxin gene in strain 96 SAC bacterial cells or spores added to sediment sample K7A prior to DNA extraction and purification. Images were generated with Photoshop version 5.02 and Freehand version 7.02 software.

and also the ubiquitous nature of botulinum cells and spores in wetland sediments. An extraction method that would selectively isolate DNA from vegetative cells, leaving dormant spores intact, would enable us to further use PCR analysis to investigate the prevalence of the botulinum toxin-producing population in wetlands in relation to environmental conditions associated with avian botulism outbreaks.

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