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Detection of the Genes Encoding Botulinum Neurotoxin Types A to E by the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) was used as the basis for the development of highly sensitive and specific diagnostic tests for organisms harboring botulinum neurotoxin type A through E genes. Synthetic DNA primers were selected from nucleic acid sequence data for *Clostridium botulinum* neurotoxins. Individual components of the PCR for each serotype (serotypes A through E) were adjusted for optimal amplification of the target fragment. Each PCR assay was tested with organisms expressing each of the botulinum neurotoxin types (types A through G), *Clostridium tetani*, genetically related nontoxigenic organisms, and unrelated strains. Each assay was specific for the intended target. The PCR reliably identified multiple strains having the same neurotoxin type. The sensitivity of the test was determined with different concentrations of genomic DNA from strains producing each toxin type. As little as 10 fg of DNA (approximately three clostridial cells) was detected. *C. botulinum* neurotoxin types A, B, and E, which are most commonly associated with human botulism, could be amplified from crude DNA extracts, from vegetative cells, and from spore preparations. This suggests that there is great potential for the PCR in the identification and detection of botulinum neurotoxin-producing strains.

Botulism has been recognized as a food-borne disease for more than 1,000 years (12). The clinical manifestations of botulism are caused by the action of neurotoxins produced by the anaerobic, spore-forming organism *Clostridium botulinum*. Botulinum neurotoxins act preferentially on cholinergic nerve endings in both humans and lower animals to prevent the release of acetylcholine and thus produce a flaccid paralysis in the host (25). These neurotoxins, which are produced by seven distinct serological types of *C. botulinum* (types A through G), are the most potent biological or chemical substances known. *C. botulinum* types A through F have been implicated as causes of human or animal disease. The toxin produced by *C. botulinum* type G has not been associated with paralytic illness but has been isolated from soil samples (11) and autopsy specimens (27).

According to *Bergey's Manual of Systematic Bacteriology*, the species *C. botulinum* includes all clostridia that produce any of the characteristic and serologically distinct botulinum neurotoxins (5). However, over the past 20 years, genetic studies have shown that *C. botulinum* is a heterogeneous species, and classifying strains on the basis of neurotoxin production alone without consideration of the physiological properties of the strains themselves is questionable (16, 18, 30, 37). In 1970, Holdeman and Brooks (14) divided *C. botulinum* into three major metabolic groups. The current nomenclature for *C. botulinum* recognizes four groups (groups I through IV) that are based principally on the ability or inability of the organisms to digest complex proteins. Each group has a phenotypically and genotypically similar nontoxigenic counterpart. For group I organisms (*C. botulinum* types A, B, and F) the nontoxigenic counterpart is *Clostridium sporogenes*, for group III organisms (*C. botulinum* types C and D) the nontoxigenic counterpart is *Clostridium novyi* type A, and for group IV isolates (*C. botulinum* type G) the nontoxigenic counterparts are *Clostridium subterminale* and *Clostridium hastiforme*. There is no proposed species name for the nontoxigenic group II strains (*C. botulinum* types B, E, and F). Recently, Suen et al. (30) proposed the new species name *Clostridium argentinense* for all organisms that produce type G botulinal toxin, as well as their genetically related nontoxigenic counterparts. This is perhaps the initial step toward a better definition of strains currently regarded as *C. botulinum*.

Regardless of the taxonomic relationships among *C. botulinum* strains and their nontoxigenic counterparts, laboratory confirmation of botulism has always demanded the detection of neurotoxin. A sensitive and specific mouse bioassay is the established method that is used almost universally. Alternatives to animal experimentation are always ethically desirable and should be encouraged. Many immunological methods for botulinum neurotoxin detection have been described previously (1, 10, 22, 32). The use of DNA-based techniques for the detection of neurotoxin producers has not been explored extensively. We have described previously the specific detection of *C. botulinum* type B by using the polymerase chain reaction (PCR) with primers deduced from protein sequence information (31). The subsequent direct sequencing of the resulting PCR products reported in this paper enabled us to design alternative type B primers. In addition, publication of the complete nucleotide sequences of the neurotoxin genes for types A (4, 33), C (13), and D (3) and the partial sequence for type E (9) allowed us to design PCR primers for each of these types.

In this paper we describe PCR protocols for the detection of botulinum neurotoxin types A through E and the specificity and sensitivity of the method for each type.

MATERIALS AND METHODS

Bacteria. The strains which we used are listed in Table 1. All clostridial isolates were inoculated into sterile cooked meat medium (Oxoid) rehydrated with either Trypticase-peptone-yeast extract-glucose-cysteine broth (10) or pep-

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TABLE 1. Isolates used in this study

Taxon	Toxin produced[a]	Strain(s)	Source[b]
C. botulinum	A	ATCC 441	ATCC
		A726	ACH
	B	NCTC 7273	NCTC
		A625, A688, A777, A805	ACH
		ARI J6621-11, ARI P8568-3A, CSL 1787	ARI
		17B	CSIRO
	C	NCTC 8264, NCTC 3732[c]	NCTC
	D	M91, Onderstepoort[c]	ARI
		NCTC 8265	NCTC
	E	ATCC 17855	ATCC
	E	Abashiri, Alaska, Beluga, EK, Iwannai, Minneapolis, Rakefisk, VH, 16/63	CSIRO
C. butyricum	E	ATCC 43755	ATCC
C. botulinum	F	NCTC 8265	NCTC
Clostridium baratii	F	ATCC 43756	ATCC
C. argentinense	G	ACM 3690	ACM
C. tetani	Tetanus	ACM 58	ACM
C. sporogenes		ACM 54, ACM 60	ACM
C. novyi type A		ACM 349	ACM
Clostridium perfringens		ACM 55	ACM
C. butyricum		ACM 2646	ACM
Bacillus cereus		ATCC 14579	ATCC
L. innocua		ACM 3178	ACM
L. monocytogenes		ACM 527	ACM
Escherichia coli		ACM 1803	ACM

[a] Only clostridial neurotoxins are indicated.
[b] ATCC, American Type Culture Collection, Rockville, Md.; ACH, Adelaide Children's Hospital, Adelaide, Australia; NCTC, National Collection of Type Cultures, London, England; ARI, Animal Research Institute, Yeerongpilly, Australia; CSIRO, CSIRO Food Research Laboratory, Sydney, Australia; ACM, Australian Collection of Microorganisms, Brisbane, Australia.
[c] Strains of *C. botulinum* types C and D that did not produce neurotoxin because of a loss of phage during serial in vitro culture (31a).

tone-yeast extract-glucose (32). Proteolytic isolates were incubated at 37°C, and nonproteolytic strains were incubated at 30°C for 24 to 48 h. When cooked meat granules were omitted from the broth, anaerobic conditions were established by using H_2-CO_2-generating envelopes placed in anaerobic jars (BBL GasPak systems). Nonclostridial isolates were grown in peptone-yeast extract-glucose broth or on nutrient agar plates (Oxoid) aerobically at 37°C for 24 h.

DNA isolation. The five DNA extraction methods described below were used to prepare templates for PCR. (i) High-molecular-weight total cellular DNAs were isolated from all bacteria studied by using a modification of the method of Marmur (21), as described by Sly et al. (26). The cells of an overnight 200-ml broth culture, collected by centrifugation at 8,000 × *g*, were lysed by using the lysis buffer described by Strom et al. (29). The concentration and purity of the DNA were determined spectrophotometrically (20). (ii) DNA was recovered from boiled cell lysates. The cells from 10-ml broth cultures were collected by pelleting them by centrifugation at 3,500 × *g*, washed twice with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (20), resuspended in 100-μl portions of sterile distilled water, and boiled for 15 min. Following centrifugation, each supernatant was collected and stored at −20°C. The supernatant was used either directly in the PCR or following purification with a commercially available DNA binding matrix (Prep-a-Gene; Bio-Rad) according to the manufacturer's instructions. (iii) The use of spore DNA as a template for PCR was investigated. Clean, cell-free spore suspensions of *C. botulinum* type A through E strains were prepared as previously described (7). Cooked meat medium cultures were monitored for sporulation by phase-contrast microscopy. Cultures in which the level of spore formation was more than 50% were selected. Vegetative cells containing spores were lysed with lysozyme (100 μg/ml), trypsin (200 μg/ml), and sodium dodecyl sulfate (SDS). Each resulting cleared lysate was centrifuged for 5 min at 8,000 × *g* to collect the free spores. The liquid supernatant was retained, and the DNA from the lysed cells was recovered by ethanol precipitation and resuspended in 20 μl of water for the PCR. The spore pellet was washed four times with sterile distilled water. Contaminating DNA from vegetative cells was removed from the spore suspension enzymatically with DNase by the method described by Ausubel et al. (2). The mixture was kept at 65°C for 15 min to ensure inactivation of the DNase and centrifuged, and the spore pellets were washed four times with distilled water. After each wash, the supernatant was stored at −20°C to check (by using the PCR) that the DNase treatment was effective. The spore pellets were lysed by the heated alkaline method of Murrell and Anand (22a), which involved suspension of the spores in 200 μl of 1 M NaOH (pH 10.0) and heating of the preparation at 60°C for 5 min. Spore debris was removed by centrifugation at 8,000 × *g* for 5 min, and the aqueous phase containing the DNA was recovered and used directly for the PCR or purified by using the DNA binding matrix. (iv) Recovery of DNA from autoclaved cells was investigated. Portions (100 μl) of cell suspensions were prepared as described above for boiled cell lysates and then autoclaved at 121°C for 15 min in 2-ml Nunc tubes. Following centrifugation, each supernatant was collected and stored at −20°C. The supernatants were used directly or after purification with the DNA binding matrix. (v) *C. botulinum* DNA was also recovered from alkaline cell lysates. Vegetative cells were collected and washed as described above for boiled cell lysates but were resuspended in 100 μl of 1 M NaOH (pH 10.0) and heated at 65°C for 5

TABLE 2. Oligonucleotide primers selected for neurotoxin types A through E

Type	Region[a]	Size (no. of nucleotides)[b]	Primer	Sequence	Melting temp (°C)[c]	Reference(s)
A	697–1154	457	A1	5′ TATGGAATAGCAATTAATCC 3′	47	4, 33
			A2	5′ GTGTAATTTACCTTAGGTAC 3′	49	
B	117–1401	1,284	B1	5′ GATGGAACCACCATTTGCT/AAAG 3′	57	36
			B2	5′ T/AACATCT/AATCAT/AATTCCT/AGG 3′	51	
B	414–1141	727	B3	5′ AGATAGACGTGTTCCACTCG 3′	55	36
			B4	5′ CTGCTATATTAGTTTCTG 3′	43	
C	1353–2351	998	C1	5′ ATATACTCCGGTTACGGCG 3′	53	13
			C2	5′ CCTGGATAACCACGTTCCC 3′	55	
D	220–623	403	D1	5′ TAAGTAAACCGCCCAGACC 3′	53	3
			D2	5′ TAGTATAGATAATGTTCCA 3′	43	
E	283–728	445	E1	5′ TATATATTAAACCAGGCGG 3′	47	9
			E2	5′ TAGAGAAATATTGGAACTG 3′	45	

[a] Positions of the first and last nucleic acids in the targeted fragment.
[b] Number of nucleotides in the fragment.
[c] Melting temperature (T_m) was calculated by using the following formula (2): T_m = [4°C (G+C content of oligonucleotide) + 2°C (A+T content of oligonucleotide)] − 5°C.

min. Each suspension was centrifuged, and the supernatant was used directly in the PCR or purified with the DNA binding matrix.

Oligonucleotide primers. All oligonucleotide primers were synthesized either at the Queensland Institute of Medical Research or at the University of Queensland Centre for Molecular Biology and Biotechnology. Most primers (Table 2) were selected from previously published nucleic acid sequences of botulinum neurotoxin genes; the exceptions were primers B3 and B4, which were selected from the sequencing experiments described in this paper.

Optimization of the PCR. "Standard" conditions for the PCR (15) were used initially for all applications, except that the recommended primer extension temperature of 72°C was decreased to 60°C to allow for the low G+C content of the genome of *C. botulinum*. The time of extension was dependent upon the target sequence. The PCR was performed with a programmable thermocycler (Hybaid, Ltd., Teddington, United Kingdom). A master mixture of reagents was prepared and then distributed in 100-μl amounts into capped polypropylene microcentrifuge tubes (DNA included, *Tth* DNA polymerase excluded). Each 100-μl PCR reaction mixture contained 1.5 to 3.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 200 μg of gelatin per ml, 0.1% Tween 20, each deoxynucleoside triphosphate (Boehringer Mannheim), at a concentration of 200 μM, 1 U of *Tth* DNA polymerase (Toyobo), usually 30 pmol of each primer, and template DNA. The template DNA was denatured at 90°C for 10 min to inactivate protease and to ensure denaturation before cycling commenced. The reaction mixture was then placed on ice, the enzyme was added, and the reaction mixture was overlaid with 70 μl of mineral oil to reduce evaporation. The PCR conditions that gave optimal specificity, fidelity, and yield of the desired product were determined empirically. The parameters examined included primer and magnesium ion concentrations and the PCR temperature cycling profile. The annealing temperature was systematically increased from 37 to 60°C, and annealing times of 1 min, 30 s, and 20 s were compared. The magnesium concentration was increased in 0.5 mM increments from 0 to 5.0 mM. Primer concentrations of 15, 30, 60, and 100 pmol were evaluated for each PCR. Finally, once the other parameters were optimized, the optimum number of cycles was determined; we started with 25 cycles and increased the number systematically to 50 cycles. Negative controls containing all of the reagents except template DNA were routinely included. One variable at a time was altered, and the effects were monitored by examining the intensity and size distribution of PCR products after separation on a 1.0% (wt/vol) agarose gel (SeaKem) run in TAE buffer (20). Photographs were taken with Polaroid type 665 film and a Polaroid model MP.4 Land camera under UV (302-nm) illumination (Spectroline model TR-302 transilluminator). Each gel well contained 10 μl of PCR mixture added to 2 μl of gel loading buffer. DNA fragments were made visible by adding ethidium bromide to a final concentration of 1 μg/ml to the molten gel.

DIG labelling of PCR products. Portions (1 μg) of the DNAs isolated from *C. botulinum* types A through E were used as target DNAs to produce digoxigenin (DIG)-labelled probes by using the method of Lo and coworkers (19). Amplification was accomplished by using the optimal conditions for each type. The DIG-11-dUTP-labelled PCR products were extracted once with chloroform, ethanol precipitated, and then dissolved in 100 μl of TE buffer. Spectrophotometric analysis was used to estimate that 10 μg of unlabelled PCR product was usually synthesized, and this value was used as the basis for calculating the amount of labelled probe required for hybridization.

Hybridization of DIG-labelled products. The PCR products required for sensitivity determinations were transferred from agarose gels to nylon filters by using a model TE80 Transvac vacuum blotting unit (Hoeffer Scientific Instruments) along with 0.4 N NaOH–0.6 M NaCl as the transfer solution. DNA dot blots were prepared by using a microfiltration apparatus (Bio-Rad) according to the manufacturer's instructions. A 5-μg portion of genomic DNA was denatured in 0.4 M NaOH and 10 mM EDTA, neutralized with 2 M cold ammonium acetate (pH 7.0), and transferred to a nylon membrane. The DNA was fixed to the membrane by exposure to UV light for 3 min.

The hybridization conditions, posthybridization wash procedures, and procedures used for visualization of the probe-target hybrid with a DIG nucleic acid detection kit (Boehringer Mannheim) were the conditions and procedures recommended by the manufacturer. Hybridization was allowed to proceed at 65°C overnight in experiments designed to determine the sensitivity of the PCR protocol. When the DIG-labelled probe was used to determine the specificity of the PCR, hybridization was carried out at 65°C for 2 h. The hybridization solution contained 100 ng of probe per ml.

Direct sequencing of PCR products. The dideoxy chain termination procedure was used to sequence a linear double-

TABLE 3. Optimal conditions for PCR

Parameter	Optimal condition for neurotoxin type:					
	A	B (primers B1 and B2)[a]	B (primers B3 and B4)[b]	C	D	E
Annealing temp and time	50°C, 20 s	46°C, 20 s	55°C, 20 s	50°C, 20 s	50°C, 20 s	50°C, 20 s
Extension temp and time	60°C, 2 min	60°C, 4 min	60°C, 2 min	60°C, 4 min	60°C, 2 min	60°C, 2 min
Denaturation temp and time	90°C, 20 s	90°C, 20 s	90°C, 20 s	90°C, 20s	90°C, 20 s	90°C, 20 s
$MgCl_2$ concn (mM)	2.5	1.5	1.5	1.5	3.0	2.0
Primer concn (pmol)	15	30	15	60	30	60
Cycle no.	30	25	25	25	30	30

[a] Amplification with primers B1 and B2.
[b] Amplification with primers B3 and B4.

stranded 1.3-kb PCR product of *C. botulinum* type B strain NCTC 7273 in this study (6). The nucleotide compositions of the initial amplification and sequencing primers used (primers B1 and B2) have been published recently (31) and are included in Table 2. The additional type B primers examined in this study (primers B3 and B4) (Table 2) were derived from the sequence information generated with sequencing primers B1 and B2 and were used to complete the sequencing of the amplification product. The PCR products were purified for sequencing by using a DNA purification matrix kit (Bio-Rad) according to the manufacturer's instructions. The eluted DNA (4 μg) was precipitated in the presence of ethanol. The resulting pellet was washed three times with 70% (wt/vol) ethanol and resuspended in 7 μl of sterile distilled water. The DNA sequence was then determined by using a Sequenase sequencing kit (U.S. Biochemicals) and the modifications described by Dorsch and Stackebrandt (6), except that ^{35}S-dATP labelling reactions were performed. Electrophoresis was performed on 8 or 4% polyacrylamide–8 M urea gels. The gels were fixed twice in 10% (vol/vol) acetic acid and 10% (vol/vol) methanol for 30 min and allowed to dry overnight. The exposure time was 72 h.

Nucleotide sequence accession number. The 1,284-bp portion of the L chain of the type B botulinum neurotoxin gene described below has been deposited in the GenBank data base under accession number Z11934.

RESULTS

Optimizing the PCR. Designing primers with the recommended G+C composition of 50 to 60 mol% (15) was, in most instances, not possible because of the low G+C content of the botulinum neurotoxin gene. A comparative nucleotide alignment of the sequences allowed us to manually select potential PCR primers. The final primers selected for each type are shown in Table 2. Each PCR was optimized with purified DNA from the strain producing the corresponding toxin, as follows: toxin A, *C. botulinum* type A strain ATCC 441; toxin B, *C. botulinum* type B strain NCTC 7273; toxin C, *C. botulinum* type C strain NCTC 8264; toxin D, *C. botulinum* type D strain M91; and toxin E, *C. botulinum* type E strain ATCC 17855. One representative for each toxin type was used to determine the optimum PCR parameters, which were subsequently confirmed by amplification of other isolates of each type listed in Table 1. The conditions that gave the optimal specificity, fidelity, and yield of each desired PCR product are summarized in Table 3. Magnesium and primer concentrations were found to have the greatest effect on the PCR. An attempt was made to shorten the PCR cycling time by (i) allowing the PCR to proceed for five cycles according to the conditions shown in Table 3 for each primer pair and changing the extension temperature to 72°C for 45 s for each cycle thereafter or (ii) changing the extension temperature to 72°C for 45 s for each cycle throughout the entire run. The best amplification was observed when a 60°C extension temperature was used throughout each cycle (Fig. 1). Primers B1 and B2 were also tested, and these primers along with primers E1 and E2 were the only primer sets which demonstrated no amplification when an extension temperature of 72°C was maintained throughout the entire PCR cycle. The yields of amplification products produced by type C primers were also poor (Fig. 1). In all instances the sensitivity of the PCR was compromised severely when an extension temperature of 72°C was used in the protocol (data not shown). We also attempted to perform a two-step PCR allowing annealing and primer extension to occur at 60°C. No visible PCR product was detected in any of these experiments (data not shown).

PCR specificity. The specificity of each PCR was assessed with purified DNAs extracted by using the method of Marmur from all of the isolates listed in Table 1; data for some of these isolates are shown in Fig. 2. Amplification products having the correct molecular weights of the targeted fragments were observed with all types tested. Amplification products having molecular weights other than the molecular weights of the target products were eliminated by increasing the annealing temperature and amplifying the targets, except with primers C1 and C2. These products were observed by using DNAs isolated from *C. botulinum* type E strain ATCC 17855, *Listeria innocua*, and *Listeria monocytogenes* and persisted when the annealing temperature was increased, even when no amplification was seen with *C. botulinum* type C strain NCTC 8264 DNA. Type E primers were able to detect the type E neurotoxin gene present in *Clostridium butyricum* ATCC 43555 (Fig. 2).

PCR amplification results were compared by using template DNAs recovered by using Marmur's method (21), crude boiled cell lysates, lysozyme- and trypsin-treated suspensions, autoclaved cells, and alkaline-lysed cells (Fig. 3). It was possible to detect specific PCR products in the supernatants of boiled and alkaline-lysed cells without purification for types A, B, and E (data not shown). However, a better yield of the desired product was obtained following purification of all prospective templates (Fig. 3). The DNA released by the rapid lysis procedures used for types C and D always required purification prior to PCR; however, the reproducibility of amplification was poor, and the yield was lower compared with DNA recovered by using Marmur's method (21) (Fig. 3). The material recovered from autoclaved cell suspensions of all types was not suitable for PCR even after purification (Fig. 3). Spore DNA was released when the heated alkaline method of Murrell and Anand (22a)

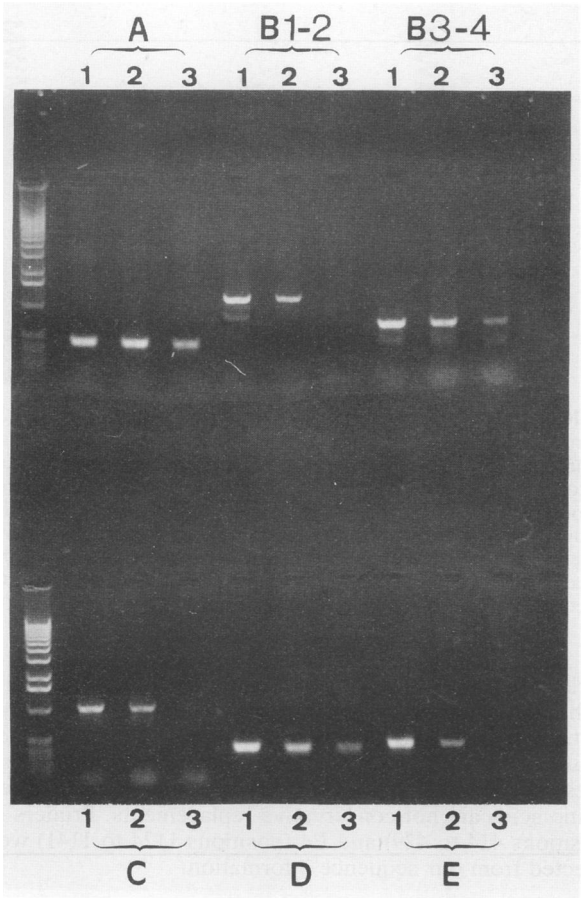

FIG. 1. Effect of varying the parameters of the PCR extension step for neurotoxin types A through E. The annealing and denaturation temperatures and times were maintained at optimum values. Lanes 1 contained PCR products obtained by using an extension temperature of 60°C for 2 min (primers A, B3 plus B4, and E) or 4 min (primers B1 plus B2 and C) for 25 cycles; lanes 2 show the results of amplification following extension at 60°C for 5 cycles and then extension at 72°C for 45 s for the remaining 20 cycles; and lanes 3 show the results for reactions in which an extension temperature of 72°C was used in all 25 cycles. The best amplification was obtained after agarose gel electrophoresis and ethidium bromide staining for all types by using an extension temperature of 60°C in all cycles (lanes 1). The molecular weight marker (lane on the left) was a commercially available 1-kb DNA ladder (Bethesda Research Laboratories). One-tenth of the PCR reaction mixture was loaded into each well of the gel.

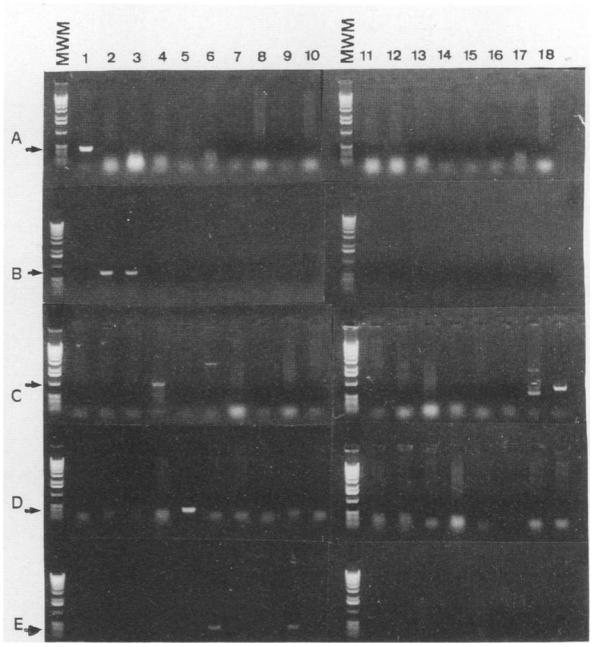

FIG. 2. Ethidium bromide-stained agarose gels of PCR products generated from template DNAs from toxigenic clostridia and other organisms. The molecular weight marker (lanes MWM) was a commercially available 1-kb DNA ladder (Bethesda Research Laboratories). One-tenth of the PCR reaction mixture was loaded into each well of the gel. The letters on the left indicate the type of PCR primer pair used in each gel. The arrows indicate the positions of the amplified targeted sequences; the type A sequence was 457 bp long, the type B sequence was 727 bp long, the type C sequence was 998 bp long, the type D sequence was 403 bp long, and the type E sequence was 445 bp long. The representative samples used were *C. botulinum* type A strain ATCC 441 (lane 1), *C. botulinum* type B proteolytic strain NCTC 7273 (lane 2), *C. botulinum* type B nonproteolytic strain 17B (lane 3), *C. botulinum* type C strain NCTC 8264 (lane 4), *C. botulinum* type D strain M91 (lane 5), *C. botulinum* type E strain ATCC 17855 (lane 6), *C. botulinum* type F strain NCTC 8265 (lane 7), *C. argentinense* type G strain ACM 3690 (lane 8), *C. butyricum* type E strain ATCC 43755 (lane 9), *Clostridium baratii* type F strain ATCC 43756 (lane 10), *C. tetani* ACM 58 (lane 11), *C. sporogenes* ACM 60 (lane 12), *C. novyi* ACM 349 (lane 13), *C. butyricum* ACM 2646 (lane 14), *E. coli* ACM 1803 (lane 15), *Bacillus cereus* ATCC 14579 (lane 16), *L. innocua* ACM 3178 (lane 17), and *L. monocytogenes* ACM 527 (lane 18).

was used; the spore DNA provided a suitable template for the amplification of type B (Fig. 4). Comparable results were obtained with type A and E primers and their respective targets (data not shown). The presence of contaminating DNA from vegetative cells was monitored by the PCR, and this DNA was shown to be effectively removed by DNase. Approximately 70% of the spores became phase dark when they were viewed by phase-contrast microscopy following heated alkaline lysis. There was no amplification when spore DNAs recovered from type C or D strains were used.

PCR sensitivity. Serial 10-fold dilutions of genomic DNAs from the *C. botulinum* types were amplified to determine the sensitivity of the PCR. The lowest amount of template that produced an observable product on an agarose gel after the optimal number of cycles for each toxin type is shown in Fig. 5. The detection levels were 1 pg (types A, B, and E) when DIG-labelled PCR products were used to probe vacuum blots and 10 fg (types A, B, and E) when initial template DNA was detected. The levels of sensitivity achieved with genomic DNAs from type C and D strains after hybridization with specific probes were poor in comparison (10 and 1 pg, respectively). Purified amplification products from type C and D isolates were also serially diluted, and the sensitivity of the PCR was determined. Visible products were produced that corresponded to 10^{-17} g of type C template and 10^{-16} g of type D template without hybridization (Fig. 5).

Specificity of DIG-labelled probes. The abilities of DIG-labelled PCR products to act as specific neurotoxin probes were evaluated by preparing genomic DNA dot blots of all of the isolates listed in Table 1. Under high-stringency conditions individual probes reacted specifically with their respective types (Fig. 6). Hybridization with the highly related tetanus toxin and hybridization with genotypically related

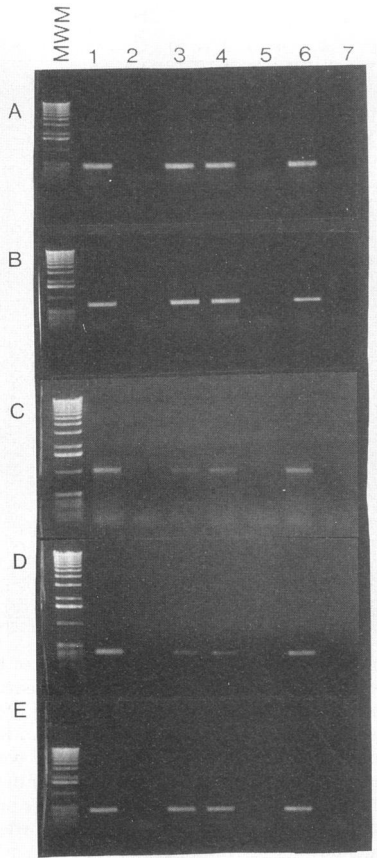

FIG. 3. Amplification of template DNA extracted by five different methods. Most samples were purified with a commercial DNA binding matrix (Bio-Rad) prior to the PCR; the only exception was DNA isolated by the method of Marmur (20). The letters on the left indicate the neurotoxin types investigated. The amount of material loaded into each well of the gel represented one-tenth of the PCR mixture volume. Lane 1, DNA extracted by the method of Marmur (20); lane 2, negative PCR control; lane 3, boiled cell lysate; lane 4, cells lysed with trypsin, lysozyme, and SDS; lane 5, autoclaved cells; lane 6, alkaline-lysed cells; lane 7, negative DNA binding matrix control. The molecular weight marker (lane MWM) was a commercially available 1-kb DNA ladder (Bethesda Research Laboratories). The bacterial strains used as the sources of DNAs were *C. botulinum* type A strain ATCC 441, *C. botulinum* type B strain NCTC 7273, *C. botulinum* type C strain NCTC 8264, *C. botulinum* type D strain M91, and *C. botulinum* type E strain ATCC 17855. Following purification, most extraction methods provided an amplifiable source of template; the only exception was the material recovered from autoclaved cells (lane 5).

clostridial species were not observed. Multiple type A, B, and E strains from geographically different sources all hybridized with their respective probes. The DNAs isolated from nontoxic derivatives of *C. botulinum* types C and D (strains NCTC 3732 and Oderstepoort, respectively) produced no PCR products and did not give a positive hybridization signal when they were probed with type C and D probes, respectively (Fig. 6).

DNA sequencing. A 1,284-bp portion of the L chain of the type B botulinum neurotoxin gene beginning at nucleotide 117 of the coding region was sequenced. The complete nucleotide sequence for the gene encoding type B neurotoxin has been reported recently (36). Five differences in

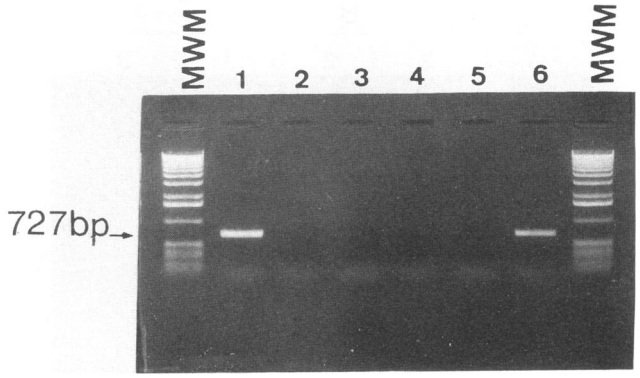

FIG. 4. Detection of *C. botulinum* type B strain NCTC 7273 spore DNA. The molecular weight marker (lanes MWM) was a commercially available 1-kb DNA ladder (Bethesda Research Laboratories). The following samples were used: PCR product from genomic DNA (lane 1), preparations from four supernatant washes following DNase treatment of spore preparations prior to spore lysis (lanes 2 through 5), and supernatant following alkaline spore lysis demonstrating a positive PCR (lane 6). One-tenth of the PCR reaction mixture was loaded into each well of the gel.

nucleotide bases were found when our data were compared with the data reported previously (Table 4), which resulted in two amino acid substitutions in the deduced protein sequence. Therefore, at amino acid residue 221 Gly substitutes for Arg, and at position 228 Ser replaces Ala. These amino acids are not conservative replacements. Primers B3 (positions 414 to 429) and B4 (positions 1124 to 1141) were selected from our sequence information.

DISCUSSION

The mouse bioassay is still the accepted standard in Australia (28) and many other countries for detecting *C. botulinum* in clinical and environmental samples and for the identification of isolates. This animal test is both sensitive and specific although not without criticism. The test can be slow, particularly when workers attempt to eliminate nonspecific deaths because of toxic substances other than botulinum neurotoxin in the sample, and often many mice are required, which presents ethical problems. The PCR technique has been used successfully to identify many organisms and usually does so more rapidly and with greater specificity and sensitivity than conventional techniques (17, 23, 34). In this study we demonstrated the utility of the PCR for the identification or detection of botulinum neurotoxin types A through E.

Sensitivity and specificity are the two most important criteria in order for any assay to become a useful and reliable diagnostic or analytical tool. Although phenotypically and genotypically diverse, the organisms that are included in the species *C. botulinum* are "united" by the ability to produce the characteristic and serologically distinct botulinum neurotoxins. It is the potency of these proteins that distinguishes *C. botulinum* as the most important of the pathogenic food-borne bacteria. Indeed, many of the current food preservation techniques are designed specifically to inhibit or destroy *C. botulinum*. It was for these reasons that the neurotoxin gene was selected as the target molecule for the PCR. Unfortunately, comparative alignment of the available nucleic acid sequence data for botulinum neurotoxins did not reveal regions that could act as "universal" botulinum

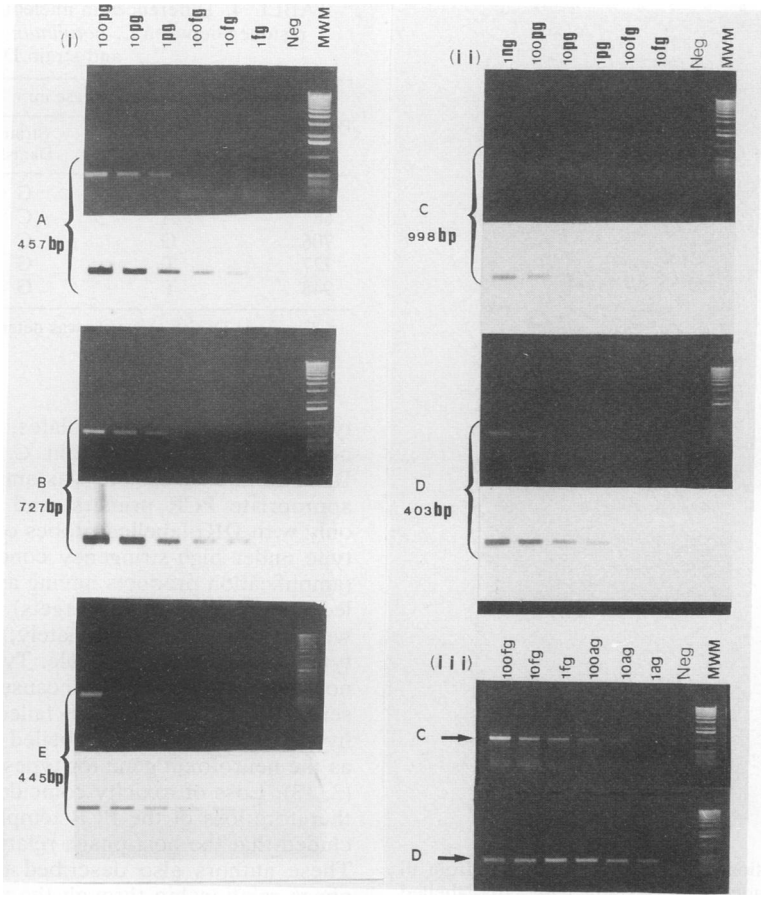

FIG. 5. Sensitivity of PCR for the detection of *C. botulinum* type A through E DNAs. The values at the top of panels i and ii are the amounts of genomic DNAs added to the reaction mixtures prior to amplification. The lowest genomic DNA amounts detected after agarose gel electrophoresis and ethidium bromide staining were 1 pg (type A, B, and E DNAs), 1 ng (type C DNA), and 100 pg (type D DNA). These values decreased to 10 fg (type A, B, and E DNAs), 10 pg (type C DNA), and 1 pg (type D DNA) when corresponding samples were subjected to Southern hybridization with the appropriate DIG-labelled DNA probe, as shown below each agarose gel. The numbers at the top of panel iii are the amounts of purified type C and D PCR products that were used as templates. A visible product that corresponded to 10 ag of type C starting template and 1 ag of type D starting template was detected. The molecular weight marker (lanes MWM) was a commercially available 1-kb DNA ladder (Bethesda Research Laboratories). The amount of material loaded into each well of the gel represented one-tenth of the PCR mixture volume.

neurotoxin primers (that is, amplify all of the serotypes of *C. botulinum* and not the highly related toxin of *C. tetani*). However, serotype-specific primers were readily distinguished. A target fragment having a different size was deliberately selected for each of the types (types A through E) for the potential development of a multiplex PCR system. This should have facilitated the use of a single PCR that could identify the toxin type on the basis of fragment size. The reaction conditions for amplification with each primer pair were examined to establish optimal performance. Magnesium and primer concentrations, in addition to the temperature cycling profile, were found to be most important parameters. Unfortunately, the different optimum requirements of the various types excluded the development of a multiplex PCR system. Attempts to shorten the PCR cycling time by increasing the extension temperature to 72°C for 45 s (the recommended optimum for *Tth* DNA polymerase) during the cycle were unsuccessful as the sensitivity of the test was compromised. The 60°C extension temperature was selected initially to compensate for the low G+C content (26 to 28 mol%) of *C. botulinum* (18). An extension temperature of 72°C is close to the previously reported melting temperature for *C. botulinum* genomic DNA (76°C) (37). At the higher extension temperature it is possible that thermal denaturation of the extending strands occurs, which is reflected by the poor yield of PCR product and thus the decreased sensitivity.

PCR specificity is dependent both on the gene selected as the target molecule and on the uniqueness of the primer sequences (15). The ability of each selected primer to bind to other regions within the neurotoxin genes of the botulinum serotypes and tetanus toxin, leading to nonspecific amplifications, was determined by computer analysis. Only those primers that exhibited specificity were synthesized. Complementarity between the two 3′ ends of a primer set has been shown to enhance primer dimer formation (35) and was avoided, where possible, when the neurotoxin primers were selected. Even in the absence of complementarity between the 3′ ends of two primers, most primer sets yield primer dimers given a sufficient number of cycles (40 to 50 cycles), high enzyme and primer concentrations, and low annealing temperature (15), conditions which were avoided during

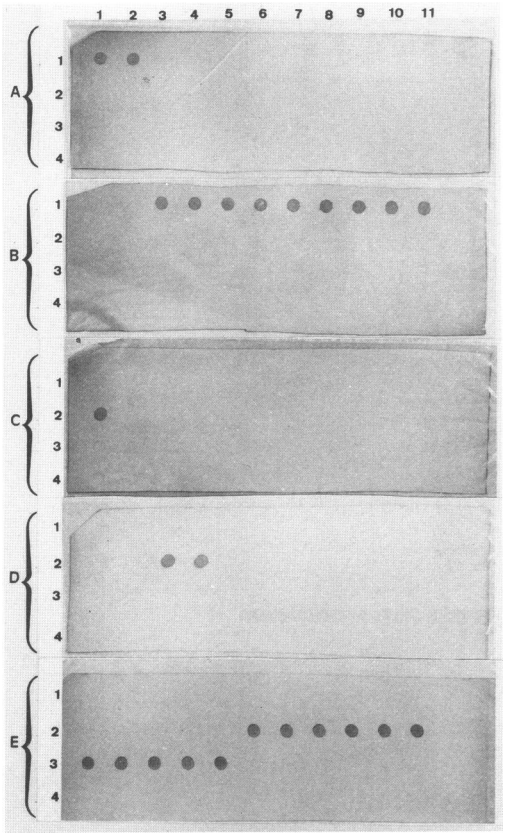

FIG. 6. Dot blot hybridization of nucleic acids extracted from all of the bacterial isolates included in this study with DIG-labelled probes for neurotoxin types A through E. The letters on the left indicate the types of specific probes tested. The vertical and horizontal numbers indicate the row and position numbers of the samples, respectively. The following samples were used for row 1 on each membrane: *C. botulinum* type A strains ATCC 441 (position 1) and A726 (position 2) and *C. botulinum* type B strains NCTC 7273 (position 3), A625 (position 4), A688 (position 5), A777 (position 6), A805 (position 7), ARI J6621-11 (position 8), ARI P8568-3A (position 9), CSL 1797 (position 10), and 17B (position 11). The following samples were used for row 2: *C. botulinum* type C strains NCTC 8264 (position 1) and NCTC 3732 (position 2), *C. botulinum* type D strains M91 (position 3), NCTC 8265 (position 4), and Oderstepoort (position 5), and *C. botulinum* type E strains ATCC 17855 (position 6), Abashiri (position 7), Alaska (position 8), Beluga (position 9), EK (position 10), and Iwannai (position 11). The following samples were used for row 3: *C. botulinum* type E strains Minneapolis (position 1), Rafefisk (position 2), VH (position 3), 16/63 (position 4), *C. butyricum* type E strain ATCC 43755 (position 5), *C. botulinum* type F strain NCTC 8265 (position 6), *C. baratii* type F strain ATCC 43756 (position 7), *C. argentinense* ACM 3690 (position 8), *C. tetani* ACM 58 (position 9), *C. novyi* ACM 349 (position 10), and *C. sporogenes* ACM 54 (position 11). The following samples were used for row 4: *C. sporogenes* ACM 60 (position 1), *C. perfringens* ACM 55 (position 2), *C. butyricum* ACM 2646 (position 3), *B. cereus* ACM 14579 (position 4), *L. innocua* ACM 3178 (position 5), *L. monocytogenes* ACM 527 (position 6), and *E. coli* ACM 1803 (position 7).

optimization of each PCR described here. It was essential to prove experimentally that the primers could amplify DNA not only from the strain used for optimization experiments but also from other *C. botulinum* isolates of the same serotype. This was confirmed by using 2 type A, 8 type B, 2 type D, and 11 type E isolates, which included the type E neurotoxin-producing strain *C. butyricum* ATCC 43255. DNA from each isolate was amplified specifically with the appropriate PCR primers, and genomic DNA hybridized only with DIG-labelled probes of the respective neurotoxin type under high-stringency conditions. No cross-reactions (amplification products having approximately the same molecular weights as the targets) between neurotoxin types were observed. Unfortunately, only one toxin-producing type C culture was available. Type C and D strains that did not produce neurotoxin because of a loss of phage during serial in vitro culture (31a) failed to react in the PCR or to hybridize with the DIG-labelled probes. This was expected as the neurotoxin gene for types C and D is phage encoded (3, 13). Loss of toxicity coincides with a loss of phage and therefore loss of the PCR template. Eklund et al. (8) concluded that the host-phage relationship is pseudolysogenic. These authors also described the instability of the host-phage relationship through the spore state of some type C and D strains, which may explain the inability of the PCR to detect type C and D neurotoxin genes in spore DNA preparations.

TABLE 4. Differences in nucleotide and deduced amino acid residues between *C. botulinum* type B strain NCTC 7273 and strain Danish[a]

Position	Nuceotide base in:		Amino acid residue in:	
	Strain NCTC 7273	Strain Danish	Strain NCTC 7273	Strain Danish
195	C	G		
687	A	C		
706	G	C	Gly	Arg
727	T	G	Ser	Ala
948	T	G		

[a] The strain Danish sequence was determined by Whelan et al. (36).

After it was shown that each PCR test repeatedly gave positive results for all available representatives of a particular type, it was important to demonstrate that DNAs from genotypically related microorganisms and members of different genera did not produce amplification products having approximately the same molecular weight as the expected target. Previously, we showed that primers B1 and B2 amplified products having approximately the same molecular weight from *C. sporogenes* and *C. butyricum* and that hybridization was essential for specificity for type B (31). No such products were produced with primers B3 and B4. Similarly, all other neurotoxin primers (primers A and C through E) were highly specific.

The DNA content of each cell type and the number of copies of the neurotoxin gene present in each genome affect the sensitivity of the PCR. The botulinum neurotoxin gene is a single-copy gene (33). Given that the molecular mass of *C. botulinum* chromosomal DNA is 1.8×10^9 Da (7), theoretically the PCR could detect, after hybridization, the equivalent of approximately three type A cells, three cells with primers B3 and B4, 3,000 type C cells, 300 type D cells, and three type E bacterial cells. Primer B3-B4 amplification was more sensitive than primer B1-B2 amplification (31). Unlike types A, B, and E, which are chromosomally encoded, types C and D are phage mediated. As the relationship between host and phage is pseudolysogenic, it is not known whether each cell in a culture broth contains the phage. Therefore, the lower levels of sensitivity reported for types C (10 pg)

and D (1 pg) than for types A, B, and E (10 fg) when genomic DNA was used as the template may be misleading as to the efficacy of the PCR for these types. In order to simulate the number of phage particles that the PCR could detect, known concentrations of purified amplification products from type C and D strains were serially diluted. Given that a single molecule of amplified product from type C and D strains corresponds to 1×10^{-18} and 4×10^{-19} g of DNA, respectively, the PCR could detect, without hybridization, the equivalent of 10 phage particles coding for either type C or D neurotoxin. Strict attention must be paid to the possibility of molecular contamination in the PCR which would lead to false-positive results. This was encountered only once during the sensitivity testing of types C and D when amplified products were used as the templates and was detected by the appearance of a positive PCR result in the negative control. The contamination was controlled by keeping the preparation in a laminar flow cabinet. It is therefore essential that negative controls be incorporated routinely into the test protocol.

While the method of Marmur (21) for genomic DNA extraction yielded large quantities of highly purified DNA, this procedure was labor intensive and time consuming. DNA suitable for the PCR does not need to be of such high quality (15). In light of this, several simple methods to obtain template DNA were investigated. DNAs recovered from lysozyme- and trypsin-treated cells, boiled cell lysates, and heated, alkaline lysates were adequate templates. Purification of the supernatant obtained with each of these methods by using a commercial DNA binding matrix is recommended for types A, B, and E and is essential for types C and D. Inhibition of the PCR presumably due to extraneous proteins or cellular debris in the supernatant was encountered without this step. DNA preparations obtained by the method of Marmur (21) were a better source of template for type C and D strains. The reason for the lower yield and inconsistent amplification of DNAs recovered from rapid type C and D DNA preparations is unknown. Inhibitors of the PCR present in the crude lysates obtained from these types of preparations may not be effectively removed when the DNA purification kit is used. The inability to recover suitable template for the PCR from autoclaved cell suspensions even after purification of the lysate was surprising. Autoclaving kills by coagulation of protoplasm and no doubt exerts a shearing effect on DNA. It was anticipated that the sheared DNA could be used as a template for the PCR as described previously (15). Prior to purification, the autoclaved cell suspension was centrifuged to sediment the particulate material, and the supernatant was retained for the PCR. DNA released from the cells during autoclaving may have remained associated with the pelleted debris or was so severely sheared by the process that it was unsuitable for the PCR or still contained substances inhibitory to the PCR that were not removed during purification of the liquid lysate. The rapid method of choice was the heated, alkaline lysis procedure, particularly for type A, B, and E neurotoxin producers. This method released DNA not only from vegetative cells but also from spores, which was important for the use of the PCR with natural samples that may be contaminated with either spores or vegetative cells. Botulinum neurotoxins can readily absorb across mucous membranes (24), which presents a major hazard for laboratory workers when *C. botulinum* isolates are handled. The combination of heat and an alkaline pH in this method readily denatures any neurotoxin present, thus reducing the health risk to laboratory workers.

In summary, conditions representing the optimum complementation between efficiency and specificity for the PCR for botulinum neurotoxin types A through E were defined. With our methods we could detect the DNA equivalent of three organisms without evidence of cross-reactions among neurotoxin producers, genotypically related nontoxigenic organisms, and the unrelated strains included in the study. Where applicable, the PCR could reliably identify multiple strains of the same neurotoxin type. In particular, the PCR protocols for neurotoxin types most commonly involved in human disease (types A, B, and E) reliably exhibited high degrees of specificity and sensitivity for their respective targets when a variety of template preparations were used, which suggests that the PCR may be a method to challenge the mouse bioassay. It should be noted that the levels of sensitivity and specificity of the PCR assays reported in this study were obtained from pure-culture analyses and may be compromised during in situ analysis of the protocols. In this case, the PCR would be challenged by inhibitory substances produced by the normal flora of a sample, as well as various other organic, chemical, and physical sample constituents. The use of the PCR protocols for botulinum neurotoxins for clinical, food, and environmental samples is currently under investigation by members of our group.

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