JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 1911–1917
0095-1137/94/$04.00+0

Vol. 32, No. 8

Detection of Type A, B, and E Botulism Neurotoxin Genes in *Clostridium botulinum* and Other *Clostridium* Species by PCR: Evidence of Unexpressed Type B Toxin Genes in Type A Toxigenic Organisms

GIOVANNA FRANCIOSA,[1] JOSEPH L. FERREIRA,[2] AND CHARLES L. HATHEWAY[3]*

Istituto Superiore di Sanitá, Laboratorio Alimenti, 00161, Rome, Italy[1]; U.S. Food and Drug Administration, Atlanta, Georgia 30309[2]; and Centers for Disease Control and Prevention, Atlanta, Georgia 30333[3]

Received 27 October 1993/Returned for modification 9 December 1993/Accepted 27 April 1994

We studied the effectiveness of the PCR in detecting the type A, B, and E botulism neurotoxin genes in 209 strains of *Clostridium botulinum* and 29 strains of other *Clostridium* spp. All 79 strains that produced type A toxin, 77 strains that produced type B toxin, and 51 organisms that produced type E toxin (46 *C. botulinum* and 5 *C. butyricum*) were PCR positive in reactions with primers targeting sequences specific for their respective toxin genes. The PCR for type A toxin was positive for one type B toxin-producing strain that produced a small amount of type A toxin in addition to a large amount of type B toxin. Surprisingly, the type B toxin gene was detected in addition to the type A toxin gene in 43 type A toxin-producing strains, only 1 of which could be shown by bioassay to produce biologically active type B toxin in culture. The type B gene was also detected in two strains of *C. subterminale*, which were determined to be nontoxigenic by bioassay. While the PCR was sensitive and specific in detecting the neurotoxin genes, the discovery of unexpressed toxin genes indicates that PCR results may not be adequate for establishing type B neurotoxigenicity.

The species *Clostridium botulinum* includes several groups of anaerobic spore-forming bacteria that produce a potent neurotoxin. The neurotoxin causes a paralytic condition in humans and various animal species which we know as botulism (13). Certain strains of at least two other species of *Clostridium*, *C. baratii* (12) and *C. butyricum* (18), have also been found to produce this neurotoxin. Seven serologic types of the neurotoxin, designated by the letters A through G, have been identified. In the United States (and apparently elsewhere), more than 99% of cases of confirmed human botulism are caused by toxins of types A, B, and E (13). The only reliable means of detecting, identifying, and typing botulism neurotoxins is by the mouse bioassay (13). Detection of toxigenicity is a requirement for the identification of *C. botulinum* as well as for determining the potentials of strains of other species for causing botulism.

The nucleotide sequences of the structural genes for botulism neurotoxins of types A (3, 25), B (17, 29), C (16), D (2), E (20, 28), and F (6, 26) have been determined. Knowledge of these sequences makes it possible to detect the toxin genes in these organisms by using the PCR technique. The Botulism Laboratory at the Centers for Disease Control and Prevention (CDC) has accumulated a large number of strains of *C. botulinum* that produce type A, B, and E toxins in the course of investigating outbreaks and individual cases of human botulism for more than 20 years. These provided the material for the collaborative effort described here, which involved CDC, the Istituto Superiore di Sanitá, and the U.S. Food and Drug Administration. We studied the efficacy of using the PCR for the identification of *Clostridium* spp. with the potential for causing botulism as a result of the presence of type A, B, and E neurotoxin genes. We tested a total of 238 strains of *Clostridium*, including 218 strains previously recorded as being producers of botulism neurotoxin.

* Corresponding author. Mailing address: NCID, DBMD, MS#CO7, Centers for Disease Control and Prevention, 1600 Clifton Road, N.E., Atlanta, GA 30333. Phone: (404) 639-3867. Fax: (404) 639-3970. Electronic mail address: CLH2@CIDDBD2.EM.CDC.GOV.

MATERIALS AND METHODS

***Clostridium* strains and culture.** Two hundred thirty-eight strains of *Clostridium* spp. were tested by PCR for the presence of type A, B, and E neurotoxin genes. All of the strains of *C. botulinum* and *C. sporogenes* isolated from specimens were recovered on egg yolk agar (EYA) plates (Carr Scarborough Microbiologicals, Inc., Decatur, Ga.) (5) on the basis of their lipase reactions. The toxigenicities of the pure isolates were determined by mouse bioassay. The strains of neurotoxigenic *C. baratii* (19) and *C. butyricum* (1) were isolated without the benefit of the lipase marker. The strains in each of the cultures had been completely characterized at the time of isolation by the methods of Dowell and Hawkins (5). The strains in reference cultures were characterized in the same manner at the time of receipt in the laboratories. The cultures were stored under refrigeration in brain storage medium (5).

Neurotoxicity bioassay. At the time of isolation from specimens or receipt from another laboratory, the toxigenicity of each strain was determined by mouse bioassay (13), and the toxin type was identified by specific neutralization of biologic activity by using monovalent botulism antitoxin reagents (available to qualified laboratories from the Biological Reagents Program, CDC). The monovalent reagents contain approximately 10 IU/ml. One international unit neutralizes approximately 10^4 mouse 50% lethal doses of neurotoxin. Type A hyperimmune horse antiserum containing approximately 500 IU/ml was used to neutralize certain cultures with high titers of neurotoxin without dilution; this would allow detection of low levels of type B toxin if it was produced concurrently with type A toxin.

ELISA for detection of neurotoxin production. After the initial phase of our study, we began saving samples from each

TABLE 1. Primers for PCR detection of type A, B, and E neurotoxin genes

Primer (PCR product size [bp])	Sequence	Location on gene
Type A (2,278)		
NKB-1	5′-GAT ACA TTT ACA AAT CCT GAA GGA GA-3′	145–173
NKB-5	5′-AAC CGT TTA ACA CCA TAA GGG ATC ATA GAA-3′	2423–2393
Type B (1,284)		
B1-a	5′-GAT GGA ACC ATT TGC TAG-3′	63–83
B2-d	5′-AAC ATC AAT ACA TAT TCC TGG-3′	1347–1327
Type B (1,450)		
B3	5′-CCA GGA ATA TGT ATT GAT GTT-3′	1327–1347
B4	5′-AAA TCA AGG AAC ACA CTA-3′	2777–2760
Type B (881)		
B5	5′-TGG ATA AGA ATA CCT AAA TAT AAG-3′	2791–2814
B6	5′-AAG CAA CTG ACA ACT ATA TGT-3′	3672–3652
Type B (3,873)		
JF-B1	5′-ATG CCA GTT ACA ATA AAT TTT AAT TAT-3′	1–30
JF-B2	5′-TTC AGT CCT CCC TTC ATC TTT AGG-3′	3873–3850
Type E (762)		
GF-1	5′-AAA AGT CAT ATC TAT GGA TA-3′	−59–−40
GF-3	5′-GTG TTA TAG TAT ACA TTG TAG TAA TCC-3′	703–677
16S rRNA (763)[a]		
BAC-3	5′-ACG GCC CAG ACT CCT ACG GGA GGC-3′	792–817
BAC-4	5′-GGG TTG CGC TCG TTG CGG CAC TTA-3′	1554–1531

[a] The locations of the primers on the 16S rRNA gene refer to the sequence as published elsewhere (27).

culture used for PCR and testing them by enzyme-linked immunosorbent assay (ELISA) for their toxigenicities. Samples of template cultures were inoculated into trypticase-peptone yeast extract glucose (TPYG) broth (Carr Scarborough Microbiologicals), and the cultures were incubated for 5 to 7 days at 35°C. Culture fluids (100 μl, undiluted) were assayed by a double-sandwich ELISA (7). Each test used a polyclonal rabbit type A or E antitoxin or goat type B antitoxin as capture antibody. Monoclonal mouse (type A), rabbit (type B), and goat (type E) antibodies were used as detection antibodies. Goat anti-mouse, goat anti-rabbit, or rabbit anti-goat immunoglobulin G-alkaline phosphatase conjugates were the indicator antibodies. All except the mouse reagent were polyclonal. The assays for type A and E toxins were type specific, but the assay for type B toxin cross-reacted at a lower intensity with cultures containing strains that produced type A toxin; this cross-reactivity between ELISAs for type A and B toxins that use polyclonal reagents has been noted elsewhere (4). On each assay plate, control cultures of *C. botulinum* strains that produce type A, B, and E neurotoxins and *C. sporogenes* were assayed. Any test culture having an absorbance at least 0.500 unit greater than that of the *C. sporogenes* control culture was considered positive. Strains that were PCR positive but ELISA negative or equivocal were retested by bioassay.

Primers. One set of oligonucleotide primers specific for the type A and type E botulism neurotoxin genes and three sets of primers specific for the type B gene were synthesized by the Biotechnology Core Facility, National Center for Infectious Diseases, CDC. An additional primer set (JF-B1 and JF-B2), which was chosen for PCR amplification of the entire type B toxin gene, was synthesized by Mary W. Trucksess, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Washington, D.C. A set of universal primers used to amplify a conserved region of the 16S rRNA genes common to many bacterial species (30), selected by Patricia Fields, CDC (unpublished data), was also synthesized by the Biotechnology Core Facility, CDC. The primer sequences, their locations on the toxin genes, and the sizes of the PCR products amplified in their respective reactions are given in Table 1.

The type A gene-specific primer sequences were selected by Naoki Kato, Gifu University, Gifu, Japan. The routinely used type B gene-specific primers (B1a and B2d) were based on the primers used by Szabo et al. (23). The additional type B gene-specific primers (B3, B4, B5, and B6) were selected from regions of the gene encoding the heavy chain of the toxin molecule. The type E gene-specific primers were selected from homologous regions of both type E neurotoxin genes of *C. botulinum* and *C. butyricum* (9). The final concentration of each primer in the reaction mixtures was 1 μM; the type E gene-specific primers and primers B1-a and B2-d, however, were used at 0.4 μM.

PCR. The reagents for PCR were as follows: (i) Tris-EDTA buffer (TE; pH 7.4) containing 0.01 M Tris and 0.001 M EDTA, prepared by CDC Scientific Resources Program, Biologic Products Branch; (ii) 10× PCR buffer (catalog no. N808-0006; Perkin-Elmer Cetus); (iii) deoxynucleotide triphosphates (catalog no. 808-0006; Perkin-Elmer Cetus); and (iv) Ampli-*Taq* DNA polymerase (catalog no. N808-1012; Perkin-Elmer Cetus).

One microliter of washed bacterial cells was used directly as the DNA source in the PCR. Cultures were removed from storage and were streaked onto EYA to observe purity, colonial morphology, and lipase activity before they were transferred to TPYG. One milliliter of an overnight (16-h) culture was centrifuged, washed in 1 ml of TE buffer, and resuspended in 1 ml of distilled water.

The 50-μl PCR volume contained 5 μl of 10× PCR buffer, a 0.2-mM concentration of each deoxynucleotide triphosphate, a 1- or 0.4-μM (see above) concentration of the primer pair targeting the desired toxin gene sequence (Table 1), 1 μl of washed cells, and 1.25 U of *Taq* polymerase. Before addition of the *Taq* polymerase, the mixture was heated at 99°C for 10 min in the thermocycler to lyse the cells. After the mixture had been cooled to 50°C, the *Taq* polymerase was added and a drop of mineral oil was overlaid on the mixture. The reaction mixture then underwent 1 cycle of 10 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C; and finally, 1 cycle of 10 min at 72°C.

Each organism was tested in PCRs for genes for type A, B, and E toxins and 16S rRNA. Products were analyzed by electrophoresis on 0.7% agarose gels, and the sizes of the fragments were determined by relating the position of the fragment on the gel to standard DNA fragments on the same gel (DNA ladder; Gibco-BRL, Gaithersburg, Md.; lambda DNA *Eco*RI *Hin*dIII digest, Sigma Chemical Co., St. Louis, Mo.). A positive PCR for the 16S rRNA gene ensured that the initial 99°C heat treatment released sufficient DNA from the cells to serve as template and that nothing was inhibitory to the PCR. This was particularly important for organisms that were negative for all three toxin genes.

PCR product-probe hybridization. The PCR products generated by using type A gene- or type B gene-specific primers with cells from type A or type B botulism neurotoxin-producing bacteria were tested by DNA hybridization with type A- and type B neurotoxin gene-specific DNA probes. The reaction products resulting from type E gene-specific primers with type E toxin-producing organisms were tested with the type E-specific DNA probe only.

DNA probes were prepared by labelling PCR-amplified products from reference strains for toxin types A, B, and E with digoxigenin-11-dUTP and using them as described previously (8). The type A gene-specific probe was a 1,358-bp fragment corresponding to nucleotide bases 34 to 1392 of strain 73A, the type B gene-specific probe was a 621-bp fragment corresponding to bases 129 to 750 of the Beans strain, and the type E gene-specific probe was an 861-bp fragment corresponding to bases −105 to 756 of the Beluga strain (the Beans and Beluga strains are well-recognized laboratory reference strains, but neither was included in the PCR study). Thus, the type A and B gene-specific probes are internal to the PCR products being tested, and the type E gene-specific PCR products are internal to the probe used for verification. Positive and negative type A, B, and E gene-specific control PCR products were tested on each hybridization filter.

RESULTS

The results of PCRs for type A, B, and E neurotoxin genes for 238 strains of *Clostridium* are summarized in Table 2. A PCR product of approximately 2,280 bp was amplified in the presence of the type A-specific primers (NKB-1 and NKB-5) with all but three strains previously identified as *C. botulinum* type A upon initial isolation. These three strains (1882, 2129, and 5113) were found to be nontoxigenic by both ELISA and bioassay. Toxigenic cultures of strains 1882 and 2129 were obtained from storage and were positive in the PCR. Other cultures of strain 5113 obtained from storage persisted in being nontoxigenic; a review of the records showed that strain 5113 was nontoxigenic upon initial isolation and should have been identified at that time as *C. sporogenes*. On the basis of those records, the organism was reassigned to the group of *C. sporogenes* strains examined in the present study (Table 2).

TABLE 2. PCR analysis for type A, B, and E botulism neurotoxin genes

Organism	No. of strains	PCR result for toxin gene:		
		A	B	E
C. botulinum type A	36	+	−	−
C. botulinum type A	43	+	+	−
C. botulinum type B	76	−	+	−
C. botulinum type Ba[a]	1	+	+	−
C. botulinum type C	1	−	−	−
C. botulinum type D	1	−	−	−
C. botulinum type E	46	−	−	+
C. botulinum type E[b]	3	−	−	−
C. botulinum type F	2	−	−	−
C. butyricum type E[c]	5	−	−	+
C. butyricum	2	−	−	−
C. argentinense type G	3	−	−	−
C. baratii type F[d]	1	−	−	−
C. subterminale	2	−	+	−
C. subterminale	4	−	−	−
C. hastiforme	1	−	−	−
C. sporogenes	9	−	−	−
C. perfringens	2	−	−	−

[a] The type Ba strain has been described previously (10, 15).
[b] These PCR-negative strains were found to be nontoxigenic by bioassay.
[c] Toxigenic strains of *C. butyricum* that produce type E neurotoxin (1, 18).
[d] Toxigenic strain of *C. baratii* that produces type F neurotoxin (19).

A PCR product of approximately 1,280 bp was amplified in the presence of the type B-specific primers B-1a and B-2b in reactions with all 77 strains previously identified as *C. botulinum* type B, regardless of their proteolytic capabilities.

A 760-bp PCR product was amplified from all type E strains except strains 7678, 8003, 8091, and 8112 in the presence of type E-specific primers. Strains 7678, 8003, and 8091 were nontoxigenic by bioassay. Although strain 8112 was toxigenic, the PCR was negative in three repetitions; a positive result was obtained with the fourth repetition.

Eight strains that produced type C, D, F, and G neurotoxins, as well as 18 of the 20 nontoxigenic organisms, were PCR negative for all three toxin genes. A PCR fragment was amplified from strains S134 and S4422 of *C. subterminale* by using type B-specific primers. The 1,280-bp fragment was consistent with the expected amplification product from the type B toxin gene. This positive test result was obtained in three repetitions with strain S134. Both strains have been repeatedly characterized and have always been nontoxigenic in toxin bioassays.

A 1,280-bp DNA product was amplified from 43 of the 79 strains of *C. botulinum* type A in the presence of the type B-specific primers B1-a and B2-d. When this product from 30 of the type A strains was tested with the PCR-generated type B-specific probe, specific hybridization was obtained, indicating the complementarities of the products with the probe (see below). Ten representative type A strains (519, 588, 593, 667, 754, 4864, 5163, 5178, 5277, and 13280; all positive in the PCR for the type B neurotoxin gene) were streaked onto EYA plates, and a well-isolated colony from each was picked to reassure that the cultures were pure. Each isolate was retested with the type A- and type B-specific primer sets, and the PCR products were amplified in both reactions with each isolate.

Typical electrophoresis gels with PCR products amplified from type A, B, and E bacterial cells serving as the source of

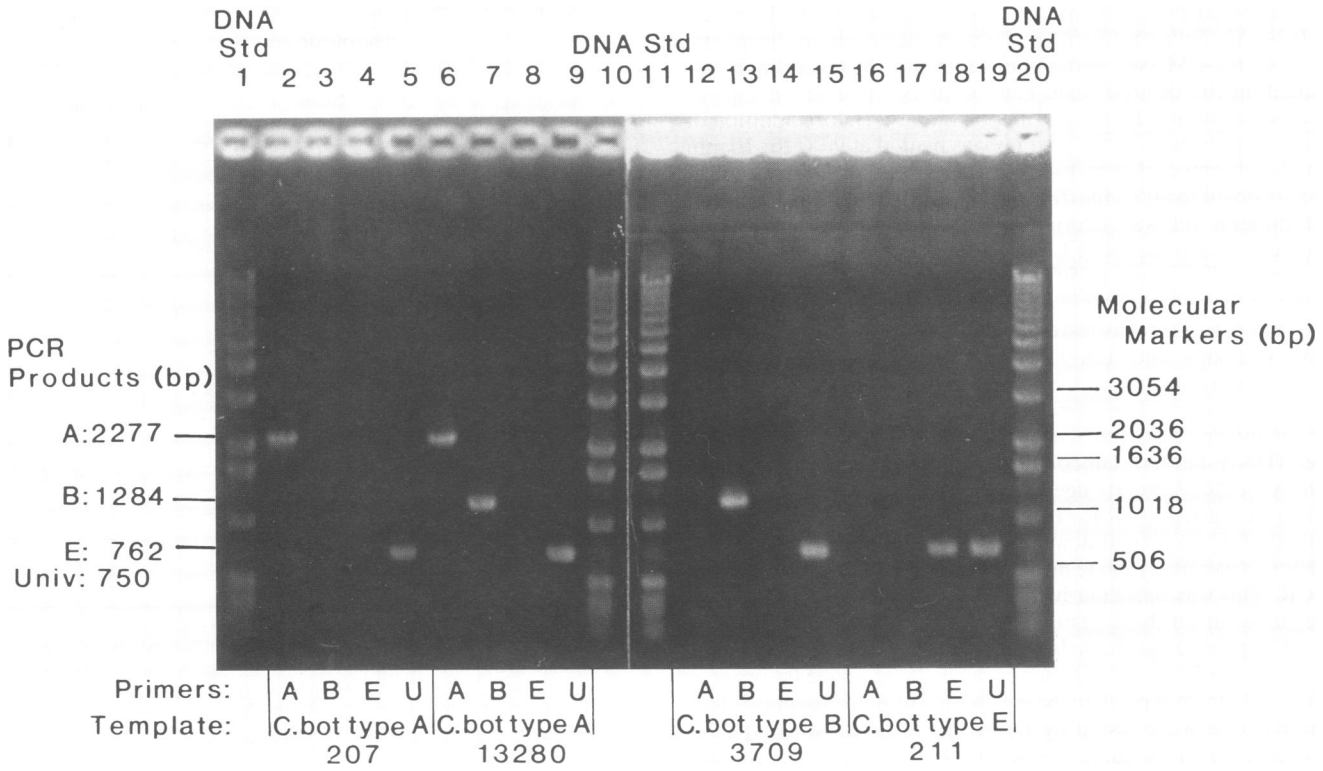

FIG. 1. Electrophoresis of PCR amplification products from DNA templates from neurotoxin type A, B, and E strains of *C. botulinum* in the presence of primers specific for the type A neurotoxin gene (NKB-1 and NKB-5; lanes 2, 6, 12, and 16), the type B neurotoxin gene (B1-a and B2d; lanes 3, 7, 13, and 17), and the type E neurotoxin gene (GF-1 and GF-3; lanes 4, 8, 14, and 18), and the universal gene (16S rRNA) (BAC-3 and BAC-4; lanes 5, 9, 15, and 19). Templates were *C. botulinum* type A strain 207 (lanes 2 to 5), *C. botulinum* type A strain 13280 (lanes 6 to 9), *C. botulinum* type B strain 3709 (lanes 12 to 15), and *C. botulinum* type E strain 211 (lanes 16 to 19). Lanes 1, 10, 11, and 20 contain DNA size standards, a 1-kb DNA ladder (Gibco-BRL).

DNA template are shown in Fig. 1. Type A strain 207 gave positive PCR results only with the type A gene-specific primers, as did 35 other type A strains. Type A strain 13280 gave positive PCRs in the presence of both type A and type B toxin gene-specific primers, in the same manner as 42 other type A strains. Type B strain 6440 gave positive results only in the presence of type B toxin gene-specific primers, and type E strain 211 was positive only in the presence of type E toxin gene-specific primers.

The isolates from type A strains 519, 588, and 667 and the type B strain 6440 were then tested by the PCR with type B toxin gene-specific primer sets B3-B4 and B5-B6 (Table 1). All four strains yielded PCR products in the reactions with the type B toxin gene-specific primers, with sizes consistent with the expected segments of the heavy-chain portion of the type B toxin gene (1,450 bp with the B3-B4 primers and 881 bp with the B5-B6 primers). PCRs with the primers JF-B1 and JF-B2, in which the entire type B toxin gene is amplified, were also performed with type A strains (519, 593, 667, 13280) and a type B strain (6440); PCR products consistent with the 3,873-bp gene were obtained from strains of each toxin type. A comparison of the amplification products obtained in PCRs with primers B1-a and B2-d and primers JF-B1 and JF-B2 with type A strain 13280 and type B strain 6440 is shown in Fig. 2.

A type A toxin gene fragment was amplified from one strain of *C. botulinum* type B by using type A toxin gene-specific primers. This strain (strain 657), initially reported as a producer of an atypical type B toxin (15), was recently discovered to produce a small amount of type A toxin in addition to type B toxin (10). We confirmed the presence of both type A and type B toxins by ELISA in cultures of this strain. The ELISA for type A toxin uses a monoclonal antibody, is specific for the type A toxin molecule, and can identify toxin A in the presence of type B toxin and other toxin-associated proteins. On the other hand, the ELISA for type B toxin cross-reacts with products of type A strains and cannot clearly identify low levels of type B toxin if it is produced by type A strains.

Verification of PCR product sequences. The identities of approximately one-half of the amplification products generated in the PCRs were verified by dot blot hybridization with the type-specific probes. Figure 3 shows filter blots with the A, B, and E toxin gene-specific probes and indicates the specificity of each. Thirty-nine type A toxin amplification products from type A organisms as well as the type A toxin amplification product from the atypical type B strain 657 were verified by hybridization with the type A toxin gene-specific probe. Thirty-eight type B toxin amplification products from PCRs with primers B1-a and B2-d from type B organisms, 30 products from type A organisms, and 1 product from 1 of the *C. subterminale* strains (S143) were verified by hybridization with the type B toxin gene-specific probe. The type E toxin gene-specific probe hybridized with 20 PCR products amplified from type E toxin-producing bacteria when the type E toxin gene-specific primers were used; 19 from *C. botulinum* and 1 from *C. butyricum* were tested.

Testing for type B toxin in cultures of type A strains. Seventeen type A strains that were positive in the type B toxin PCR were tested for type B toxin by bioassay. After addition of

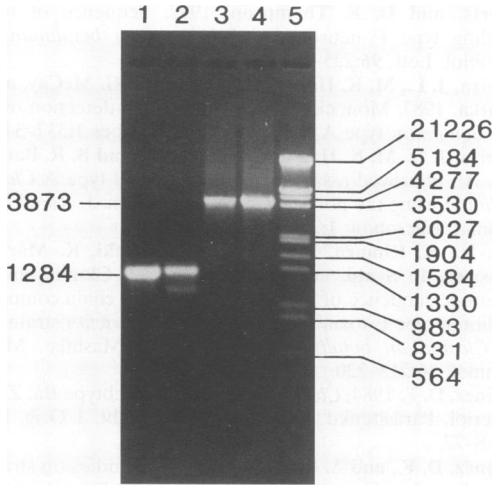

FIG. 2. Amplification products corresponding to the light-chain fragment and the entire *C. botulinum* type B neurotoxin gene from PCRs by using DNA template from *C. botulinum* type A strain 13280 (lanes 1 and 3) and *C. botulinum* type B strain 6440 (lanes 2 and 4). Lanes 1 and 2, primer set B1-a and B2-d; lanes 3 and 4, primer set JF-B1 and JF-B2; lane 5, DNA standard *Eco*RI and *Hin*dIII digest of lambda phage DNA (Sigma Chemical Co.).

50 IU of type A antitoxin to 0.4 ml of undiluted cell-free culture fluid, only one strain, strain 588, remained toxic. The untreated culture (0.4 ml) was lethal at a dilution of 1:100,000; after the addition of type A antitoxin, it was lethal at a dilution of 1:10. The residual toxicity was neutralized by the addition of an additional 1 IU of type B antitoxin. The culture contained approximately 500,000 mouse lethal doses of type A toxin per ml and 25 lethal doses of type B toxin per ml.

DISCUSSION

We previously reported the use of PCR for the identification of *C. botulinum* type A (8). Szabo et al. (24) recently reported that PCR is satisfactory for detecting the genes encoding neurotoxin types A to E on the basis of their study using 25 toxigenic strains. They used purified DNAs from the bacterial cells, and they used different PCR conditions for each set of primers. In our investigation, 200 strains of *C. botulinum* producing toxin types A, B, and E and 5 strains of *C. butyricum* were studied. Detection of type C or type D toxin genes was not pursued because very few strains of these types were available. We simplified the procedure by using heated, washed cells from an overnight culture of each strain as template DNA.

Our study showed that all type A, B, and E toxigenic organisms reacted in PCRs specific for their toxin genes. However, using the same type B gene-specific primers used by Szabo et al. (23, 24), we obtained positive results in PCRs for 44 organisms (42 *C. botulinum* type A and 2 *C. subterminale*) that apparently do not produce type B toxin. This was not due to a lack of specificity of the primers, since the type B toxin-specific probe hybridized with products amplified by four different pairs of primers corresponding to different segments of the published sequence of the type B toxin gene. It appears that the type B toxin gene is present in many organisms that do not produce type B toxin. Thus, we cannot suggest a modification that would render the test specific for only type B toxigenic organisms.

We demonstrated type B toxin in the culture of type A strain 588 which had been recognized since its isolation in 1976 as a type A toxin-producing strain. A number of strains that produce more than one type of neurotoxin, generally a preponderance of one type and a minor amount of a second type, have been recognized over the years. Thus, neurotoxin types Af (11), Bf (14), Ab (21), and Ba (10) have been designated. Strain 588 would correspond to type Ab, according to this convention. The PCR results with the type Ba strain (657) showed the presence of the genes for both type A and type B toxins. It is possible that organisms that produce more than one type of toxin may cause a more severe illness because of synergism between the toxins.

The finding of the type B toxin gene in two strains of *C. subterminale* was surprising. It would be less surprising to find it in organisms identified as *C. sporogenes*, which resemble proteolytic *C. botulinum* in all respects except toxigenicity. We can theorize that such organisms were derived from toxigenic organisms, but lost their toxigenicities because of a mutation that rendered the gene product biologically inactive or the gene incapable of transcription. None of the nine *C. sporogenes* strains yielded positive PCR results with the primers used. The only neurotoxigenic organisms resembling *C. subterminale* produce type G toxin (22).

The type B genes (and their promoter regions) present in the *C. botulinum* type A and *C. subterminale* strains need to be sequenced and compared with the genes present in organisms that produce the toxin. Perhaps this same approach should be followed for strains that produce two types of toxin; it might provide answers for the quantitative differences observed. The presence or absence of mRNA or inactive toxin protein needs to be demonstrated.

The presence of unexpressed toxin genes presents a problem for the practical use of PCR for investigating botulism and for identifying unknown strains. Still, PCR would be a useful procedure for laboratories without facilities and reagents for toxin testing. The type A and type E toxin-specific reactions

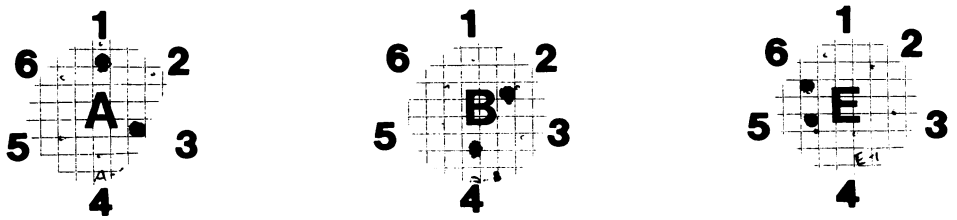

FIG. 3. PCR products bound to nitrocellulose filters and subsequently hybridized to digoxigenin-labeled probes synthesized from reference strains of *C. botulinum* expressing type A, B, and E neurotoxins. Disks indicated by letter designate probe type. PCR spots: 1, 13280 (type A), toxin A-specific primers; 2, 13280 (type A), toxin B-specific primers; 3, 62A (type A), toxin A-specific primers; 4, 6440 (type B), toxin B-specific primers; 5, 211 (type E), toxin E-specific primers; 6, 5262 (*C. butyricum* type E), toxin E-specific primers.

appear to be reliable and exclusive. The toxigenicities of strains yielding positive results in PCRs for type B toxin may have to be verified. However, strains that react in PCRs for both type A and type B toxin might generally be considered to be type A toxin-producing strains. Organisms with characteristics consistent with *C. botulinum* (lipase positive, anaerobic, sporulating, saccharolytic) that react only in the type B toxin-specific PCR will probably be type B toxigenic organisms.

At present, the laboratory investigation of botulism involves (i) testing of serum, stool, food, or gastric samples for toxin, (ii) culturing of samples and enrichment of cultures on EYA and isolation of lipase-positive organisms, and (iii) testing of isolated organisms for their toxigenicities (13). If PCR were incorporated into the methodology, PCRs would be performed on the suspended samples, on the enrichment cultures, and on isolated organisms. Toxin assays on patient serum would be necessary because circulating toxin is the most conclusive evidence for confirming the diagnosis, and the organisms do not appear in the circulation. Detection of toxin in food is necessary for identifying the vehicle because the mere presence of the organism is not a significant finding.

Processing and culturing of samples would be the same for either conventional or PCR methodologies. The costs of materials by either method would not be of primary consideration, while the labor requirement would be greater for PCR. Twenty-one samples tested in the PCRs for the four genes can be processed in 1 week, while more than 100 samples can be tested by bioassay in a similar time. PCR would require overnight incubation of an inoculated specimen or of a transferred culture and then a full day of PCR and electrophoresis operations. Although the bioassay is referred to as a 4-day test, the outcome is usually apparent within 24 h.

The main advantage to PCR would be the lessening or elimination of animal testing. At present, methods for investigating botulism cannot be replaced with PCR, although PCR may be useful as an adjunct procedure.

ACKNOWLEDGMENTS

We thank Paolo Aureli for overall support and advice; George Killgore, Loretta McCroskey, Mary Ann Lambert-Fair, David Whaley, Orjan Olsvik, Tatjana Popovic, Patricia Fields, Fred Quinn, and Mark Hemphill for help, advice, and encouragement in the laboratory; and Enrico De Bernart for providing the software for recording the experimental data.

We acknowledge financial support provided by a grant from the National Research Council of Italy.

REFERENCES

1. **Aureli, P., L. Fenicia, B. Pasolini, M. Gianfranceschi, L. M. McCroskey, and C. L. Hatheway.** 1986. Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. J. Infect. Dis. **154:**201–206.
2. **Binz, T., H. Kurazono, M. R. Popoff, M. W. Eklund, G. Sakaguchi, S. Kozaki, K. Krieglstein, A. Henschen, D. M. Gill, and H. Niemann.** 1990. Nucleotide sequence of the gene encoding for *Clostridium botulinum* neurotoxin type D. Nucleic Acids Res. **18:**5556.
3. **Binz, T., H. Kurazono, M. Wille, J. Frevert, K. Wernars, and H. Niemann.** 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. **265:**9153–9158.
4. **Dezfulian, M., C. L. Hatheway, R. H. Yolken, and J. G. Bartlett.** 1984. Enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* type A and type B toxins in stool samples from infants with botulism. J. Clin. Microbiol. **20:**379–383.
5. **Dowell, V. R., Jr., and T. M. Hawkins.** 1974. Laboratory methods in anaerobic bacteriology. Center for Disease Control, Atlanta.
6. **East, A. K., P. T. Richardson, D. Allaway, M. D. Collins, T. A. Roberts, and D. E. Thompson.** 1992. Sequence of the gene encoding type F neurotoxin of *Clostridium botulinum*. FEMS Microbiol. Lett. **96:**225–230.
7. **Ferreira, J. L., M. K. Hamdy, A. L. Herd, S. G. McCay, and F. A. Zapatka.** 1987. Monoclonal antibody for the detection of *Clostridium botulinum* type A toxin. Mol. Cell. Probes **1:**337–345.
8. **Ferreira, J. L., M. K. Hamdy, S. G. McCay, and B. R. Baumstark.** 1992. An improved assay for identification of type A *Clostridium botulinum* using the polymerase chain reaction. J. Rapid Methods Automat. Microbiol. **1:**29–39.
9. **Fujii, N., K. Kimura, T. Yashiki, K. Tsuzuki, K. Moriishi, N. Yokosawa, B. Syuto, and K. Oguma.** 1992. Cloning and whole nucleotide sequence of the gene for the light chain component of botulinum type E toxin from *Clostridium butyricum* strain BL6340 and *Clostridium botulinum* type E strain Mashike. Microbiol. Immunol. **36:**213–220.
10. **Giménez, D. F.** 1984. *Clostridium botulinum* subtype Ba. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **257:**68–72.
11. **Giménez, D. F., and A. S. Ciccarelli.** 1970. Studies on strain 84 of *Clostridium botulinum*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **215:**212–220.
12. **Hall, J. D., L. M. McCroskey, B. J. Pincomb, and C. L. Hatheway.** 1985. Isolation of an organism resembling *Clostridium barati* which produces type F botulinal toxin from an infant with botulism. J. Clin. Microbiol. **21:**654–655.
13. **Hatheway, C. L.** 1988. Botulism, p. 111–133. *In* A. Balows, W. J. Hausler, Jr., M. Ohashi, and A. Turano (ed.), Laboratory diagnosis of infectious diseases: principles and practice, vol. 1. Springer-Verlag, New York.
14. **Hatheway, C. L., and L. M. McCroskey.** 1989. Unusual neurotoxigenic clostridia recovered from human fecal specimens in the investigation of botulism, p. 477–481. *In* T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita, and A. Uchida (ed.), Proceedings of the 5th International Symposium on Microbial Ecology: recent advances in microbial ecology. Japan Scientific Societies Press, Tokyo.
15. **Hatheway, C. L., L. M. McCroskey, G. L. Lombard, and V. R. Dowell, Jr.** 1981. Atypical toxin variant of *Clostridium botulinum* type B associated with infant botulism. J. Clin. Microbiol. **14:**607–611.
16. **Hauser, D., M. W. Eklund, H. Kurazono, T. Binz, H. Niemann, D. M. Gill, P. Boquet, and M. R. Popoff.** 1990. Nucleotide sequence of *Clostridium botulinum* C1 neurotoxin. Nucleic Acids Res. **18:**4924.
17. **Hutson, R. A., M. D. Collins, A. K. East, and D. E. Thompson.** 1994. Nucleotide sequence of the gene coding for non-proteolytic *Clostridium botulinum* type B neurotoxin: comparison with other clostridial neurotoxins. Curr. Microbiol. **28:**101–110.
18. **McCroskey, L. M., C. L. Hatheway, L. Fenicia, B. Pasolini, and P. Aureli.** 1986. Characterization of an organism that produces type E botulinal toxin but which resembles *Clostridium butyricum* from the feces of an infant with type E botulism. J. Clin. Microbiol. **23:**201–202.
19. **McCroskey, L. M., C. L. Hatheway, B. A. Woodruff, J. A. Greenberg, and P. Jurgenson.** 1991. Type F botulism due to neurotoxigenic *Clostridium baratii* from an unknown source in an adult. J. Clin. Microbiol. **29:**2618–2620.
20. **Poulet, S., D. Hauser, M. Quanz, H. Niemann, and M. R. Popoff.** 1992. Sequences of the botulinal neurotoxin E derived from *Clostridium botulinum* type E (strain Beluga) and *Clostridium butyricum* (strains ATCC 43181 and ATCC 43755). Biochem. Biophys. Res. Commun. **183:**107–113.
21. **Poumeyrol, M., J. Billon, F. DeLille, C. Haas, A. Marmonier, and M. Sebald.** 1983. Intoxication botulique mortelle due à une suoche de *Clostridium botulinum* de type AB. Med. Malad. Infect. **13:**750–754.
22. **Suen, J. C., C. L. Hatheway, A. G. Steigerwalt, and D. J. Brenner.** 1988. *Clostridium argentinense*, sp. nov: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. Int. J. Syst. Bacteriol. **38:**375–381.

23. **Szabo, E. A., J. M. Pemberton, and P. M. Desmarchelier.** 1992. Specific detection of *Clostridium botulinum* type B by using the polymerase chain reaction. Appl. Environ. Microbiol. **58:**418–420.
24. **Szabo, E. A., J. M. Pemberton, and P. M. DesMarchelier.** 1993. Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. Appl. Environ. Microbiol. **59:**3011–3020.
25. **Thompson, D. E., J. K. Brehm, J. D. Oultram, T. J. Swinfield, C. C. Shone, T. Atkinson, J. Melling, and N. P. Minton.** 1990. The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. Eur. J. Biochem. **189:**73–81.
26. **Thompson, D. E., R. A. Hutson, A. K. East, D. Allaway, M. D. Collins, and P. T. Richardson.** 1993. Nucleotide sequence of the gene coding for *Clostridium barati* type F neurotoxin: comparison with other clostridial neurotoxins. FEMS Microbiol. Lett. **108:** 175–182.
27. **Van Der Meer, J. R., W. Ludwig, and W. M. DeVos.** 1993. Characterization of a ribosomal RNA gene cluster from *Clostridium tyrobutyricum*: phylogenetic positioning based on the 16s and 23s nucleotide sequences. Syst. Appl. Microbiol. **16:**201–207.
28. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, T. Atkinson, and N. P. Minton.** 1992. The complete amino acid sequence of *Clostridium botulinum* type E neurotoxin, derived by nucleotide-sequence analysis of the encoding gene. Eur. J. Biochem. **204:**657–667.
29. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, J. K. Brehm, T. Atkinson, and N. P. Minton.** 1992. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire sequence. Appl. Environ. Microbiol. **58:**2345–2354.
30. **Wilson, K. H., R. B. Blitchington, and R. C. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. J. Clin. Microbiol. **28:**1942–1946.