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PCR for Detection of *Clostridium botulinum* Type C in Avian and Environmental Samples

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A PCR was developed and applied for the detection of *Clostridium botulinum* type C in 18 avian and environmental samples collected during an outbreak of avian botulism, and the results were compared with those obtained by conventional methodologies based on the mouse bioassay. PCR and mouse bioassay results compared well (100%) after the enrichment of samples, but PCR results directly indicated the presence of this microorganism in six samples, while only one of these contained the type C botulinal neurotoxin before enrichment. The PCR assay was sensitive (limit of detection between 15 and 15 × 10^3 spores per PCR), specific (no amplification products were obtained with other clostridia), and rapid, since sonicated and heated samples provided enough template for amplification without any DNA purification. Eleven isolates of *C. botulinum* type C were recovered from mallards (*Anas platyrhynchos*), grey herons (*Ardea cinerea*), and mud during investigation of this outbreak.

Botulism in animals has been known since the type C and D toxins, two of the seven antigenically different neurotoxins (A through G) produced by *Clostridium botulinum*, were originally recognized as the cause of the disease which had affected chickens and cattle, respectively, in the United States, Australia, and South Africa (17).

Among animals, birds are especially susceptible to botulism, perhaps because they feed on insects, fly larvae, invertebrate carcasses, and spoiled feeds. All of these may harbor spores of *C. botulinum*, often contaminating the soil, and the toxins produced after spore germination in the favorable anaerobic conditions (required for growth of this microorganism) of soil and water bottoms. Spores can multiply and produce toxin in vivo as well (21). Although many neurotoxigenic clostridia are found in the soil, *C. botulinum* type C is mainly involved in avian botulism (23). Like other botulinum toxins, type C neurotoxin acts by blocking the release of acetylcholine neurotransmitter at the cholinergic synapses and causes the well-known neuroparalytic disease which can lead to the deaths of animals (7). Different molecular targets have recently been found on the presynaptic membranes for the botulinum toxins. Only the type C neurotoxin cleaves syntaxin; this could be related to its specific action against birds (24, 28).

In the summer of 1994, a number of bird deaths in the natural oasis of Scandiano (Reggio Emilia) near a river estuary was recorded. The animals (ducks, herons, gulls, and other wild fauna) presented signs presumptive of botulism, such as abnormal posture of the head, weakness, and flaccid paralysis; moreover, other outbreaks of avian botulism had previously been reported in nearby areas (2, 3, 5). Therefore, botulism was immediately suspected and attempts were made to detect the botulinum neurotoxin and causative agent in animals and the environment. The detection of botulinum toxin in food, feed, environmental, or clinical specimens, traditionally performed by the mouse bioassay, is essential for confirmation of both human and animal botulism. However, isolation of the microorganism producing it is also suitable, especially when toxin detection is troublesome, i.e., in samples containing other toxic substances or very low levels of neurotoxin. This is also achieved by demonstration of the botulinal toxin in enrichment cultures and subsequent recovery of the neurotoxigenic organism in pure culture (16).

PCR, a molecular genetic technique for in vitro amplification of specific DNA segments, has recently been used for detecting botulinum toxin genes as an alternative to the conventional methods used in botulism investigations (12–14, 29). Szabo et al. analyzed a case of type B botulism in a horse by PCR (30).

In this study, we used PCR for the detection and identification of *C. botulinum* type C from environmental samples and avian specimens collected during this outbreak and compared the results with those obtained by conventional methodologies based on the mouse bioassay. The gene encoding type C neurotoxin, which is carried by a bacteriophage inserted into the genome of *C. botulinum* as a prophage (11), had been already sequenced (18); on the basis of this sequence, we selected two primers for amplification of a gene fragment by PCR. The isolated neurotoxigenic microorganisms were also characterized.

MATERIALS AND METHODS

Bacterial strains. The clostridial strains used in this study are listed in Table 1. American Type Culture Collection strains, stored in brain storage medium at 4°C, were kindly provided by C. L. Hatheway, Botulism Laboratory, Centers for Disease Control and Prevention, Atlanta, Ga.; all of them had previously been characterized and tested by PCR for the presence or absence of type A, B, and E botulism neurotoxin genes (14).

Culture media. Isolates were picked from egg yolk agar (EYA) plates (9) and inoculated into TPGY broth (5% Trypticase, 0.5% peptone, 0.4% glucose, 2% yeast extract, 0.1% sodium thioglycolate [pH 7.0]). Broth cultures were incubated at 37°C for 24 h (proteolytic strains) and at 28°C for 24 to 48 h (nonproteolytic strains) under anaerobic conditions (Whitley Anaerobic Cabinet MK.III). One cooked-meat (CM) broth culture (Oxoid) of the *C. botulinum* type C strain (ISS CL102) used as the reference strain in this study was maintained as a sporulated culture at 4°C.

Avian specimens and soil samples. One mallard (*Anas platyrhynchos*) and two grey herons (*Ardea cinerea*), dead after presenting signs suggestive of botulism, were sent frozen to our laboratory from the Veterinary Public Service of Reggio Emilia. The autopsy samples from the birds examined in this investigation were intestinal contents, stomachs, spleens, and livers. Intestinal specimens from the

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TABLE 1. *Clostridium* strains tested by PCR in this study

Strain	Species	Botulinum toxin produced	Source[a]
H 207 (Hall)	*C. botulinum*	A	CDC
H 4847 (ATCC 27563)	*C. botulinum*	A	CDC
H 7634 (62A)	*C. botulinum*	A	CDC
H 4848 (ATCC 25765)	*C. botulinum*	B	CDC
H 17783 (ATCC 17783)	*C. botulinum*	B	CDC
CL102	*C. botulinum*	C	ISS
H 2059	*C. botulinum*	D	CDC
H 211	*C. botulinum*	E	CDC
CL 21[b]	*C. butyricum*	E	ISS
H RH84	*C. botulinum*	F	CDC
H 714	*C. botulinum*	G	CDC
H 19494	*C. sporogenes*		CDC
CL 02 (ATCC 3624)	*C. perfringens*		ATCC
H S0134	*C. subterminale*		CDC
H 4858	*C. hastiforme*		CDC

[a] CDC, Centers for Disease Control and Prevention; ISS, Istituto Superiore di Sanità, Rome, Italy; ATCC, American Type Culture Collection.

[b] Data for this strain are taken from reference 1.

autopsies of two other mallards and serum samples from two ill herons maintained at −20°C were also received at our laboratory. Two soil samples and two mud samples (about 500 g each) were collected from the environment where the outbreak occurred, placed in sterile glass jars, and sent to our laboratory.

Toxin detection in avian specimens. The presence of botulinum neurotoxin in available sera and organs from birds was detected by the mouse bioassay described by Hatheway (16). The organs were mixed with appropriate volumes of 0.2 M gelatin-phosphate buffer (pH 6.5), ground, and held overnight at 4°C; after cold centrifugation at 12,000 rpm (centrifuge model PM180R; ALC International, Milan, Italy) for 20 min to sediment the particulate matter, including bacterial cells, 0.5 ml of the fluid supernatants was injected intraperitoneally into two mice to detect the presence of any botulinum toxin. Serum samples (0.5 ml) were directly injected into mice. When mice died, neutralization was carried out by adding to separate samples of supernatants or sera a 0.25 volume of polyvalent antitoxin against type A through F toxins and monovalent antitoxins against type C and D toxins (horse serum antitoxins, 10 IU/ml; Centers for Disease Control and Prevention). Then two mice were injected with each antitoxin-sample mixture.

Organism detection. (i) Conventional culturing. Parts of the sediments from animal tissues and of soil and mud samples (~10 g each) were inoculated into CM broth. Cultures were treated at 60°C for 1 h and incubated anaerobically at 37°C for 7 days (20). EYA plates, previously incubated under anaerobic conditions at room temperature, were streaked with CM cultures toxic for mice and incubated anaerobically for 48 h at 37°C. Single colonies were picked up and grown in TPGY broth at 37°C for 24 to 48 h under anaerobic conditions.

(ii) PCR. PCR to detect the *C. botulinum* type C toxin gene was performed at three steps: directly from animal samples suspended in gelatin-phosphate buffer and from soil samples suspended in distilled water; from CM enrichment broth cultures of animal and environmental samples; and from single colonies isolated from EYA plates and cultured in TPGY broth.

Oligonucleotide primers (CI and CII) were selected from the published sequence (18) for the amplification of a segment of the type C botulinum toxin gene in which no high-level DNA homology with other botulinum toxin genes was present. Primer CI (5′-GCGGCACAAGAAGGATTTG-3′) was located between bp 747 and 765, and primer CII (5′-CGCCGTAACCGGAGTATAT-3′) was located between bp 1343 and 1361 (within the coding region of the type C neurotoxin gene sequence). The primers were synthesized at the Istituto Superiore di Sanità. Each PCR mixture contained, in a total volume of 50 μl, 1× buffer II (10 mM Tris-HCl, 50 mM KCl [pH 8.3]; Perkin-Elmer Cetus), 2 mM $MgCl_2$ (Perkin-Elmer Cetus), and 2′-deoxynucleoside 5′-triphosphates (Pharmacia) at a concentration of 200 μM each. The final concentration of primers in the reaction mixture was 1 μM.

Amplification was carried out by directly using a crude lysate of samples without any DNA purification. One milliliter of the different liquids collected at the three steps was centrifuged and washed five times with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer (27); the pellet was suspended in 1 ml of sterile distilled water and sonicated for 10 min (model T460; TransSonic, Singen, Germany) to facilitate lysis, except for 24-h TPGY broth cultures. Five microliters of each suspension in distilled water was directly added to the PCR mixture. After overlaying the mixtures with a drop of mineral oil (Sigma), PCR tubes were preheated at 99°C for 10 min in a thermocycler (model PT100; M. J. Research, Inc., Watertown, Mass.) to enhance DNA release from organisms. *Taq* polymerase (1.25 U; Perkin-Elmer Cetus) was added. PCR mixtures were subjected to the following thermal treatments: denaturation at 95°C for 10 min; 30 cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and extension at 60°C for 4 min; and a final extension at 60°C for 15 min.

C. botulinum type C (ISS CL102) was always included in experiments as a positive control; no DNA source was added in the negative control.

Ten microliters of PCR products was analyzed by electrophoresis through a 1% agarose gel and by staining with ethidium bromide (27).

Sensitivity testing of PCR with spores. Spores of the reference strain (ISS CL102), maintained in CM broth, were prepared by the method of Doyle (10). After being washed, spores were suspended in sterile distilled water; the suspension was 10-fold serially diluted in distilled water and enumerated by the three-tube most-probable-number method (26). TPGY broth tubes inoculated with the initial suspension and serial dilutions were heat treated at 70°C for 10 min and incubated at 37°C for 2 weeks. All growth-positive tubes were centrifuged, and supernatants were tested for type C botulinal toxin by the mouse bioassay. The same suspensions used for enumeration were sonicated for 10 min, and 5 μl of undiluted suspension and of 10^{-1} to 10^{-5} dilutions was added to PCR reaction mixtures, heated at 99°C for 10 min, and subjected to the PCR protocol described above.

Identification of isolated strains. Colonies isolated by conventional methods (16) and yielding a positive PCR product were first tested for the production of type C neurotoxin by the mouse neutralization test (16) and additionally characterized by lipase and lecithinase reactions, sugar fermentation patterns, acid production, and proteolytic and hemolytic activities for species identification (9). Acid production was determined by gas-liquid chromatographic analysis of peptone-glucose-yeast extract broth cultures anaerobically incubated for 5 days at 37°C (22).

RESULTS

PCR specificity and sensitivity. Amplification of a segment of the type C neurotoxin gene by CI and CII primers was demonstrated first with reference strain ISS CL102. After optimization of the PCR conditions, the specificity of the reaction was confirmed by testing 14 strains of clostridia (Table 1), some of which were producers of other types of botulinum neurotoxins. None of them showed amplification products.

Sensitivity testing of the PCR protocol with spores of the type C reference strain showed that at least 15 spores in the reaction mixture provided enough DNA template for amplification after ultrasonic and thermal pretreatments. However, when more than 15×10^3 spores were added to the reaction mixture, no amplification product was formed (Fig. 1). Spore debris and/or excess of calcium ions released from the spore cortex into the reaction mixtures, possibly inhibiting the *Taq* polymerase activity by competition with magnesium ions in the buffer, may have adversely affected DNA amplification.

Spores were chosen to test the effectiveness of ultrasound and heat treatments for lysis of this organism, since they are the most resistant form of *C. botulinum*. They are also the form expected to be found in environmental samples and in tissues and old cultures in which organisms are no longer actively growing. Twenty-four-hour broth cultures containing mostly vegetative forms of this microorganism did not need to be

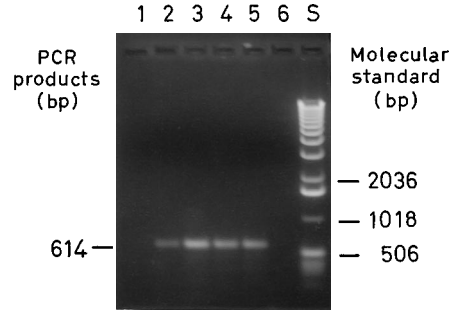

FIG. 1. Sensitivity of this PCR assay with the CI and CII primer set. The numbers of ultrasonically pretreated spores of *C. botulinum* type C (CL102) per PCR mixture were 15×10^4 (lane 1), 15×10^3 (lane 2), 15×10^2 (lane 3), 15×10 (lane 4), 15 (lane 5), and <15 (lane 6). Lane S, molecular size standards (1-kb DNA ladder; Gibco-BRL).

TABLE 2. Comparison of the results obtained by type C toxin detection[a] and PCR directly from samples and from CM enrichment cultures

Sample	Detection			
	Before enrichment		After enrichment	
	Toxin	PCR	Toxin	PCR
Soil 1	NT[b]	−	+	+
Soil 2	NT	−	−	−
Mud 1	NT	+	+	+
Mud 2	NT	−	+	+
Mallard 1 intestine	+	+	+	+
Mallard 2 intestine	−	+	+	+
Mallard 3				
Intestine	−	+	+	+
Spleen	−	−	+	+
Liver	−	+	+	+
Stomach	−	−	+	+
Grey heron 1				
Intestine	−	−	−	−
Spleen	−	−	−	−
Liver	−	−	−	−
Stomach	−	−	+	+
Grey heron 2				
Intestine	−	−	−	−
Spleen	−	+	+	+
Liver	−	−	−	−
Stomach	−	−	−	−
Grey heron 3 serum	−	NT	NT	NT
Grey heron 4 serum	−	NT	NT	NT

[a] By mouse neutralization test with 10 IU of monospecific antiserum per ml.
[b] NT, not tested.

sonicated, since thermal pretreatment alone was sufficient to release the DNA for amplification (data not shown).

Toxin and organism detection. The results obtained by PCR and conventional methods based on the mouse bioassay are summarized in Table 2. Type C neurotoxin was directly detected by the mouse test in only 1 of the 16 animal specimens analyzed, i.e., the intestinal contents of a mallard. Toxin has been demonstrated to bind very quickly to its neurological targets (15); this could be the reason why its detection in these specimens, including sera, was so rare. No other botulinum toxin was detected in any sample. Environmental samples were not assayed for the presence of neurotoxin, since this is not required by conventional methodologies.

A PCR product of the expected size (~600 bp) was obtained from five avian specimens, including the one positive for type C toxin detection; these results presumably indicated the presence of *C. botulinum* type C even in samples which did not contain neurotoxin. PCRs directly performed on environmental samples were negative, except for one mud sample. The available amounts of avian sera were insufficient to try to detect *C. botulinum* by PCR, as suggested by Szabo et al. (30).

However, after enrichment in CM broth, eight avian organ specimens and three environmental samples contained the type C neurotoxin and hence the microorganism producing it (Table 2); all of them yielded amplification products of the expected size by PCR, thus showing 100% correlation between PCR and the mouse bioassay at this step.

The presumptive presence of inhibitory substances or large numbers of other bacteria might have limited the sensitivity of this PCR assay when performed directly on environmental and animal samples which became positive by PCR after enrichment. Spore numbers of $>15 \times 10^3$ in the PCR reaction mixtures containing these samples may be also speculated.

Isolation and characterization of neurotoxigenic organisms. Single-colony isolates from EYA plates were tested by PCR. Those yielding the 614-bp PCR product were confirmed to produce the type C neurotoxin by the mouse neutralization test. A total of 11 neurotoxigenic strains were isolated from eight samples and phenotypically characterized. All were nonproteolytic and strongly hemolytic on blood agar plates (9) and showed the same sugar fermentation and acid production patterns. The sugars strongly fermented were glucose and trehalose, and those fermented more weakly were maltose, sucrose, glycerol, and arabinose. Gas-liquid chromatographic analysis showed the presence of acetic, propionic, and butyric acids. Furthermore, both lipase and lecithinase reactions were positive. On the basis of these results, each strain was classified as *C. botulinum* type C (19).

DISCUSSION

Botulism occurs more frequently in animals than it does in humans (6). A large number of animals may be affected, with both environmental and economic impact. Other outbreaks of animal botulism have previously been recorded in Italy (2–5). All occurred during the summer, when the decreased levels of water in canals cause the deaths of various living organisms and consequently the proliferation of anaerobic bacteria. Birds contribute to the spread of microorganisms. Rapid detection of the causative agent may be useful in order to immunize domestic animals living in the same area or to prevent epizootics among the wild fauna. During investigation of this outbreak of avian botulism, botulinum toxin type C was identified. Therefore, we developed a PCR method to detect the microorganism producing it either directly in bird and environmental samples or in enrichment cultures.

So far, PCR technology has been successfully applied in the detection of type A through E botulism neurotoxin genes, even directly in food, soil, and clinical specimens (12–14, 29, 30). Although it offers many advantages, such as rapidity, specificity, and sensitivity, the amplification of part of the genes does not necessarily demonstrate the presence of neurotoxin in the matrix; since in most cases human botulism follows the direct ingestion of botulinum toxin with contaminated food, this may be a limit of the technique when applied, for instance, to the identification of the causative source. However, the case is different for an investigation of animal botulism. Whether the disease in animals is an infection, an intoxication, or both is still to be clarified, but some evidence shows that in vivo production of the botulinum toxin by *C. botulinum* spores is likely frequent in animals (8). Therefore, isolation of *C. botulinum* from affected animals and their habitat is desirable to establish an eventual cause-effect relationship.

C. botulinum type C is a difficult organism to isolate (16) because of some of its features, such as the requirement for strict anaerobiosis and the instability of toxigenicity due to the loss of the phage carrying the neurotoxin gene after several transfers in cultural media. Our PCR method allowed us to detect a specific segment of the type C neurotoxin gene directly from some specimens which were nontoxic by the mouse bioassay and from all toxic enrichment cultures in a short time (~6 h). Indeed, no DNA extraction from samples was necessary, since ultrasonic and thermal pretreatments were sufficient to release enough DNA template for amplification even from spores. In our procedure, the sonication pretreatment likely sensitizes the spores to subsequent heating, avoiding time-consuming DNA extraction procedures.

PCR also helped in the recovery of toxigenic organisms from EYA plates; at least 10 colonies from each plate were tested simultaneously by this method, with savings of laboratory animals and antitoxin reagents. Usually, the majority of isolates are nontoxigenic; by PCR, we screened many colonies and positively identified those possessing the toxin gene. Eleven toxigenic isolates were recovered in total; all had the same phenotypic features and were characterized as *C. botulinum* type C. Conventional methodologies for the isolation of *C. botulinum* type C from EYA plates count on positive lipase and lecithinase reactions as markers; however, the latter is variable (25) and a positive lipase reaction alone does not distinguish this type from other *C. botulinum* types or from other *Clostridium* species.

In conclusion, our PCR protocol provides a good alternative to conventional methodologies for routinely screening a large number of samples; genetic features of the isolated strains can also be confirmed by this DNA-based technique.

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