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Specific Detection of *Clostridium botulinum* Type B by Using the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) and a radiolabeled oligonucleotide probe were used to specifically detect proteolytic and nonproteolytic *Clostridium botulinum* type B. Two synthetic primers deduced from the amino acid sequence data of type B neurotoxin were used to amplify a 1.5-kbp fragment corresponding to the light chain of the toxin. Although, nonspecific priming was observed when the PCR protocol was tested with other clostridial species, only the PCR product from *C. botulinum* type B isolates reacted with the radiolabeled internal probe. As little as 100 fg of DNA (approximately 35 clostridial cells) could be detected after only 25 amplification cycles.

The severe neuroparalytic illness caused by *Clostridium botulinum* neurotoxins types A to G necessitates a rapid diagnosis and the immediate implementation of the appropriate control measures by health authorities during suspected foodborne outbreaks. The diagnosis of botulism is confirmed by clinical observation together with the detection of *C. botulinum* toxin or cells. Presently, the only method of sufficient sensitivity for the detection of BotNTs is the mouse lethality test or bioassay (8). However, this assay takes up to 3 days to complete and the culture of *C. botulinum* prior to the bioassay may take up to 7 days depending on competing microflora and *C. botulinum* neurotoxin type (8). In addition, bioassays are considered undesirable and unethical today. Alternative in vitro immunological methods for toxin detection, which include electroimmunodiffusion (13), reversed passive latex haemagglutination (8), radioimmunoassay (1), and enzyme-linked immunosorbent assays (6, 7, 15), have been described previously. Only the ELISA procedure has demonstrated sensitivity approaching that of the bioassay, but none has been accepted for commercial production. This study reports the preliminary development of a sensitive and specific detection test for *C. botulinum* type B by using the polymerase chain reaction (PCR) (14) and a radiolabeled oligonucleotide probe.

Ten isolates of *C. botulinum* type B, representatives of toxin types A, C, D, E, and F, and closely related species were used in this study. The culturing and maintenance of strains was as previously described (7).

Chromosomal DNA was isolated from all bacteria by the method of Marmur (12). The concentration and purity of the DNA were determined spectrophotometrically (11). Crude, boiled (15 min) cell lysates were also used as a source of template DNA. PCR amplification was performed on DNA extracts by using a programmable cycling Autogene (Grant) waterbath. Two 21-mer oligonucleotide primers were deduced from the available published amino acid sequence data of *C. botulinum* neurotoxin type B (3). The upstream primer [B1; 5′-GATGGAACCACCATTTGC(T/A)AG-3′] was based on the amino acid sequence data of the light chain from the wobble position of Met-20 to Arg-27. The downstream primer [B2; 5′-(T/A)ACATC(T/A)ATACA(T/A)ATTCCTGG-3′] was derived from amino acid sequence data of the heavy chain from Pro-3 to Val-19. An internal 39-mer probe (B3; 5′-TATTATAAGGCTTTCAAAATAACAGATAGAATTTGGATA-3′), which corresponds to Try-32 to Ile-44 of the light chain of BotNT type B was also synthesized. At positions of degeneracy, the choice of nucleotide was made on the basis of codon usage by clostridial genes (16) and comparison of the codon bias of the related tetanus toxin gene (5). All oligonucleotides were synthesized by the Queensland Institute of Medical Research. PCR amplification proceeded by using standard conditions (9). Denaturation, annealing, and extension temperatures and times were 94°C for 30 s, 37°C for 20 s, and 60°C for 4 min, respectively. A final extension period of 10 min was carried out after 25 cycles. Negative controls containing all reagents except template DNA were routinely included. PCR products were examined on a 1.0% (wt/vol) agarose gel (SeaKem) run in TAE buffer (11) and visualized by the method of Ausubel et al. (2). PCR products were transferred from the agarose gel to nylon filters with a TE 80 Transvac Vacuum blotting unit (Hoeffer Scientific Instruments) with 0.4 N NaOH–0.6 M NaCl as the transfer solution.

The specificity and sensitivity of the PCR was tested by hybridization of PCR products from clostridial species with an internal oligonucleotide probe labeled at the 5′ end with [γ-^{32}P]ATP (Bresatech) by using bacteriophage T4 polynucleotide kinase (New England BioLabs) (11). The Sephadex G-50 spun column procedure was used to separate labeled probe from unincorporated nucleotides (11). Prehybridization (37°C for 1 h), hybridization (63°C overnight), and posthybridization washes were performed as previously described (2). After washing, the filters were air dried. Autoradiograms were exposed for 36 h at −70°C by using Fuji RX film and intensifying screens.

A vector-free biotinylated probe was also produced by the method of Lo et al. (10) with *C. botulinum* NCTC 7273 DNA and used to determine the specificity and sensitivity of the PCR. Hybridization conditions, posthybridization washes, and visualization of the biotin-11-dUTP-labeled PCR probe-target hybrid with the Blu-Gene nonradioactive nucleic acid detection system (Bio-Rad Laboratories) were performed according to the manufacturers' instructions. Hybridizations were carried out at 42°C overnight and at 42°C for 4 h for sensitivity and specificity determinations, respectively.

C. botulinum type B genomic DNAs from all proteolytic

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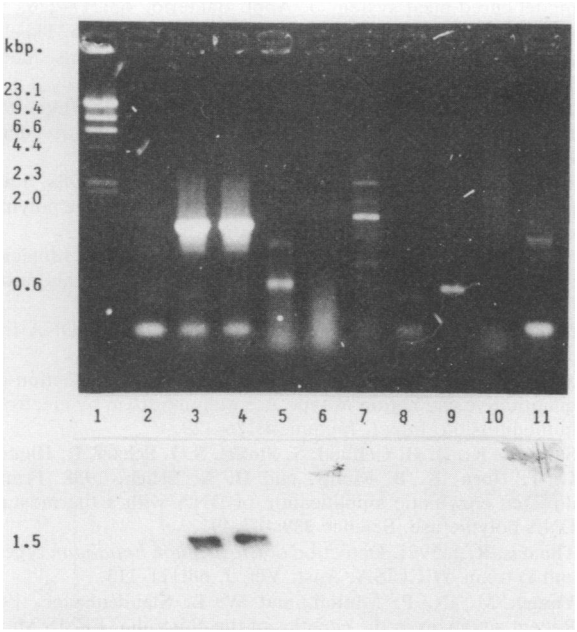

FIG. 1. Ethidium bromide-stained agarose gels of PCRs with template DNAs from *C. botulinum* and other clostridia. The representative samples were phage lambda DNA digested with *Hin*dIII (lane 1), *C. botulinum* type A ATCC 441 (lane 2), *C. botulinum* type B (proteolytic) NCTC 7273 (lane 3), *C. botulinum* type B (nonproteolytic) 17 B (lane 4), *C. botulinum* type C NCTC 8264 (lane 5), *C. botulinum* type D NCTC 8265 (lane 6), *C. botulinum* type E Naniamo (lane 7), *C. botulinum* type F NCTC 10281 (lane 8), *C. tetani* UQM 58 (lane 9), *C. sporogenes* UQM 60 (lane 10), and *C. butyricum* UQM 2646 (lane 11). The amount of material loaded into each well of the gel represented 1/10 of the PCR reaction volume. Reaction products were analyzed by Southern transfer of agarose gels and hybridized with an oligonucleotide internal probe labeled at the 5′ end with [γ-^{32}P]ATP. Only the PCR product from *C. botulinum* type B isolates reacted with the radiolabeled probe.

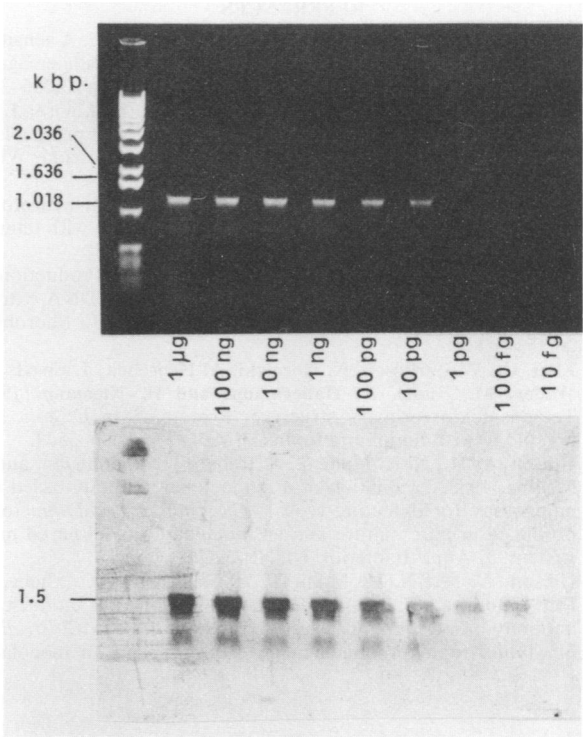

FIG. 2. Sensitivity of PCR for the detection of *C. botulinum* DNA. The numbers between the top and bottom of the figure represent the amount of DNA contained in 10 µl of the reaction mixture prior to amplification. The lowest DNA concentration detected after agarose gel electrophoresis and ethidium bromide staining was 10 pg (top). This limit was reduced to 100 fg when corresponding samples were submitted to Southern hybridization with a biotinylated DNA probe derived from a PCR product (bottom). The molecular weight standard was a commercially available 1-kb DNA ladder (Bio-Rad Laboratories). One-tenth of the PCR reaction volume was loaded into each well of the gel.

and nonproteolytic strains tested were amplified with primers B1 and B2 and produced a single fragment of approximately 1.5 kbp (Fig. 1). Vacuum blots of electrophoresed PCR products hybridized to the labeled internal probe (B3), confirming that the amplified fragment corresponded to the expected portion of the light chain of the type B toxin gene.

The specificity of the PCR was assessed with purified DNA extracted from *C. botulinum* toxin types A to F, *C. tetani*, and nontoxigenic clostridia (Fig. 1). Purified DNAs from *C. botulinum* type E, *C. sporogenes* UQM 60 and *C. butyricum* UQM 2646 also produced PCR products of approximately 1.5 kbp. When the annealing temperature was increased to 50°C, nonspecific amplification was still observed with *C. sporogenes* and *C. butyricum*. However, only the PCR product from *C. botulinum* type B isolates reacted with the radiolabeled internal probe (Fig. 1) or the biotinylated probe under the specified hybridization temperature and time (results not shown).

Serial 10-fold dilutions of *C. botulinum* NCTC 7273 genomic DNA (1 µg to 1 fg) were amplified to determine the sensitivity of the PCR. The lowest amount of template that produced an observable product on an agarose gel after 25 cycles was 10 pg (Fig. 2). This detection level was decreased to 100 fg when the biotinylated PCR product was used to probe vacuum blots (Fig. 2). Given that the molecular mass of *C. botulinum* chromosomal DNA is 1.8×10^9 Da (4), 100 fg corresponds to approximately 30 to 35 cells. Probing with the radiolabeled internal oligonucleotide was found to be less sensitive, reducing the detection level to 1 pg (results not shown).

The present results, therefore, demonstrate that PCR is both a specific and sensitive test for the reliable identification of proteolytic and nonproteolytic *C. botulinum* type B. Unlike the bioassay, the current PCR protocol takes 7 h. If a crude, boiled cell lysate is used, this is a simple and rapid procedure which also destroys the heat-labile neurotoxin that may be present, thus reducing some of the hazards associated with the handling of *C. botulinum*. The application of this methodology for the identification of *C. botulinum* directly from food and clinical samples is in progress.

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REFERENCES

1. **Ashton, A. C., J. S. Crowther, and J. O. Dolly.** 1985. A sensitive and useful radioimmunoassay for neurotoxin and its haemagglutinin complex from *Clostridium botulinum*. Toxicon **23**:235–246.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. Green Publishing Associates and Wiley Interscience, New York.
3. **DasGupta, B. R., and A. Datta.** 1988. Botulinum neurotoxin type B (strain 657): partial sequence and similarity with tetanus toxin. Biochemie **70**:811–817.
4. **Durban, E., E. M. Durban, and N. Grecz.** 1974. Production of spore spheroplasts of *Clostridium botulinum* and DNA extraction for density gradient centrifugation. Can. J. Microbiol. **20**:353–358.
5. **Eisel, U., W. Jarausch, K. Goretzki, A. Henschen, J. Engels, U. Weller, M. Hudel, E. Habermann, and H. Niemann.** 1986. Tetanus toxin: primary structure, expression in *E. coli*, and homology with botulinum toxins. EMBO J. **5**:2495–2502.
6. **Gibson, A. M., N. K. Modi, T. A. Roberts, P. Hambleton, and J. Melling.** 1988. Evaluation of a monoclonal antibody-based immunoassay for detecting type B *Clostridium botulinum* toxin produced in pure culture and an inoculated model cured meat system. J. Appl. Bacteriol. **64**:285–291.
7. **Gibson, A. M., N. K. Modi, T. A. Roberts, C. C. Shone, P. Hambleton, and J. Melling.** 1987. Evaluation of a monoclonal antibody-based immunoassay for detecting type A *Clostridium botulinum* toxin produced in pure culture and an inoculated model cured meat system. J. Appl. Bacteriol. **63**:217–226.
8. **Hauschild, A. H. W.** 1989. *Clostridium botulinum*, p. 111–189. *In* M. P. Doyle (ed.), Food-borne bacterial pathogens. Marcel Dekker, Inc., New York.
9. **Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White.** 1990. PCR protocols—a guide to methods and applications. Academic Press, Inc., New York.
10. **Lo, Y.-M.D., W. Z. Mehl, and K. A. Fleming.** 1988. Rapid production of vector-free biotinylated probes using the polymerase chain reaction. Nucleic Acids Res. **16**:8719.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Marmur, J.** 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. **3**:208–218.
13. **Miller, L. G., and A. W. Anderson.** 1971. Rapid detection and quantitative estimation of type A botulinum toxin by electroimmunodiffusion. Infect. Immun. **4**:126–129.
14. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
15. **Thomas, R. J.** 1991. Detection of *Clostridium botulinum* types C and D toxin by ELISA. Aust. Vet. J. **68**:111–113.
16. **Young, M., N. P. Minton, and W. L. Staudenbauer.** 1989. Recent advances in the genetics of the clostridia. FEMS Microbiol. Rev. **63**:301–326.