



Bacteriological Analytical Manual

Chapter 17: *Clostridium botulinum*

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Introduction

Clostridium botulinum is an anaerobic, rod-shaped sporeforming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 (2) involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism.

Antigenic types of *C. botulinum* are identified by the complete neutralization of their toxins using the homologous antitoxin. Cross-neutralization of a specific toxin by heterologous antitoxins does not occur or is minimal. There are seven recognized antigenic types: A through G. Cultures of five of these types apparently produce only one type of toxin but all are given type designations corresponding to their toxin production. Types C and D cross-react with antitoxins to each other because they each produce more than one toxin and have at least one common toxin component. Type C produces predominantly C₁ toxin with lesser amounts of D and C₂, or only C₂, and type D produces predominantly type D toxin along with smaller amounts of C₁ and C₂. Mixed toxin production by a single strain of *C. botulinum* may be more common than previously realized. There is a slight reciprocal cross-neutralization with types E and F, and recently a strain of *C. botulinum* was shown to produce a mixture of predominantly type A toxin, with a small amount of type F.

Aside from toxin type, *C. botulinum* can be differentiated into general groups on the basis of cultural, biochemical, and physiological characteristics. Cultures producing types C and D toxins are not proteolytic on coagulated egg white or meat and have a common metabolic pattern which sets them apart from the others. All cultures that produce type A toxin and some that produce B and F toxins are proteolytic. All type E strains and the remaining B and F strains are nonproteolytic, with carbohydrate metabolic patterns differing from the C and D nonproteolytic groups. Strains that produce type G toxin have not been studied in sufficient detail for effective and satisfactory characterization.

C. botulinum is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to contaminated fish or other seafoods. Types A and B are most commonly encountered in foods associated with soil contamination. In the United States, home-canned

vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of foodborne illness caused by these types.

Measures to prevent botulism include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all botulinal spores in the food. Heat processing is the most common method of destruction. Properly processed canned foods will not contain viable *C. botulinum*. Home-canned foods are more often a source of botulism than are commercially canned foods, which probably reflects the commercial canners' great awareness and better control of the required heat treatment.

A food may contain viable *C. botulinum* and still not be capable of causing botulism. If the organisms do not grow, no toxin is produced. Although many foods satisfy the nutritional requirements for the growth of *C. botulinum*, not all of them provide the necessary anaerobic conditions. Both nutritional and anaerobic requirements are supplied by many canned foods and by various meat and fish products. Growth in otherwise suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity, a high concentration of NaCl, an inhibitory concentration of NaNO₂ or other preservative, or two or more of these conditions in combination. Refrigeration will not prevent growth and toxin formation by nonproteolytic strains unless the temperature is precisely controlled and kept below 3°C. Foods processed to prevent spoilage but not usually refrigerated are the most common vehicles of botulism.

Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for nonproteolytic strains it is 26-28°C. Nonproteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the nonproteolytics do not manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form. These and other differences can be important in epidemiological and laboratory considerations of botulism outbreaks. Clinical diagnosis of botulism is most effectively confirmed by identifying botulinal toxin in the blood, feces, or vomitus of the patient. Specimens must be collected before botulinal antitoxin is administered to the patient. Identifying the causative food is most important in preventing additional cases of botulism. See Examination of Canned Foods, Chapter 21.

Botulism in infants 6 weeks to 1 year of age was first recognized as a distinct clinical entity in 1976. This form of botulism results from growth and toxin production by *C. botulinum* within the intestinal tract of infants rather than from ingestion of a food with preformed toxin. It is usually caused by *C. botulinum* types A or B, but a few cases have been caused by other types. Infant botulism has been diagnosed in most U.S. states and in every populated continent except Africa (1).

Constipation almost always occurs in infant botulism and usually precedes characteristic signs of neuromuscular paralysis by a few days or weeks. Illnesses have a broad range of severity. Some infants show only mild weakness, lethargy, and reduced feeding and do not require hospitalization. Many have shown more severe symptoms such as weakened suck, swallowing, and cry; generalized muscle weakness; and diminished gag reflex with a pooling of oral secretions. Generalized muscle weakness and loss of head control in some infants reaches such a degree of severity that the patient appears "floppy." In some hospitalized cases, respiratory arrest has occurred, but most were successfully resuscitated, and with intense supportive care have ultimately recovered. As a result, the case-fatality rate (2%) for this form of botulism is low. Recovery usually requires at least several weeks of hospitalization (1).

Honey, a known source of *C. botulinum* spores, has been implicated in some cases of infant botulism. In studies of honey, up to 13% of the test samples contained low numbers of *C. botulinum* spores (3). For this reason, the FDA, the Centers for Disease Control and Prevention (CDC), and the American Academy of Pediatrics recommend not feeding honey to infants under one year old.

The mouse bioassay is a functional assay that detects biologically active toxin. The assay requires a three part approach: toxin screening, toxin titer, and finally toxin neutralization using monovalent antitoxins. The process requires two days of analysis at each step.

Recently, rapid, alternative, in-vitro procedures have been developed for the detection of types A, B, E, and F botulinal toxin producing organisms and their toxins. The toxins generated in culture media can be detected using ELISA techniques such as the DIG-ELISA and the amp-ELISA. Biologically active and non-active toxins are detected since the assay detects the toxin antigen. The ELISA assays require one day of analysis. The toxin genes of viable organisms can be detected using the polymerase chain reaction technique and require one days of analysis after overnight incubation of botulinal spores or vegetative cells. In-vitro assays that are positive are confirmed using the mouse bioassay.

I. Mouse Bioassay for *Clostridium botulinum* Toxin

A. Equipment and Materials

1. Refrigerator
2. Clean dry towels
3. Bunsen burner
4. Sterile can opener (bacteriological or puncture type)
5. Sterile mortar and pestle
6. Sterile forceps
7. Sterile cotton-plugged pipets
8. Mechanical pipetting device (**NEVER** pipet by mouth)
9. Sterile culture tubes (at least a few should be screw-cap tubes)
10. Anaerobic jars (GasPak or Case-nitrogen replacement)
11. Transfer loops
12. Incubators, 35 and 28°C

13. Sterile, reserve sample jars
14. Culture tube racks
15. Microscope slides
16. Microscope, phase-contrast or bright-field
17. Sterile petri dishes, 100 mm
18. Centrifuge tubes
19. Centrifuge, refrigerated, high-speed
20. Trypsin (1:250; Difco Laboratories, Detroit, MI)
21. Syringes, 1 and or 3 ml, sterile, with 25 gauge, 5/8 inch needles for injecting mice
22. Mice, 16-24 g (for routine work, up to 34 g)
23. Mouse cages, feed, water bottles, etc.
24. Millipore filters: 0.45 µm pore size

B. [Media](#) and [Reagents](#)

1. Alcoholic solution of iodine (4% iodine in 70% ethanol) ([R18](#))
2. Chopped liver broth ([M38](#)) or cooked meat medium ([M42](#))
3. Trypticase-peptone-glucose-yeast extract (TPGY) ([M151](#)) broth or with trypsin (TPGYT) ([M151a](#))
4. Liver-veal-egg yolk agar ([M84](#)) or anaerobic egg yolk agar ([M12](#))
5. Sterile, gel-phosphate buffer, pH 6.2 ([R29](#))
6. Absolute ethanol
7. Gram stain reagents ([R32](#)), crystal violet ([R16](#)), or methylene blue ([R45](#)) solutions
8. Sterile physiological saline solution ([R63](#))
9. Monovalent antitoxin preparations, types A-F (obtain from CDC)
10. Trypsin solution (prepared from Difco 1:250)
11. 1 N Sodium hydroxide solution ([R73](#))
12. 1 N Hydrochloric acid solution ([R36](#))

C. Sample preparation

Preliminary examination. Refrigerate samples until testing, except unopened canned foods, which need not be refrigerated unless badly swollen and in danger of bursting. Before testing, record product designation, manufacturer's name or home canner, source of sample, type of container and size, labeling, manufacturer's batch, lot or production code, and condition of container. Clean and mark container with laboratory identification codes.

Solid and liquid foods. Aseptically transfer foods with little or no free liquid to sterile mortar. Add equal amount of gel-phosphate buffer solution and grind with sterile pestle before inoculation. Alternatively, inoculate small pieces of product directly into enrichment broth with sterile forceps. Inoculate liquid foods directly into enrichment broth with sterile pipets. Reserve sample; after culturing, aseptically remove reserve portion to sterile sample jar for tests which may be needed later. Refrigerate reserve sample.

Opening of canned foods (see Chapter 21).

Examine product for appearance and odor. Note any evidence of decomposition. **DO NOT TASTE** the product under any circumstances. Record the findings.

D. Detection of viable *C. botulinum*

1. **Enrichment.** Remove dissolved oxygen from enrichment media by steaming 10-15 min and cooling quickly without agitation before inoculation.

Inoculate 2 tubes of cooked meat medium with 1-2 g solid or 1-2 ml liquid food per 15 ml enrichment broth. Incubate at 35°C.

Inoculate 2 tubes of TPGY broth as above. Incubate at 28°C. Use TPGYT as alternative only when organism involved is strongly suspected of being a nonproteolytic strain of types B, E, or F.

Introduce inoculum slowly beneath surface of broth to bottom of tube. After 5 days of incubation, examine enrichment cultures. Check for turbidity, gas production, and digestion of meat particles. Note the odor.

Examine cultures microscopically by wet mount under high-power phase contrast, or a smear stained by Gram reagent, crystal violet, or methylene blue under bright-field illumination. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells. A typical clostridial cell resembles a tennis racket. At this time test each enrichment culture for toxin, and if present, determine toxin type according to procedure in F, below. Usually, a 5-day incubation is the period of active growth giving the highest concentration of botulinal toxin. If enrichment culture shows no growth at 5 days, incubate an additional 10 days to detect possible delayed germination of injured spores before discarding sample as sterile. For pure culture isolation save enrichment culture at peak sporulation and keep under refrigeration.

2. **Isolation of pure cultures.** *C. botulinum* is more readily isolated from the mixed flora of an enrichment culture or original specimen if sporulation has been good.

Pre-treatment of specimens for streaking. Add equal volume of filter-sterilized absolute alcohol to 1 or 2 ml of enrichment culture in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. To isolate from sample, take 1 or 2 ml of retained portion, and add an equal volume of filter-sterilized absolute alcohol in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. Alternatively, heat 1 or 2 ml of enrichment culture or sample to destroy vegetative cells (80°C for 10-15 min). **DO NOT** use heat treatment for nonproteolytic types of *C. botulinum*.

Plating of treated cultures. With inoculating loop, streak 1 or 2 loopfuls of ethanol or heat-treated cultures to either liver- veal-egg yolk agar or anaerobic egg yolk agar (or both) to obtain isolated colonies. If necessary, dilute culture to obtain well-separated colonies. Dry agar plates well before use to prevent spreading of colonies. Incubate streaked plates at 35°C for about 48 h under anaerobic conditions. A Case anaerobic jar or the GasPak system is adequate to obtain anaerobiosis; however, other systems may be used.

E. Selection of typical *C. botulinum* colonies

Selection. Select about 10 well-separated typical colonies, which may be raised or flat, smooth or rough. Colonies commonly show some spreading and have an irregular edge. On egg yolk medium, they usually exhibit surface iridescence when examined by oblique light. This luster zone, often referred to as a pearly layer, usually extends beyond and follows the irregular contour of the colony. Besides the pearly zone, colonies of *C. botulinum* types C, D, and E are ordinarily surrounded by a wide zone (2-4 mm) of yellow precipitate. Colonies of types A and B generally show a smaller zone of precipitation. Considerable difficulty may be experienced in picking toxic colonies since certain other members of the genus *Clostridium* produce colonies with similar morphological characteristics but do not produce toxins.

Inoculation. Use sterile transfer loop to inoculate each selected colony into tube of sterile broth. Inoculate *C. botulinum* type E into TPGY broth. Inoculate other toxin types of *C. botulinum* into chopped liver broth or cooked meat medium. Incubate as described in D-1, above, for 5 days. Test for toxin production as described in F, below. To determine toxin type, **see** F-3, below.

Isolation of pure culture. Restreak toxic culture in duplicate on egg yolk agar medium. Incubate one plate anaerobically at 35°C. Incubate second plate aerobically at 35°C. If colonies typical of *C. botulinum* are found only on anaerobic plate (no growth on aerobic plate), the culture may be pure. Failure to isolate *C. botulinum* from at least one of the selected colonies means that its population in relation to the mixed flora is probably low. Repeated serial transfer through additional enrichment steps may increase the numbers sufficiently to permit isolation. Store pure culture in sporulated state either under refrigeration, on glass beads, or lyophilized.

F. Detection and identification of botulinal toxin

1. **Preparation of food sample.** Culture one portion of sample for detection of viable *C. botulinum*; remove another portion for toxicity testing, and store remainder in refrigerator. Centrifuge samples containing suspended solids under refrigeration and use supernatant fluid for toxin assay. Extract solid foods with

equal volume of gel-phosphate buffer, pH 6.2, by macerating food and buffer with pre-chilled mortar and pestle. Centrifuge macerated sample under refrigeration and use supernatant fluid for toxin assay. Rinse empty containers suspected of having held toxic foods with a few milliliters of gel-phosphate buffer. Use as little buffer as possible to avoid diluting toxin beyond detection. To avoid or minimize nonspecific death of mice, filter supernatant fluid through a millipore filter before injecting mice. For non-proteolytic samples or cultures, trypsinize after filtration.

2. Determination of toxicity in food samples or cultures

Trypsin treatment. Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Therefore, treat a portion of food supernatant fluid, liquid food, or TPGY culture with trypsin before testing for toxin. Do not treat TPGYT culture with trypsin since this medium already contains trypsin and further treatment may degrade any fully activated toxin that is present. Adjust portion of supernatant fluid, if necessary, to pH 6.2 with 1 N NaOH or HCl. Add 0.2 ml aqueous trypsin solution to 1.8 ml of each supernatant fluid to be tested for toxicity. (To prepare trypsin solution, place 0.5 g of Difco 1:250 trypsin in clean culture tube and add 10 ml distilled water, shake, and warm to dissolve. Analysts who are allergic to trypsin should weigh it in a hood or wear a face mask.) Incubate trypsin-treated preparation at 35-37°C for 1 h with occasional gentle agitation.

Toxicity testing. Conduct parallel tests with trypsin-treated materials and untreated duplicates. Dilute a portion of untreated sample fluid or culture to 1:5, 1:10, and 1:100 in gel-phosphate buffer. Make the same dilutions of each trypsinized sample fluid or culture. Inject each of separate pairs of mice intraperitoneally (i.p.) with 0.5 ml untreated undiluted fluid and 0.5 ml of each dilution of untreated test sample, using a 1 or 3 ml syringe with 5/8 inch, 25 gauge needle. Repeat this procedure with trypsin-treated duplicate samples. Heat 1.5 ml of untreated supernatant fluid or culture for 10 min at 100°C. Cool heated sample and inject each of a pair of mice with 0.5 ml undiluted fluid. These mice should not die, because botulinal toxin, if present, will be inactivated by heating.

Observe all mice periodically for 48 h for symptoms of botulism. Record symptoms and deaths. Typical botulism signs in mice begin usually in the first 24 h with ruffling of fur, followed in sequence by labored breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure. Death of mice without clinical symptoms of botulism is not sufficient evidence that injected material contained botulinal toxin. On occasion, death occurs from other chemicals present in injected fluid, or from trauma.

If after 48 h of observation, all mice except those receiving the heated preparation have died, repeat the toxicity test, using higher dilutions of supernatant fluids or cultures. It is necessary to have dilutions that kill and dilutions that do not kill in order to establish an endpoint or the minimum lethal dose (MLD) as an estimate of the amount of toxin present. The MLD is contained in the highest dilution killing both mice (or all mice inoculated). From these data, the number of MLD/ml can be calculated.

3. **Typing of toxin.** Rehydrate antitoxins with sterile physiological saline. **Do not use glycerin water.** Dilute monovalent antitoxins to types A, B, E, and F in

physiological saline to contain 1 international unit (IU) per 0.5 ml. Prepare enough of these antitoxin solutions to inject 0.5 ml of antitoxin into each of 2 mice for each dilution of toxic preparation to be tested. Use the toxic preparation that gave the higher MLD, either untreated or trypsinized. Prepare dilutions of the toxic sample to cover at least 10, 100, and 1000 MLD below the previously determined endpoint of toxicity if possible (see 2, above). The untreated toxic preparation can be the same as that used for testing toxicity. If a trypsinized preparation was the most lethal, it will be necessary to prepare a freshly trypsinized fluid. The continued action of trypsin may destroy the toxin.

Inject the mice with the monovalent antitoxins, as described above, 30 min to 1 h before challenging them with i.p. injection of the toxic preparations. Inject pairs of mice (protected by specific monovalent antitoxin injection) i.p. with each dilution of the toxic preparation. Also inject a pair of unprotected mice (no injection of antitoxin) with each toxic dilution as a control. The use of 4 monovalent antitoxins (types A, B, E, and F) for the unknown toxic sample prepared at 3 dilutions requires a total of 30 mice — 6 mice for each antitoxin (24 mice) plus 2 unprotected mice for each of the 3 dilutions (6 mice) as controls. Observe mice for 48 h for symptoms of botulism and record deaths. If test results indicate that toxin was not neutralized, repeat test, using monovalent antitoxins to types C and D, plus polyvalent antitoxin pool of types A through F.

II. Mouse Screening Procedure for *Clostridium botulinum* Type E Spores in Smoked Fish

A. Equipment and Materials

1. 12 mice (16-24 g, or up to 34 g) per subsample (24 or more required for positives)
2. Types A, B, E antisera
3. Saline, sterile, 0.85% NaCl ([R63](#))
4. Trypsin (Difco); 1:250, 5% solution
5. Syringes, 1 and 3 ml, 25 gauge, 5/8 inch needle
6. Incubator 28°C
7. TPGY medium ([M151](#))
8. Water bath, 37°C
9. Gel-phosphate diluent ([R29](#))

10. Centrifuge, refrigerated

11. Plastic bags, strong and water-tight

B. Procedure

Incubation. Place each smoked fish subsample (which may consist of 1 or more fish, depending on size, and may be either vacuum-packed or bulk-smoked fish) in a strong water-tight plastic bag. Add freshly steamed and cooled TPGY broth to subsample. **NOTE:** Add enough TPGY broth to completely cover fish. Squeeze bag to expel as much air as possible and seal it with hot-iron bag sealer or other air-tight closure device. Incubate at 28°C for 5 days. Precautions should be taken during incubation period since bag may swell and split from gas formation.

Cultures. At end of incubation period, centrifuge 20 ml of TPGY culture from each subsample at 7500 × *g* rpm for 20 min. Use refrigerated centrifuge. Determine pH of TPGY. If above 6.5, adjust to 6.0-6.2 with HCl. Refrigerate for overnight storage.

Trypsinization. To 3.6 ml of culture, adjusted to pH 6.0-6.2, add 0.4 ml of 5% solution of trypsin. Incubate at 35-37°C for 1 h. Remove culture and let cool to room temperature before injecting mice. Trypsinized extract cannot be stored overnight.

Toxicity screening. Dilute trypsinized and nontrypsinized broth cultures to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (**NOTE:** Do not store trypsinized material overnight.) Inject mice i.p. with 0.5 ml of each dilution. Inject 2 mice per dilution, i.e., trypsinized and nontrypsinized (total 12 mice per subsample). Observe mice for botulism symptoms and record condition of mice at frequent intervals for 48 h. If no deaths occur, no further tests are indicated. Deaths are presumptive evidence of toxin and should be confirmed.

Confirmation with protected mice. Dilute new portion of nontrypsinized or trypsinized culture (whichever showed the highest titer) to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.) Inject 6 mice i.p. with 0.5 ml of 1:5 saline dilution of type E antiserum. These will be compared to 6 mice without this protection (controls). After 30 min, inject 0.5 ml of each dilution into 2 mice protected with antiserum and into 2 mice not so protected. Record their condition at intervals up to 48 h. If unprotected mice die and protected mice live, the presence of type E toxin is indicated. If all protected mice die, repeat confirmation with higher dilutions of toxic culture in type E-protected mice and with mice protected against *C. botulinum* types A and/or B antiserum. If all antiserum-protected mice die, send toxic culture media on dry ice to Division of Microbiological Studies (HFS-516), FDA, **5100 Paint Branch Pkwy, College Park, MD 20740**, for further tests. Isolate and identify cultures from samples containing toxin of type E, if possible.

Obtain *C. botulinum* antisera from Centers for Disease Control and Prevention, Atlanta, GA 30333, USA. Reconstitute lyophilized antisera with sterile saline. Dilute sera 1:5 with sterile saline for mouse injection.

If you have questions about the method, contact **Shashi Sharma**, FDA. Telephone **(240)-402-1570**.

General Hints Regarding *C. botulinum* Toxin Analysis

1. The first 24 hours are the most important time regarding symptoms and death of mice: 98-99% of animals die within 24 hours. Typical symptoms of botulism and death may occur within 4 to 6 hours.
2. If deaths occur after 24 hours, be very suspicious, unless typical botulism symptoms are clearly evident.
3. If deaths occur in mice injected with the 1:2 or 1:5 dilution but not with any higher dilution, be very suspicious. Deaths may have been from nonspecific causes.
4. Mice can be marked on tails with dye to represent various dilutions. Dye does not come off easily.
5. Mice injected with botulinal toxin may become hyperactive before symptoms occur.
6. Food and water may be given to the mice right away; it will not interfere with the test.
7. Rehydrated antitoxin may be kept up to 6 months under refrigeration, and may be frozen indefinitely.
8. TPGY medium is relatively stable and can be kept 2-3 weeks under refrigeration.
9. With cooked meat medium, vortex tubes completely; toxin may adhere to meat particles.
10. Trypsin is not filtered. Use 0.5 g in 10 ml of distilled water. It can be kept up to 1 week under refrigeration.

Interpretation of Data (NOTE: Laboratory tests are designed to identify botulinal toxin and/or organisms in foods)

1. Toxin in a food means that the product, if consumed without thorough heating, could cause botulism.
2. Viable *C. botulinum* but no toxin in foods is not proof that the food in question caused botulism.
3. The presence of toxin in food is required for an outbreak of botulism to occur.
4. Ingested organisms may be found in the alimentary tract, but are considered to be unable to multiply and produce toxin in vivo, except in infants.
5. Presence of botulinal toxin and/or organisms in low-acid (i.e., above pH 4.6) canned foods means that the items were underprocessed or were contaminated through post-processing leakage.

- Swollen cans are more likely than flat cans to contain botulinal toxin since the organism produces gas during growth.
 - Presence of toxin in a flat can may imply that the seams were loose enough to allow gas to escape.
 - Botulinal toxin in canned foods is usually of a type A or a proteolytic type B strain, since spores of the proteolytics can be among the more heat-resistant.
 - Spores of nonproteolytics, types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.
6. The protection of mice from botulism and death with one of the monovalent botulinal antitoxins confirms the presence of botulinal toxin and determines the serological type of toxin in a sample.
 7. The following reasons may explain why deaths occur in mice that are protected by one of the monovalent antitoxins:
 - There may be too much toxin in the sample.
 - More than one kind of toxin may be present.
 - Deaths may be due to some other cause.

Retesting at higher dilutions of toxic fluids is required, and mixtures of antitoxins must be used in place of monovalent antiserum. Some other toxic material, which is not heat-labile, could be responsible if both heated and unheated fluids cause death. The heat-stable toxic substance could possibly mask botulinal toxin.

Safety Precautions for the *Clostridium botulinum* Laboratory

1. Place biohazard signs on doors to restrict entrance and keep the number of people in the laboratory to a minimum.
2. All workers in the laboratory should wear laboratory coats and safety glasses.
3. Use 1% hypochlorite solution to wipe laboratory table tops before and after work.
4. **NEVER PIPETTE ANYTHING BY MOUTH. USE MECHANICAL PIPETTORS.**
5. Use a biohazard hood for transfer of toxic material, if possible.
6. Centrifuge toxic materials in a hermetically closed centrifuge with safety cups.
7. Personally take all toxic material to the autoclave and see that it is sterilized immediately.
8. Do not work alone in the laboratory or animal rooms after hours or on weekends.

9. Have an eye wash fountain and foot-pedaled faucet available for hand washing.
10. No eating and drinking in the laboratory when someone works with toxins.
11. In a very visible location, list phone numbers where therapeutic antitoxin can be obtained in case of emergency. **THIS IS VERY IMPORTANT!**
12. Reduce clutter in the laboratory to a minimum and place equipment and other materials in their proper place after use.

References

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III. Amplified ELISA Procedure for Detection of Botulinal Toxins A, B, E, and F from Culture. Contact Joseph L. Ferreira (404 253-2216) for questions about method.

These toxins can be detected using an amplified ELISA procedure that has a detection limit of approximately 10 MLD/mL. Toxic cultures may be more antigenic than purified toxins and the level of detection using the ELISA may be more sensitive than the mouse bioassay. Both TPGY and CMM are tested since more toxin may be generated in one medium compared to the other and the mouse bioassay, which is needed for confirmation of ELISA tests, also utilizes these media.

A. Equipment and Materials

1. Microplate, Dynex Immulon II U-bottom, cat. No. 3655
2. Microtiter pipettors to deliver from 0.1- 2.0, 2-20, and 50-200 μ l.

3. Multichannel pipettor, 8 or 12 place 50-200 μ l
4. Pipets, disposable 1,5,10 ml
5. Glass test tubes 13X100 mm, 15X150 mm
6. Incubator, 35°C
7. Refrigerated centrifuge
8. Microplate washer
9. Microplate shaker
10. Microplate reader (read 490 and 630 nm reference)
11. Microtiter plate seals
12. Multichannel pipet reservoirs

B. Media and Reagents

1. Tryptone-peptone-glucose-yeast extract broth (TPGY).
2. Cooked meat medium (CMM).
3. 0.05M bicarbonate buffer: 0.8g Na_2CO_3 + 1.47g NaHCO_3 in 500 ml distilled H_2O , pH 9.6.
4. 1% Casein buffer: Add 10.0g vitamin-free casein + 7.65 g NaCl, 0.724g Na_2HPO_4 (anhydrous), 0.21g KH_2PO_4 to 900 ml H_2O , and 3 ml of 1 M NaOH. Heat with stirring to ~ 80°C to dissolve casein. Check pH and adjust to 7.9 with 1 M NaOH, q.s. to 1 liter. Sterilize at 121°C for 20 min. Final pH is ~7.4-7.6.
5. Goat type A or E, rabbit type B, or horse F antitoxin.
6. Goat type A, B, E, or F biotinylated antitoxin
7. Tris buffered NaCl-0.005% Tween 20 (TBST): 6.04g Tris base, 8.76g NaCl, Distilled H_2O 900 ml, dissolve Tris and NaCl, pH adjust to 7.5 at 25°C with 2 M HCl, add 50 μ l of Tween-20 and q.s. to 1 liter.
8. Extravidin-alkaline phosphatase conjugate (Sigma)
9. Amplified ELISA substrate system (GibCo)
10. 0.3 M H_2SO_4 : dilute concentrated acid (MW 98, specific gravity 1.84, purity 96-98%) by adding 1 ml to 59 ml of distilled H_2O .
11. Botulinal complex toxin standards A, B, E, and F. (Metabiotics Inc., Madison, WI)

C. Amplified ELISA Procedure

1. **Preparation of samples.** Food samples or anaerobic isolates picked from agar plates are inoculated into TPGY (**without trypsin**) and CMM as recommended in Chapter 17 of the Bacteriological Analytical Manual (2001). TPGY broth and cooked meat media are incubated for 5 days at 26°C and 35°C respectively. Cultures are centrifuged at 7,000 × g and 4°C for 30 min, supernatant pH is adjusted to 7.4-7.6 using 1 N NaOH or 1N HCl. Samples and controls are analyzed **in duplicate** for TPGY and for CMM. Analyze undiluted and 1:5 dilutions of each culture supernatant. 1:5= 0.2 ml culture + 0.8 ml casein buffer.
2. **Preparation of microtiter plates.** Coat each well of the microtiter plate with 100 µl of appropriate dilution of goat type A, E, or F or rabbit type B antitoxin diluted in bicarbonate buffer. Prepare the number of needed microtiter plate wells to test the sample. Dilute the stock antitoxins according to the accompanying directions. Store plate with coating buffer overnight at 4°C with plastic seal cover on top of plate to prevent drying.
3. **ELISA analysis of culture media.**

- a. Remove plate from 4°C storage and wash plate 5 times in Tris buffered saline (TBST) with 45 second hold between each aspiration. Use a commercial plate washer or other mechanical device; avoid using a squeeze bottle to wash.
- b. Block plate in casein buffer with by filling all wells to the top of the plate (~300 µl/well) and incubate for 60-90 min at 35°C. Prepare the sample and control dilutions while the plate is being blocked.

Negative controls: Duplicate wells with all reagents except toxin (undiluted sterile CMM and TPGY broth).

Positive controls: Test standard toxins type A, B, E, and F diluted in sterile TPGY and CMM (pH 7.6) at a concentration of 2 ng/ml (~2-60 LD₅₀/ng depending on toxin type).

- c. Wash the blocked plate as above and then add the toxic samples and controls (100 µl/well). Work from the left side of the plate to the right side when adding the reagents.
- d. Incubate toxin-containing samples and controls for 2 hr. at 35°C. Prepare the type A, B, E, and F biotin-labeled antibody reagents according to directions while incubating the samples. Do not make more than you need!
- e. Wash plate 5 times in TBST as above.
- f. Add the diluted biotin-labeled goat antibody (100 µl/well) and incubate for 60 min at 35°C.
- g. Wash plate 5 times in TBST as above.

- h. Add the streptavidin-alkaline phosphatase conjugate diluted 1:10,000 in casein buffer (100 µl/well), and incubate for 60 min at 35°C.
- i. Wash 5 times in TBST with a final 10 minute soak (the last buffer wash is not aspirated). After 10 minute soak, discard the wash and tamp the plate several times on a paper towel to remove wash buffer.
- j. Add 50 µl of the GIBCO substrate solution, incubate 12.5 min at room temperature on plate shaker (~100 rpm) then add 50 µl of the GIBCO amplifier and incubate for approximately an additional 10 min. without shaking. The plate should be taken to the plate reader immediately after addition of the amplifier reagent and be ready to read the reactions. Read absorbance at 490 nm with 630 nm subtraction (reference filter) to account for plate absorbance. The analysis can be stopped at any time (2-15 min) after addition of the amplifier when positive controls give appropriate sensitivity (absorbance ≥ 1.0) and negative controls are acceptable (absorbance not greater than ~ 0.30). The reaction can be stopped with 50 µl of 0.3 M H₂SO₄ and the absorbance read up to two hours later.

Results: A positive test is an absorbance value that is >0.20 above the absorbance observed in the negative controls (sterile uninoculated TPGY broth or CMM).

D. Confirmation of positive ELISA samples.

The ELISA is used for screening culture media that may contain type A, B, E, and/or F botulin toxins. Samples that are positive using the ELISA must be confirmed using the mouse bioassay.

Flow Diagram for Amp-ELISA

Day 1

Coat microtiter plates with capture IgG and store overnight at 4°C.

Day 2

1. Wash plates, block, put on toxic samples and controls, 2 hr incubate.
2. Wash, put on biotinylated IgG's, 1 hr incubate.
3. Wash, put on the Extravidin conjugate, 1 hr incubate.
4. Wash, put on Gibco substrate, 12.5 min incubate
5. Put on Gibco amplifier, 2-10 min incubate.
6. Read plates on microplate reader

References

1. Ferreira, J L., Maslanka, S, Johnson, E., and Goodnough, M. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked Immunosorbent assay: collaborative study. *JAOAC International* **86**:314-331.
2. Solomon, H. and Lilly, T. 2001. FDA Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum*.
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A modification of the method described above is available in Laboratory Information Bulletin (LIB) No. 4292. The LIB describes a modification that uses digoxigenin labeled IgGs to detect type A, B, E, and F botulinal toxins. The digoxigenin label substitutes for the biotin label in the amplified ELISA and is detected using an anti-digoxigenin horse radish peroxidase conjugate and TMB substrate.

IV. Detection of Type A, B, E, and F *Clostridium botulinum* Toxins Using Digoxigenin-labeled IgGs and the ELISA (DIG-ELISA).

Contact J. L. Ferreira (FDA) 404 253-2216, S. Sharma (FDA) 301 436-1570, S. Maslanka (CDC) 404 639-0895, or J. Andreadis (CDC) for questions regarding this method.

This method is a modification of the amplified-ELISA (amp-ELISA). Digoxigenin-labeled antitoxin IgG's are substituted for biotin-labeled IgG's and anti-digoxigenin horse radish peroxidase conjugate (HRP) is substituted for the streptavidin-alkaline phosphatase used in the amp-ELISA. An appropriate substrate (TMB) is used for the HRP enzyme. The A, B, E, and F botulinal toxins are detected at approximately 10 MLD/mL (0.12-0.25 ng/mL). Toxic cultures may be more antigenic than purified toxins and the level of detection using the DIG-ELISA may be more sensitive than the mouse bioassay. Both TPGY and CMM are tested since more toxin may be generated in one medium compared to the other and the confirmatory mouse bioassay also utilizes these media. Very toxic cultures (greater than approximately 10,000 MLD/mL) may give a positive absorbance for more than one toxin type in the amp-ELISA as well as the DIG-ELISA (crossing between types). Generally, a 10-fold dilution will show that the true toxin type will have a very high absorbance and the crossing type will have a negative absorbance. In either case the toxic sample must be confirmed using the mouse bioassay.

A. Equipment and Materials

1. Microplate, Dynex Immulon II U-bottom, cat. No. 3655

2. Microtiter pipettors to deliver from 0.1- 2.0, 2-20, and 50-200 μ l.
3. Multichannel pipettor, 8 or 12 place 50-200 μ l
4. Pipets, disposable 1,5,10 ml
5. Glass test tubes 13X100 mm, 15X150 mm
6. Incubator, 35°C
7. Refrigerated centrifuge
8. Microplate washer
9. Microplate shaker
10. Microplate reader (read 450 nm)
11. Microtiter plate seals
12. Multichannel pipet reservoirs

B. [Media](#) and [Reagents](#)

1. Tryptone-peptone-glucose-yeast extract broth (TPGY).
2. Cooked meat medium (CMM).
3. 0.05M bicarbonate buffer: 0.8g Na_2CO_3 + 1.47g NaHCO_3 in 500 ml distilled H_2O , pH 9.6. Capsules to prepare 100 ml volume are available from Sigma.
4. 1% Casein buffer: Add 10.0g vitamin-free casein (Research Organics) + 7.65g NaCl , 0.724g Na_2HPO_4 (anhydrous), 0.21g KH_2PO_4 to 900 ml H_2O , and 3 ml of 1 M NaOH . Heat with stirring to $\sim 80^\circ\text{C}$ to dissolve casein. Check pH and adjust to 7.9 with 1 M NaOH , q.s. to 1 liter. Sterilize at 121°C for 20 min. Final pH is ~ 7.4 -7.6. Casein blocker ready to use product is available from Pierce that gives slightly lower absorbance values than in-house prepared casein buffer. (SRL, Atlanta, GA).
5. Goat type A, B, E, or F digoxigenin-labeled antitoxin (SRL, Atlanta, GA).
6. Phosphate buffered saline with 0.005% Tween 20 wash buffer (PBST).
1.2 g Na_2HPO_4 (anhydrous), 0.22g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 8.5g NaCl per liter distilled H_2O . Adjust pH to 7.5 Add 50 μ l of Tween 20/L PBS. Sterilize at 121°C for 20 min. 10 \times PBST: 12.0g Na_2HPO_4 (anhydrous), 2.2g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 85.0g NaCl per liter distilled H_2O . Adjust pH to 7.5 Add 500 μ l of Tween 20/L PBS. 1 \times PBST is then prepared by adding 100 ml of 10X PBST to 900 ml of distilled H_2O and mixing before use. 10X PBS is available commercially from GibCo.
7. Anti-digoxigenin HRP poly conjugate (Roche Applied Science).
8. Tetra methyl benzidine (Ultra-TMB) (Pierce).

9. 0.5 M H₂SO₄.
10. Botulinal complex toxin standards A, B, E, and F. (Metabiologics Inc., Madison, WI)

C. DIG-ELISA Procedure

1. Preparation of samples.

- a. **Cultural sample preparation.** Food samples or anaerobic isolates picked from agar plates are inoculated into TPGY (**without trypsin**) and CMM as recommended in Chapter 17 of the Bacteriological Analytical Manual (2001). TPGY broth and cooked meat media are incubated for 5 days at 26°C and 35°C respectively. Cultures are centrifuged at 7,000 × g and 4°C for 30 min, supernatant pH is adjusted to 7.4-7.6 using 1 N NaOH or 1N HCl. Samples and controls are analyzed **in duplicate** for TPGY and for CMM. Analyze undiluted and 1:5 dilutions of each culture supernatant. 1:5= 0.2 ml culture + 0.8 ml casein buffer.
 - b. **Food sample preparation.** If a food has a liquid packing medium, the liquid may be removed, centrifuged as above to remove solids and/or fats and the supernatant/ aqueous layer directly analyzed by ELISA after pH adjustment to 7.4-7.6. If the food is a solid or semi-solid, the toxin must be extracted. An equal amount of food (20 g) and casein buffer (20 mL) are mixed by grinding with a mortar and pestle or by other means to mix the food and buffer. The food-buffer slurry (1:2 dilution) is centrifuged at 7,000 × g for 30 min at 4°C. The aqueous supernatant is removed and adjusted to pH 7.4-7.6 if necessary using 1 N NaOH or 1 N HCl. Some foods such as Honey may also require dilution to remove ELISA inhibitors. Honey has previously been tested at a 1:5 dilution with satisfactory results. Normal food that does not contain botulinal toxin can be spiked with known standard toxin(s) at 2ng toxin/mL (~100 MLD/mL) of the food extract in casein buffer to monitor the possible inhibition of the ELISA by the food. Botulinal neurotoxin standards were diluted in casein buffer and used as controls or for spiking foods prior to analysis.
2. **Preparation of microtiter plates.** Coat each well of the microtiter plate with 100 µl of appropriate dilution of goat type A, E, or F or rabbit type B antitoxin diluted in bicarbonate buffer. Prepare the number of needed microtiter plate wells to test the sample. Dilute the stock antitoxins according to the accompanying directions. Store plate with coating buffer overnight at 4°C with plastic seal cover on top of plate to prevent evaporation.
 3. **ELISA analysis of samples.**
 - a. Remove plate from 4°C storage and wash plate 5 times in PBST with 45 second hold between each aspiration. Use a commercial plate washer or other mechanical device; avoid using a squeeze bottle to wash.

- b. Block plate in casein buffer with by filling all wells to the top of the plate (~300 μ l/well) and incubate for 60-90 min at 35°C. Prepare the sample and control dilutions while the plate is being blocked.

Negative controls: Duplicate wells are tested with all reagents except toxin (pH adjusted undiluted sterile CMM and TPGY broth if used and casein control). Casein buffer control is used as a system control.

Positive controls: Duplicate wells are tested using standard toxins type A, B, E, and F diluted in pH adjusted sterile TPGY and CMM (if used) at a concentration of 2 ng/mL. The LD₅₀/ng will vary depending on toxin type.

ELISA Food Inhibition controls: Type A, B, E, and F neurotoxins can be used to spike a food at 2 ng/mL of the supernatant obtained from the food-casein buffer slurry. Duplicate wells are tested for each toxin type. Results are compared to the positive control that consists of toxin spiked into casein to demonstrate if the product inhibits the ELISA. The product may be diluted further to remove inhibitory substances but will lower the sensitivity of the test.

- c. Wash the blocked plate as above and then add the toxic samples and controls (100 μ l/well). Work from the left side of the plate to the right side when adding the reagents.
- d. Incubate toxin-containing samples and controls for 2 hr. at 35°C. Prepare the type A, B, E, and F digoxigenin-labeled antibody reagents according to directions while incubating the samples. Do not make more than you need!
- e. Wash plate 5 times in PBST as above.
- f. Add the diluted digoxigenin-labeled goat antibody (100 μ l/well) and incubate for 60 min at 35°C.
- g. Wash plate 5 times in PBST as above.
- h. Add the anti-digoxigenin poly HRP conjugate diluted 1:5,000 in casein buffer (100 μ l/well), and incubate for 60 min at 35°C.
- i. Wash 5 times in PBST then tamp the plate several times on a paper towel to remove any residual wash buffer.
- j. Add 100 μ l of the TMB (substrate at room temperature) solution, incubate 20-30 min at 35°C. Positive sample wells will begin to turn a blue-green color. High toxin samples will develop color within a few minutes. The analysis can be stopped with 100 μ l of stop reagent at any time (within 20-30 min) after addition of the substrate when positive controls give appropriate sensitivity (absorbance \geq 1.0) and negative controls are acceptable (absorbance not greater than \sim 0.39). The plate should be taken to the plate reader immediately after addition of the stop solution. Measure absorbance at 450 nm on microplate reader.

Results: A positive test is an absorbance value that is >0.20 above the absorbance observed in the negative controls (sterile uninoculated TPGY broth or CMM or negative food sample). As in any ELISA, higher background absorbance will result if plates are insufficiently washed.

- D. **Confirmation of positive ELISA samples.** The DIG-ELISA was designed for screening TPGY and CMM culture media that may contain type A, B, E, and/or F botulinal toxins. Some food matrices may be inhibitory to the test or may generate false positive results. Samples that are positive or are inhibitory to the DIG-ELISA test must be confirmed using the mouse bioassay.

References

1. Ferreira, J L., Maslanka, S, Johnson, E., and Goodnough, M. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked Immunosorbent assay: collaborative study. *JAOAC International* **86**:314-331.
2. Solomon, H. and Lilly, T. 2001. FDA Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum*.
3. Ferreira, J.L., Maslanka, S., Andreadis J. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled IgGs and the ELISA. FDA/ORA Laboratory Information Bulletin Vol. 18, Number 10, 4292:1-10.

Flow Diagram for DIG-ELISA

Day 1

Coat microtiter plates with capture IgG and store overnight at 4°C.

Day 2

1. Wash plates, block, put on toxic samples and controls, 2 hr incubate.
2. Wash, put on digoxigenin-labeled IgG's, 1 hr incubate.
3. Wash, put on the anti-digoxigenin HRP conjugate, 1 hr incubate.
4. Wash, put on TMB substrate, 20-30 min incubate.
5. Stop the reaction with stop reagent.
6. Measure absorbance on plates with microplate reader at 450 nm.

V. Specific Detection of Clostridium botulinum Types A, B, E, and F Using the Polymerase Chain Reaction (PCR).

For additional information on this PCR method, contact Kathy E. Craven or Joseph L. Ferreira at FDA, ORA, Southeast Regional Laboratory, 60-8th Street, N.E., Atlanta, GA 30309. Telephone: (404) 253-1200; FAX: (404)253-1210.

Clostridium botulinum organisms generally produce one of four neurotoxin types (A, B, E, and F) associated with human illness. Neurotoxin type determination is important in determining the identification of the bacterium. A PCR method was developed to identify 24 hour botulinal cultures as potential type A, B, E and F neurotoxin producers as well as culture of other clostridial species which also produce botulinal neurotoxins. Components of the PCR and amplification conditions were adjusted for optimal amplification of toxin gene target regions enabling the simultaneous testing for types A, B, E, and F in a single thermal cycler. Each primer set was specific for its corresponding toxin type. Additionally, a DNA extraction procedure was included to remove inhibitory substances that may affect amplification. This procedure is rapid, sensitive, and specific for the identification of toxigenic *C. botulinum*.

Because of the severity of neuromuscular illness caused by botulinal neurotoxin, a rapid diagnosis for the specific toxin type is necessary during illness outbreaks suspected of being foodborne. The PCR technique has also been used to detect multiple botulinal toxin-producing types within a single PCR assay (4,6). The PCR assay for the toxin gene type is determined after a 24-hour anaerobic culture to obtain vegetative cells. ELISA procedures may require up to five days of culture growth before toxin is detected (5,9). The PCR method may also be used in conjunction with the mouse bioassay to determine toxin type. For example, a culture that is PCR positive for the type A toxin gene would require mouse protection/testing confirmation only for toxin type A.

A. Equipment and Materials

1. Programmable automatic thermocycler
2. Horizontal gel electrophoresis apparatus
3. Electrophoresis constant-voltage power supply
4. Heating plate
5. Incubators, 35°C
6. Water bath, 37°C and 60°C
7. Freezer, -20 and -70°C
8. Speed Vacuum, optional

9. Microwave
10. Sterile disposable inoculating loops
11. Microcentrifuge tubes, 1.5 and Thin Walled PCR reaction tubes, 0.2 ml or 0.5 ml
12. Variable digital micropipettors (e.g., 0.5-20 μ l, 20-200 μ l, 100-1,000 μ l)
13. Aerosol-resistant pipet tips
14. Microcentrifuge
15. UV transilluminator
16. Polaroid camera and Polaroid film 3000 ISO or comparable Gel Documentation System

B. [Media](#) and [Reagents](#)

Molecular biology grade reagents are recommended and are available from various manufacturers.

1. Tryptone-peptone glucose yeast extract broth (TPGY).
2. Phosphate-buffered saline, pH 7.4 (PBS)
3. Tris EDTA, pH 8.0 (1X TE). 10mM Tris-HCL, 1mM EDTA, pH 8.0 in distilled water
4. Proteinase K- 10 mg Proteinase K/ml 1 \times TE
5. Lysozyme-10 mg Lysozyme/ml 1 \times TE
6. 3 M Sodium Acetate, pH 5.2
7. 95% ethanol
8. 2'-Deoxynucleoside-5'-triphosphates (dATP, dCTP, dGTP, dTTP); stock solution 2.5 mM of each dNTP
9. *Taq* DNA polymerase (available from various vendors) or Amplitaq® (Perkin-Elmer)
10. 10 \times Reaction Buffer B-500mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), 1.0 % Triton X-100
11. 15 mM MgCl₂
12. *Clostridium botulinum* neurotoxin oligonucleotide primers types A, B, E, and F, 10 μ M stock solutions (2).
13. Light mineral oil, optional

14. Sterile deionized water, RNase and DNase free
15. 10× TBE (0.9 M Tris-borate, 0.02 M EDTA, pH 8.3)
16. Agarose (nucleic acid electrophoresis grade)
17. Ethidium bromide solution, 10 mg/ml
18. 6× sample loading buffer
19. DNA molecular weight markers (e.g., 123 bp ladder or 100 bp ladder)

C. Procedure for amplification of *C. botulinum* neurotoxin A, B, E, and F gene fragments from presumptive *C. botulinum* isolates using TPBY enrichment broth

Food sample preparation and enrichment (Chapter 17, Part I Mouse Bioassay, Section D).

1. **DNA isolation Procedures.** Boil sterile 10 ml portions of Tryptone-Peptide-Glucose-Yeast Extract Broth (TPGY) in a water bath for 10 min and quickly cool to room temperature just prior to use. Inoculate TPGY with presumptive *C. botulinum* isolates using a disposable sterile inoculating loop and incubate overnight at 35° C. Remove a 1.4 ml aliquot from each of the cultures and dispense into separate sterile micro-centrifuge tubes. Centrifuge at 14,000 × g for 2 min and discard supernatant. Wash the bacterial pellets in 1.0 ml PBS, pH 7.4 and centrifuge at 14,000 × g for 2 min. Discard supernatant and resuspend pellets in 400 µl PBS and 100 µl of 10 mg lysozyme/ml 10mM Tris, 1 mM EDTA, pH 7.4 (TE). Incubate for 15 min at 37° C in a water bath, inverting tubes every 5-7 min during incubation. Add 10 µl of 10 mg Proteinase K/ml TE to suspensions and incubated for 1 h in a 60° C water bath. Invert tubes every 10-15 min during the incubation period. Boil suspensions for 10 min in a water bath and centrifuge for 2 min at 14,000 rpm. Transfer supernatants to sterile 1.5 ml micro-centrifuge tubes. Add 50 µl aliquot of 3 M Sodium acetate and 1.0 ml of 95% ethanol to supernatants, mix by inversion, and cool at -70 °C (or -20°C) for 30 min. Centrifuge the ethanol-salt preparations at 14,000 rpm. Discard supernatants and dry pellets using a DNA Speed-Vacuum (Savant Instruments, Inc., Holbrook, NY). Re hydrate pellets in 200-µl sterile TE buffer and store immediately at -20° C until PCR analysis is performed.
2. **Alternative DNA isolation/preparation procedures.** Cell lysis by boiling can also be performed to simplify the procedure. *C. botulinum* cultures are grown 24 hours as previously described. Remove a 1.4 ml aliquot and centrifuge at 14,000 × g for 2 min. Boil the suspension in a water bath for 10 min and centrifuge at 14,000 × g for 2 min to remove cell debris. Remove the supernatants and place into a sterile microcentrifuge tube. Store at -20°C until PCR analysis is performed. Commercial DNA extraction kits such as Gene Clean II (BIO 101, Inc., La Jolla, CA) and S&S Elu-Quick (Schleicher & Schuell, Keene, NH) may be used if the cells are sufficiently lysed. Manufacturers' protocol supplied with kits are followed. The method used for lysis of gram positive organisms prior to extraction of the DNA for PCR is important. Unless DNA concentrations are determined before PCR analysis, it may be necessary to test dilutions of the DNA sample to avoid false negative results caused by too little or too much DNA when

using commercially available kits. We recommend the use of no more than 344 ng of total DNA be used for the PCR analysis.

Note: DNA purification before amplification is recommended to reduce the possibility of inhibitory substances in cultures from affecting the PCR and to increase the concentration of target DNA. Purification of DNA removes inhibitory substances that may affect PCR amplification. Simple boiling of the cell culture may not remove all inhibitors from the PCR DNA preparation for all cultures. No PCR inhibition was observed due to the TPGY medium itself. The use of the described extraction procedure that incorporates Proteinase K and lysozyme consistently lysed *C. botulinum* cells (2). The amount of isolated DNA yielding positive results using this amplification method ranged from approximately 0.34 ng- 5,160 ng DNA per 100- μ l total volume PCR reaction. Using DNA concentrations outside this range may result in false negative results.

This method is rapid and reliable for the identification of type A, B, E and F toxin-producing clostridial strains. PCR results for typing clostridial toxin genes were obtained in approximately 4 hours following a 24-hour incubation of the culture. This method is not limited by culture production of the neurotoxin which requires up to five days incubation prior to analysis by ELISA or the mouse bioassay (3,5). The PCR products also can be toxin gene typed or confirmed by using type-specific oligonucleotide or polynucleotide DNA probes.

Oligonucleotide Primers. Desalted oligonucleotide primers are obtained from commercial suppliers. Primers were derived from published DNA sequences for *C. botulinum* structural genes encoding types A, B, E, and F neurotoxins (1, 3, 7, 8). The forward (F) and reverse (R) PCR primer sequences are:

Type A

F 5' -GTG ATA CAA CCA GAT GGT AGT TAT AG -3'
R 5' -AAA AAA CAA GTC CCA ATT ATT AAC TTT -3'

Type B

F 5' -GAG ATG TTT GTG AAT ATT ATG ATC CAG -3'
R 5' -GTT CAT GCA TTA ATA TCA AGG CTG G -3'

Type E

F 5' -CCA GGC GGT TGT CAA GAA TTT TAT -3'
R 5' -TCA AAT AAA TCA GGC TCT GCT CCC -3'

Type F

F 5' -GCT TCA TTA AAG AAC GGA AGC AGT GCT -3'
R 5' -GTG GCG CCT TTG TAC CTT TTC TAG G -3'

PCR reaction preparation. Primer sets for each of the types are used in separate PCR reactions. PCR reactions are performed in a 100 μ l volume mixture containing , 1 \times PCR buffer [10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100], 2.5 mM MgCl₂, 0.5 μ M concentration of each primer set (A, B, E, or F), 200 μ M concentration of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 2.5 U *Taq* DNA polymerase, and 2 μ l of sample DNA.

If necessary add approx. 50-70 μ l of sterile mineral oil. Thermal cyclers equipped with heated covers will not require the addition of a mineral oil overlay. If PCR reaction volumes are decreased to 50 μ l, the amount of template should be decreased to 1.0 μ l.

Note: It is recommended to add sample DNA to the PCR reaction mixture last in order to decrease potential contamination of PCR reagents. Positive and negative controls should be included in each analysis. Negative controls containing all of the reagents but lacking template DNA processed as described above are used to monitor for contamination with *C. botulinum* amplicons.

Temperature cycling. PCR conditions for simultaneous amplification of toxin gene fragments A, B, E, and F are:

One cycle at 95°C for 5 min
 Thirty cycles of 94 °C for 1 min (denaturation)
 60°C for 1 min (annealing)
 72°C for 1 min (extension)
 Final incubation of 72 °C for 10 min
 Holding temperature of 4°C

Multiplex PCR for the amplification of A and E or B and F toxin gene fragments has been performed successfully using these primers but with lower PCR product yields (4). These four primer pairs can not be used together in one multiplex reaction because the primers are incompatible.

Agarose gel analysis of PCR products. Prepare a 1.2-1.5 % agarose gel in 0.5 \times TBE containing 0.5 μ g ethidium bromide/ml agarose. Agarose may be melted in 0.5 \times TBE using a microwave. Cast gel and allow to solidify. Mix 10 μ l portions of PCR products with approximately 2.0 μ l 6 \times gel loading dye and load onto gel submerged in 1 \times TBE. An appropriate molecular weight marker must be included on each gel in order to determine the approximate molecular weight of PCR products. Molecular weight markers should contain fragments which bracket the target sequence size. Apply a constant voltage of 10 V/cm and allow amplified fragments to migrate until appropriate band separation is achieved.

A short-wave UV light is used to visualize bands relative to the molecular weight marker. Predicted fragment lengths for each toxin gene fragment are: Type A, 983-bp; Type B, 492-bp; Type E, 410-bp, and Type F, 1137-bp. Photographs of the gels are used to document the results using either a polaroid camera or a comparable gel documentation system.

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