AOAC Official Method 977.26 *Clostridium botulinum* and Its Toxins in Foods

Microbiological Method
First Action 1977
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A. Principle

Mice injected intraperitoneally (IP) with food extract containing one minimum lethal dose (MLD) of botulinum toxin die within 48 h after exhibiting sequence of symptoms characteristic of botulinum intoxication. Homologous antitoxin will protect mice from symptoms while other antitoxins will not, thus determining serological type. Viable spores in food will grow in suitable culture medium and produce toxin, which is detected and typed.

B. Apparatus

(**a**) *Can opener.*—*See* **972.44C**(**a**) (*see* 17.6.01).

(**b**) *Anaerobic jars.*—GasPak (Becton Dickinson Microbiology Systems, 7 Lovton Circle, Sparks, MD 21152, USA, No. 219511) or Case-nitrogen replacement.

(**c**) *Petri dishes.*—100 mm diameter. Dry prepared plates ca 24 h at 35°C before streaking.

(**d**) *Centrifuge.*—High-speed, refrigerated.

(**e**) *Syringes.*—1.0 or 3.0 mL with 25 gauge $\frac{5}{8}$ in. (15.9 mm) needles for inoculating mice.

C. Media and Reagents

(**a**) *Cooked meat broth.*—Use either liver or heart medium. (*1*) *Chopped liver broth.*—Grind 500 g fresh beef liver into 800 mL H_2O. Heat to bp and simmer 1 h. Cool, adjust to pH 7.0, and boil 10 min. Filter through cheesecloth, pressing out excess liquid. To broth add 10 g peptone, 1 g K_2HPO_4, and 1 g soluble starch. Adjust to pH 7.0 and dilute to 1 L with H_2O. Filter through coarse paper. (If desired, broth and liver may be stored separately in freezer for future use.) To 18 or 20 ×150 mm test tubes, add liver to height of 1–2 cm and 10–12 mL liquid. Autoclave 20 min at 121°C. (*2*) *Cooked meat medium.*—Use commercial medium of following formula: 454 g beef heart, 20 g proteose peptone, 2 g dextrose, and 5 g NaCl. Suspend 12.5 g medium in 100 mL cold H_2O. Mix thoroughly and let stand until particles are thoroughly wetted (ca 15 min). (Alternatively, add 1.25 g solid medium into test tubes, add 10 mL cold H_2O, and mix thoroughly to wet all particles.) Autoclave 15 min at 121°C. Final pH should be 7.2 ± 0.1.

(**b**) *Trypticase–peptone–glucose–yeast extract (TPGY) broth or with trypsin* (*TPGYT*).—Use TPGYT as alternative only when organism involved is strongly suspected to be nonproteolytic strain of Type B, E, or F. Dissolve 50 g Trypticase, 5 g Bacto-peptone, 20 g yeast extract, 4 g dextrose, and 1 g sodium thioglycollate in 1 L H_2O, and dispense 15 mL portions into 20 × 150 mm culture tubes or 100 mL portions into 6 fl oz prescription bottles. Autoclave 10 min (tubes) or 15 min (bottles) at 121°C. Final pH should be 7.0 ± 0.1. Refrigerate and discard if not used within 2 weeks. Immediately before use, steam or boil 10–15 min to remove O_2, cool quickly, and aseptically add 1.0 mL trypsin solution/15 mL broth.

Prepare trypsin solution by dissolving 1.5 g trypsin (Difco 1:250, No. 0152) in 100 mL H_2O. Sterilize by filtering through 0.45 µm Millipore or equivalent filter, and refrigerate.

(**c**) *Liver–veal–egg yolk agar or anaerobic egg yolk agar.*—(*1*) *Liver–veal–egg yolk agar* (*LVEY*).—Wash 2 or 3 eggs with stiff brush, and drain. Soak eggs in 0.1% $HgCl_2$ solution (w/v) 1 h. Drain $HgCl_2$ solution and replace with 70% alcohol, soaking 30 min. Remove eggs, crack aseptically, and discard whites. Remove yolk with syringe, place in sterile container, and add equal volume sterile 0.85% aqueous NaCl solution (w/v). Mix thoroughly. To each 500 mL prepared sterile commercial dehydrated liver veal agar at 50°C, add 40 mL egg yolk–NaCl suspension. Mix thoroughly and pour plates. Dry plates 2 days at room temperature or 24 h at 35°C. Discard contaminated plates, and store sterile plates in refrigerator. (*2*) *Anaerobic egg agar.*—Dissolve 5 g yeast extract, 5 g tryptone, 20 g proteose peptone, 5 g NaCl, and 20 g agar in 1 L H_2O. Adjust to pH 7.0, dispense 500 mL into 1 L flask, and autoclave 20 min at 121°C. To 500 mL melted agar at 45–50°C, add 40 mL egg yolk–NaCl suspension, prepared as in (*1*). Mix and pour plates immediately. Dry and store sterile plates as in (*1*).

(**d**) *Gel–phosphate buffer.*—pH 6.2. Dissolve 2 g gelatin and 4 g Na_2HPO_4 in 1 L H_2O with gentle heat. Dispense into 100 mL milk dilution bottle. Autoclave 20 min at 121°C.

(**e**) *Clostridium botulinum antitoxin preparations.*—Types A through F or polyvalent A–F. Available from Biological Products Branch, Office of Scientific Services, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA.

D. Preparation of Test Portion

(**a**) *Preliminary examination.*—Keep test samples refrigerated. Unopened canned foods, unless badly swollen and in danger of bursting, need not be refrigerated. Record code and condition of container. Clean and identify container.

(**b**) *Solid foods.*—Aseptically transfer portion, with little or no free liquid, to sterile mortar. Add equal amount sterile gel–phosphate buffer, **C**(**d**), and grind with sterile pestle. Alternatively, inoculate small pieces of test sample with sterile forceps directly into enrichment broth.

(**c**) *Liquid foods.*—Inoculate with sterile pipets directly into enrichment broth.

(**d**) *Canned foods.*—Prepare, disinfect with alcohol–iodine solution, and open cans as in **972.44D** (*see* 17.6.01). If can has swelled, position can so vertical side seam is away from operator. If can has buckled ends, chill before opening, and flame cautiously to avoid bursting can.

(**e**) *Visual examination.*—Note appearance, odor, and any evidence of decomposition. *Do not taste product under any circumstances.*

(**f**) *Reserve test portion.*—After culturing, aseptically remove portion to sterile jars for further tests which may be needed later.

E. Detection of Viable C. botulinum

(**a**) *Enrichment.*—Remove dissolved O_2 from media before inoculation by steaming 10–15 min and cooling quickly without agitation. Inoculate 2 tubes of cooked meat broth, **C**(**a**), with 1–2 g solid or 1–2 mL liquid food or extract/15 mL broth, introducing inoculum slowly beneath surface of broth. Incubate at 35°C. Similarly, inoculate 2 tubes of TPGY broth, **C**(**b**), and incubate at 26°C.

(**b**) *Examination.*—After 5 days, examine cultures for turbidity, gas production, digestion of meat particles, and odor. Also examine microscopically by wet mount under high power phase contrast or by bright field illumination of smear stained by Gram stain, crystal violet, or methylene blue. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells.

(**c**) *Further treatment.*—Usually 5 day incubation produces active growth and highest concentration of toxin, as well as peak

sporulation. Retain culture in refrigerator for pure culture isolation. If there is no growth after 5 days, incubate additional 10 days to detect possible delayed germination of *C. botulinum* spores before discarding culture as sterile.

F. Isolation of Pure Cultures

If good sporulation has occurred, *C. botulinum* is more readily isolated from mixed flora in enrichment culture or from original sample.

(**a**) *Pretreatment.*—Add equal volume filter-sterilized absolute alcohol to 1–2 mL culture or test portion in sterile screw-cap tube. Mix well and incubate at room temperature 1 h. Alternatively, heat 1–2 mL enrichment culture 10–15 min at 80°C to destroy vegetative cells. (Do not use heat treatment for nonproteolytic type *C. botulinum.*)

(**b**) *Plating.*—With inoculating loop, streak 1 or 2 loopfuls of alcohol or heat-treated cultures, diluted if necessary, to either or both liver–veal–egg yolk agar or anaerobic egg yolk agar dried plates in manner to obtain isolated colonies. Incubate plates ca 48 h at 35°C under anaerobic conditions of Case anaerobic jar or GasPak systems, or equivalents.

(**c**) *Selection of colonies.*—Typical colonies are raised or flat, smooth or rough, and commonly show some spreading and have irregular edge. On egg yolk media, colonies usually exhibit surface iridescence when examined by oblique light. This luster zone is referred to as "pearly layer.'' Zone usually extends beyond and follows irregular contour of colony. Besides pearly zone, colonies of Types C, D, and E are ordinarily surrounded by wide (2–4 mm) zone of yellow precipitate. Colonies of Types A and B generally show smaller zone of precipitation. Not all typical colonies will produce toxin. Some members of genus *Clostridium* have typical morphological characteristics but do not produce toxins.

(**d**) *Cultures.*—With sterile transfer loop, inoculate each of 10 selected colonies into tube of sterile medium: (*1*) TPGY broth for *C. botulinum* Type E, incubating 5 days at 26°C; and (*2*) cooked meat broth for other toxin types, incubating 5 days at 35°C. Use cultures for confirmation as in (**e**) and for detection and identification of toxin as in **G**.

(**e**) *Confirmation.*—Streak culture from (**d**) in duplicate on egg yolk agar plates, incubating one plate anaerobically and other plate aerobically at 35°C. If colonies typical of *C. botulinum* are found on anaerobic plate and no growth is found on aerobic plate, culture may be pure. Failure to isolate *C. botulinum* from one of selected colonies may indicate that its population relative to mixed flora is low. Repeated serial transfers through additional enrichment steps, **E**(**a**), may increase numbers sufficiently to permit isolation. Store pure culture, (**d**), either under refrigeration, on glass beads, or lyophilized.

G. Detection of Toxin

(**a**) *Preparation of test sample.*—Extract solid foods with equal volume gel–phosphate buffer, **C**(**d**), macerating with sterile, pre-chilled mortar and pestle. Centrifuge extract and liquid foods containing suspended solids under refrigeration. Rinse empty containers suspected of having held toxic foods with few mL gel–phosphate buffer. Use minimum volume to avoid dilution of toxin.

(**b**) *Trypsin treatment.*—Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Do not use trypsin treatment with TPGYT culture which already contains trypsin. Further treatment may degrade any fully activated toxin present in culture.

Adjust portion of food supernate, (**a**), liquid food, or cooked meat culture, if necessary, to pH 6.2 with 1M NaOH or HCl. Prepare saturated trypsin solution by dispersing 1 g trypsin (Difco 1:250) in 10 mL H_2O in clean culture tube. Mix 0.2 mL trypsin solution with 1.8 mL liquid to be tested. Incubate 1 h at 37°C with occasional gentle agitation.

(**c**) *Toxicity testing.*—Conduct each test in duplicate, i.e., on trypsin-treated and untreated materials. Dilute portions of untreated and treated food supernate, liquid food, or culture 1:2, 1:10, and 1:100, respectively, with gel–phosphate buffer. Inject separate pairs of mice, ca 15–20 g, IP with original and diluted fluids, treated and untreated, using syringe, **B**(**e**). Heat 1.5 mL original untreated fluid 10 min at 100°C for control. Cool and inject pair of mice each with 0.5 mL heated fluid. These mice should not die because botulinum toxin, if present, is inactivated by this heat treatment.

Observe mice periodically for 48 h, recording symptoms and time of deaths. Typical symptoms of botulism usually begin within 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 without symptoms of botulism is not sufficient evidence that injected material contained botulinum toxin. Deaths may occur from chemicals present in fluid or from trauma.

If after 48 h, all but mice receiving heated preparation have died, repeat toxicity test, using higher dilutions of fluids. It is necessary to have dilutions that kill as well as dilutions that do not kill to establish an end point or MLD as estimate of amount of toxin present. MLD is contained in highest dilution killing both (or all) mice inoculated. Calculate MLD/mL.

H. Typing of Toxin

Dilute monovalent antitoxins to Types A, B, E, and F in 0.85% aqueous NaCl solution to concentration of 1 International Unit/0.5 mL. Prepare enough diluted antitoxin to inject 0.5 mL into each of 2 mice for each dilution of preparation to be tested.

Use toxic preparation which gave greatest number of MLD, either treated or untreated. If untreated, same preparation can be used as was used for toxicity testing; if trypsinized preparation was most lethal, prepare freshly trypsinized fluid because continued action of trypsin may destroy toxin. Prepare dilutions to cover range of at least 10, 100, and 1000 MLD below previously determined end point of toxicity.

Inject several groups of mice IP, each mouse receiving 0.5 mL of one of diluted antitoxins, 30–60 min before challenging them with IP injection of toxic preparations.

Inject pairs of mice protected by specific monovalent antitoxin injection IP with each dilution of toxic preparation. Also inject pair of unprotected mice (no injection of antitoxin) with each toxic dilution as control. (This protocol requires 30 mice: 3 pairs for each of the 4 monovalent antitoxins [A, B, E, and F], each pair to receive challenge of one of the 3 dilutions of toxic preparation [$2 \times 3 \times 4 = 24$] plus one pair of unprotected mice for each dilution of toxic material as control [$2 \times 3 = 6$]).

Observe mice 48 h for symptoms of botulism and record time of deaths. If 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.

I. Interpretation

Toxin in food means that product, if consumed without thorough heating, could cause botulism. Presence of toxin in food is required for botulism to occur. Viable *C. botulinum*, but no toxin, in food is not proof that food in question caused botulism. Ingested organisms may be found in alimentary tract, but are considered to be unable to multiply and produce toxin *in vivo.*

Presence of botulinum toxin and/or organisms in low-acid (pH >4.6) canned foods means that items were underprocessed or were contaminated through post-processing leakage. Swollen cans are more likely than flat cans to contain botulinum toxin because organism produces gas during growth. Presence of toxin in flat can may imply that seams were loose enough to let gas escape. Toxin in canned foods is usually of Type A or of proteolytic Type B strain, because spores of proteolytics can be among more heat-resistant bacterial spores. Spores of nonproteolytics, Types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.

Protection of mice from botulism and death with one of monovalent botulinum antitoxins confirms presence of botulinum toxin and determines serological type of toxin in test sample.

If mice are not protected by one of monovalent antitoxins, there may be too much toxin in test sample, there may be more than one kind of toxin present, or deaths may be due to some other cause. In such cases, retesting at higher dilutions of test fluids is required and mixtures of antitoxins must be used in place of monovalent antiserum. If mice are still not protected, some other toxic material, which is not heat labile, could be responsible if both heated and unheated fluids cause death. It is also possible that a heat-stable toxic substance could mask the botulinum toxin.

References: *JAOAC* **60**, 541(1977); **73**, 166(1990).