AOAC Official Method 976.30
Clostridium perfringens in Foods

Microbiological Method
First Action 1976
Final Action 1979

(Applicable to examination of outbreak foods in which relatively large numbers of vegetative cells are expected to be present.)

A. Apparatus

(**a**) *Pipets.*—1.0 mL serological with 0.1 mL graduations and 10.0 mL with 1.0 mL graduations.

(**b**) *Colony counter.*—Quebec, or equivalent, dark field model.

(**c**) *High-speed blender.*—Waring, or equivalent, multi-speed model, with low-speed operation at 13 000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each test sample.

(**d**) *Anaerobic jars.*—BBL GasPak jars equipped with GasPak H + CO_2 generator envelopes are recommended. Anaero-jar with replacement of air by purified N_2 or N_2–CO_2 (9 + 1) is satisfactory.

(**e**) *Freezer, ultra-low temperature.*—Capable of maintaining temperature of −68°C.

(**f**) *Shipping container.*—Heavy duty styrofoam, including hermetically sealable metal canister (friction-fit paint can is satisfactory).

B. Reagents

(**a**) *Peptone dilution water.*—Dissolve 2.0 g peptone (Difco 0118) in 2 L H_2O for each test sample, and adjust to pH 7.0 ± 0.1. Dispense enough volume in 175 mL (6 oz) bottles to give 90 ± 1 mL and in 750 mL Erlenmeyers to give 450 ± 5 mL after autoclaving 15 min at 121°C.

(**b**) *Nitrite test reagents.*—(*1*) *Reagent A.*—Dissolve 8 g sulfanilic acid in 1 L 5M CH_3COOH (2 + 5). (*2*) *Reagent B.*—Dissolve 5 g α-naphthol in 1 L 5M CH_3COOH.

(**c**) *Buffered glycerol–salt solution.*—Dissolve 4.2 g NaCl in 900 mL H_2O. Add 12.4 g anhydrous K_2HPO_4, 4.0 g anhydrous KH_2PO_4, and 100 mL glycerol. Mix well to dissolve, and adjust pH to 7.2. Autoclave 15 min at 121°C. For double-strength glycerol solution (20%), use 200 mL glycerol and 800 mL H_2O.

C. Culture Media

(Sizes of culture media containers [test tubes, flasks, and Petri dishes] are specified for each medium. All media except tryptose–sulfite–cycloserine [TSC] agar are incubated in air at 35°C. Media not used 4 h after preparation must be heated 10 min in boiling H_2O or flowing steam to expel O_2 and cooled rapidly in tap H_2O without agitation just before use.)

(**a**) *TSC agar.*—15.0 g tryptose, 20.0 g agar, 5.0 g soytone, 5.0 g yeast extract, 1.0 g sodium metabisulfite, and 1.0 g ferric ammonium citrate (NF Brown Pearls) diluted to 1 L with H_2O (SFP agar base, Difco 0811-01 [repaced by 0811], is satisfactory). Adjust to pH 7.6 ± 0.1, dispense 250 mL portions into 500 mL flasks, and sterilize 15 min at 121°C. Before plating, add 20.0 mL 0.5% filter-sterilized solution of D-cycloserine to each 250 mL sterile melted medium at 50°C. To make egg yolk-containing plates, add 20 mL 50% egg yolk emulsion, (**c**), to 250 mL sterile medium containing D-cycloserine. Dispense 15 mL portions into 100 × 15 mm sterile Petri dishes. Cover plates with towel and let dry overnight at room temperature before use.

(**b**) D-*Cycloserine solution.*—Dissolve 1 g D-cycloserine (Sigma Chemical Co., No. C6880 or Serva Feinbiochemicia, Heidelberg, Germany) without heating in 200 mL 0.05M phosphate buffer (pH 8.0± 0.1) and sterilize by filtering through 0.45 μm membrane filter.

(**c**) *Egg yolk emulsion.*—Wash fresh eggs with stiff brush and drain. Soak 1 h in 70% alcohol. Aseptically remove yolk and mix with equal volume sterile 0.85% NaCl aqueous solution (w/v). Store at 4°C.

(**d**) *Buffered motility–nitrate medium.*—3.0 g beef extract, 5.0 g peptone, 5.0 g KNO_3, 2.5 g Na_2HPO_4, 3.0 g agar, 5.0 g galactose, and 5.0 g glycerol diluted to 1 L with H_2O. Adjust to pH 7.3 ± 0.1, dispense 11 mL portions into 150 × 16 mm tubes, and sterilize 15 min at 121°C.

(**e**) *Lactose–gelatin medium.*—15.0 g tryptose, 10.0 g yeast extract, 10.0 g lactose, 5.0 g Na_2HPO_4, 0.05 g phenol red, and 120.0 g gelatin diluted to 1 L with H_2O. Adjust to pH 7.5± 0.1 before adding lactose and phenol red. Dispense 10 mL portions into 150 × 16 mm screw-cap tubes and sterilize 15 min at 121°C.

(**f**) *Sporulation broth.*—15.0 g Polypeptone™, 3.0 g yeast extract, 3.0 g soluble starch, 0.1 g $MgSO_4$, 1.0 g sodium thioglycollate, and 11.0 g Na_2HPO_4 diluted to 1 L with H_2O. Adjust to pH 7.8 ±0.1, dispense 15 mL portions into 150 × 20 mm screw-cap tubes, and sterilize 15 min at 121°C.

(**g**) *Polypeptone–yeast extract (PY) medium.*—20.0 g Polypeptone, 5.0 g yeast extract, and 5.0 g NaCl diluted to 1 L with H_2O. Adjust to pH 6.9 ± 0.1, dispense 9 mL portions into 125 × 16 mm screw-cap tubes, and sterilize 15 min at 121°C.

(**h**) *Fluid thioglycollate medium.*—(Becton Dickinson Microbiology Systems, 7 Lovton Circle, Sparks, MD 21152, USA, No. 11260 or Difco.) Dispense 10 mL portions into 150 × 16 mm screw-cap tubes. Sterilize 15 min at 121°C and cool quickly. Final pH is 7.1 ± 0.1.

D. Preparation of Test Portion

(**a**) *For storage and shipping.*—Using aseptic technique, transfer 50 g test portion to sterile container such as Whirl-Pak plastic bag and add 50 g sterile buffered glycerol–salt solution. Mix well by kneading bag or stirring with sterile pipet. Let solution penetrate solid foods 10 min before freezing. Treat liquid test samples such as beef juice or gravy with double-strength (20% glycerol) solution to obtain final concentration of 10% glycerol. Freeze test samples as quickly as possible in ultra-low temperature freezer at −68°C or, alternatively, by placing in sealable metal canister and storing with solid CO_2 in insulated shipping container. To ship laboratory samples, place in sealable metal canister and pack in well-insulated styrofoam shipping carton with sufficient solid CO_2 to keep laboratory samples frozen during transit. Ship by most rapid means possible. Upon receipt, transfer laboratory samples to ultra-low temperature freezer at −68°C or replenish solid CO_2 in shipping carton to maintain temperature at ca −56°C until laboratory samples can be examined. Thaw laboratory samples and proceed as in (**b**) without delay.

(**b**) *For analysis.*—Using aseptic technique, weigh 50 g food sample into sterile blender jar. Add 450 mL peptone dilution H_2O and homogenize 2 min at low speed (13 000 rpm). Use this 1:10 dilution to prepare serial dilutions from 10^{-2} to 10^{-6} by transferring 10 mL of 1:10 dilution to 90 mL dilution blank, mixing well with gentle shaking, and continuing until 10^{-6} dilution is reached.

E. Plate Count Technique

Pour ca 5 mL TSC agar without egg yolk into each of ten 100 × 15 mm Petri dishes and spread evenly by rapidly rotating dish. When agar has solidified, label plates and aseptically pipet 1 mL of each dilution of homogenate in duplicate onto agar surface in center of dish.

Pour additional 15 mL TSC agar without egg yolk into dish and mix well with inoculum by gently rotating dish.

Alternatively, with sterile glass rod spreader, spread 0.1 mL dilution over previously poured plates of TSC agar containing egg yolk emulsion. Let plates absorb inoculum 5–10 min; then overlay with 10 mL TSC agar without egg yolk. (TSC agar containing egg yolk is preferred for foods which may also contain other sulfite-reducing *Clostridium* spp.)

When agar has solidified, place plates in upright position in anaerobic jar. Produce anaerobic conditions, and incubate jar 20 h at 35°C for TSC agar without egg yolk and 24 h at 35°C for TSC agar with egg yolk. After incubation, remove plates from jar and observe macroscopically for growth and black colony production. Select plates showing estimated 20–200 black colonies. Using Quebec colony counter with piece of white tissue paper over counting area, count black colonies and calculate number of *Clostridium* spp./g food. *C. perfringens* colonies in medium containing egg yolk are black and usually surrounded by 2–4 mm zone of white precipitate due to lecithinase activity. However, because a few strains are weak or negative for lecithinase, count any black colonies suspected to be *C. perfringens* and confirm identity as in **F**.

F. Confirmation Technique

Select 10 characteristic colonies from countable plates (20–200 colonies), inoculate each into tube of fluid thioglycollate medium, and incubate 18–24 h at 35°C. Make Gram-stained smear of fluid thioglycollate cultures and check for purity and presence of short, thick, Gram-positive bacillus characteristic of *C. perfringens.* Streak contaminated cultures on TSC agar containing egg yolk and incubate plates anaerobically 24 h at 35°C to obtain pure cultures. Stab-inoculate buffered motility–nitrate and lactose gelatin media with 2 mm loopfuls of pure fluid thioglycollate culture or portion of isolated colony from TSC agar plate. Inoculate sporulation broth with 1 mL fluid thioglycollate culture and incubate 24 h at 35°C. Examine tubes of buffered motility–nitrate medium by transmitted light for type of growth along stab. Nonmotile organisms produce growth only in and along line of stab. Motile organisms produce diffuse growth out into medium away from stab.

Test buffered motility–nitrate medium for presence of nitrite by adding 0.5 mL Reagent A and 0.2 mL Reagent B. Orange color, which develops within 15 min, indicates presence of nitrites. If no color develops, add few grains of powdered Zn metal, and let stand 10 min. No color change after addition of Zn indicates that nitrates are completely reduced; change to orange color indicates that organism is incapable of reducing nitrates.

Examine lactose–gelatin medium for gas and color change from red to yellow, indicating that lactose is fermented with production of acid. Chill tubes 1 h at 5°C and check for gelatin liquefaction. If medium solidifies, reincubate additional 24 h at 35°C and repeat test for gelatin liquefaction. Make Gram-stained smear from sporulation broth and examine microscopically for spores. Report whether or not spores are produced. Store sporulated cultures at 4°C if further testing of isolates is desired.

Nonmotile, Gram-positive bacilli, which produce black colonies in TSC agar, reduce nitrates to nitrites, produce acid and gas from lactose, and liquefy gelatin within 48 h are provisionally identified as *C. perfringens*.

Organisms suspected to be *C. perfringens* that do not meet criteria stated above must be confirmed by further testing. Subculture into fluid thioglycollate medium isolates that do not liquefy gelatin or which are atypical in other respects. Incubate 24 h at 35°C, make Gram-stained smear, and check for purity. Inoculate one tube of PY medium, **C**(**g**), containing 1% salicin and one tube containing 1% raffinose with 0.1 mL fluid thioglycollate culture. Incubate media 24 h at 35°C and check PY–salicin for acid and gas. Transfer 1.0 mL culture to test tube and add 1–2 drops 0.04% phenol red. Yellow color indicates acid is produced from salicin. (Salicin usually is not fermented by *C. perfringens* but is rapidly fermented with production of acid and gas by closely related species.) Reincubate media additional 48 h and test both media for production of acid. Acid is usually produced from raffinose by *C. perfringens* but not by closely related species. Acid is produced from salicin in PY medium by a few strains of *C. perfringens.*

Calculate number of *C. perfringens* in test sample on basis of percent colonies tested that are confirmed as *C. perfringens.* [*Example:* If average plate count of 10^{-4} dilution was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*, number of *C. perfringens*/g food is $85 \times (8/10) \times 10\,000 = 680\,000$.] (*Note:* Dilution factor with plates containing egg yolk is 10-fold higher than dilution plated.)

References: *JAOAC* **59**, 606(1976); **61**, 785(1978).