These mice should not die because botulinum toxin, if present, is inactivated by this heat treatment.

Observe mice periodically for 72 hr, recording symptoms and time of deaths. Typical symptoms of botulism usually begin within 24 hr 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 chems present in fluid or from trauma.

If after 72 hr, all but mice receiving heated prep have died, repeat toxicity test, using higher dilns of fluids. It is necessary to have dilns that kill as well as dilns that do not kill to establish an end point or MLD (min. lethal dose) as est. of amt of toxin present. MLD is contained in highest diln killing both (or all) mice inoculated. Calc. MLD/mL.

46.090  
**Typing of Toxin**

Dil. monovalent antitoxins to types A, B, E, and F in 0.85% NaCl soln to concn of 1 International Unit/0.5 mL. Prep. enough dill antitoxin to inject 0.5 mL into each of 2 mice for each diln of prep to be tested.

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

Inject several groups of mice IP, each mouse receiving 0.5 mL of 1 of dill antitoxins, 30–60 min before challenging them with IP injection of toxic preps.

Inject pairs of mice protected by specific monovalent antitoxin injection IP with each diln of toxic prep. Also inject pair of unprotected mice (no injection of antitoxin) with each toxic diln 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 1 of the 3 dilns of toxic prep (2 x 3 x 4 = 24) plus 1 pair of unprotected mice for each diln of toxic material as control (2 x 3 = 6).

Observe mice 72 hr 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 thru F.

46.091  
**Interpretation**

Toxin in foods means that product, if consumed without thor 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 thru post-processing leakage. Swollen cans are more likely than flat cans to contain botulinum toxin since 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, since 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 1 of monovalent botulinum antitoxins confirms presence of botulinum toxin and does serological type of toxin in sample. If mice are not protected by 1 of monovalent antitoxins, there may be too much toxin in sample, there may be more than 1 kind of toxin present, or deaths may be due to some other cause. In such cases, retesting at higher dilns of test fluids is required and mixts 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 heat stable toxic substance could mask botulinum toxin.

Ref.: JAOAC 60, 541 (1977).

**Clostridium perfringens in Foods**

**Microbiological Method**

**Final Action**

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

46.092  
**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 equiv., dark field model.

(c) High-speed blender.—Waring Blender, or equiv., 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 sample.

(d) Anaerobic jars.—BBL Gas-Pak jars equipped with Gas-Pak H CO2 generator envelopes are recommended. Anaero-jar (Pfizer Diagnostics, 1407 N Dayton St, Chicago, IL 60622) with replacement of air by purified N or N2–CO2 (9+ 1) is satisfactory.

(e) Freezer, ultra-low temperature.—REVCO Model ULT-107 (REVCO, Inc., 1100 Memorial Dr, W Columbia, SC 29169), or equiv., capable of maintaining temp. of −68°.

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

46.093  
**Reagents**

(a) Peptone dilution water.—Dissolve 2.0 g peptone (Difco B118) in 2 L H2O for each sample, and adjust to pH 7.0 ± 0.1. Dispense enough vol. 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°.

(b) Nitrite test reagents.—(1) Reagent A.—Dissolve 8 g sulfanilic acid in 1 L 5N HNO3 (2 + 5). (2) Reagent B.—Dissolve 5 g o-naphthol in 1 L 5N HNO3.

(c) Buffered glycerol-salt soln.—Dissolve 4.2 g NaCl in 900 mL H2O. Add 12.4 g anhyd. K2HPO4, 4.0 g anhyd. KH2PO4, and 100 mL glycerol. Mix well to dissolve, and adjust pH to 7.2. Autoclave 15 min at 121°. For double-strength glycerol soln (20%), use 200 mL glycerol and 800 mL H2O.

46.094  
**Culture Media**

(Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified for each medium. All media except tryptose-sulfite-cytochrome (TSC) agar are incubated in air at 35°. Media not used <6 hr after prep must be heated 10 min in boiling H2O or flowing steam to expel O2 and cooled rapidly in tap H2O without agitation just before use.)

(a) Tryptose-sulfite-cytochrome agar.—15.0 g tryptose, 20.0 g agar, 5.0 g soytone, 5.0 g yeast ext, 1.0 g Na metabisulfite, and 1.0 g ferric ammonium citrate (NF Brown Pearls) dill’d to 1 L with H2O (SFPagar base, Difco 0811-01, is satisfactory). Adjust to pH 7.6 ± 0.1, dispense 250 mL portions into 500 mL flasks, and sterilize 15 min at 121°. Before plating, add 20.0 mL 0.5% filter-sterilized soln of D-cytochrome to each 250 mL sterile melted medium at 50°. To make egg yolk-contg plates, add 20 mL 50% egg yolk emulsion, (e), to 250 mL sterile medium contg D-cytochrome. Dispense 15 mL portions
into 100 × 15 mm sterile petri dishes. Cover plates with towel and let dry overnight at room temp. before use.

(b) d-Cycloserine soln.—Dissolve 1 g d-cycloserine (Sigma Chemical Co. or Serva Feinbiochemica, Heidelberg, West Germany) without heating in 200 mL 0.05M phosphate buffer (pH 8.0 ± 0.1) and sterilize by filtering thru 0.45 μm membrane filter.

(c) Egg yolk emulsion.—Wash fresh eggs with stiff brush and drain. Soak 1 hr in 70% alcohol. Aseptically remove yolk and mix with equal vol. sterile 0.85% NaCl soln. Store at 4°C.

(d) Buffered motility-nitrate medium.—3.0 g beef extract, 5.0 g peptone, 5.0 g KNO3, 2.5 g Na2HPO4, 3.0 g agar, 5.0 g galactose, and 5.0 g glycerol dild to 1 L with H2O. 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 tryptone, 20.0 g yeast ext, 10.0 g lactose, 5.0 g Na2HPO4, 0.05 g phenol red, and 120.0 g gelatin dild to 1 L with H2O. 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 sol. starch, 0.1 g MgSO4·7H2O, 1.0 g Na thioglycollate, and 11.0 g Na2HPO4 dild to 1 L with H2O. Adjust to pH 6.9 ± 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 ext, and 5.0 g NaCl dild to 1 L with H2O. 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.—(BBL-01-140, Difco 0256, Oxoid CM173). 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.

46.095 Preparation of Sample

(a) For storage and shipping.—Using aseptic technic, transfer 50 g sample to sterile container such as Whirl-Pak plastic bag and add 50 g sterile buffered glycerol-salt soln. Mix well by kneading bag or stirring with sterile pipet. Let soln percolate solid foods 10 min before freezing. Treat liq. samples such as beef juice or gravy with double-strength (20% glycerol) soln to obtain final concn of 10% glycerol. Freeze samples as quickly as possible in ultra-temp. freezer at −68°C or, alternatively, by placing in scalable metal canister and storing with solid CO2 in insulated shipping container. To ship samples, place in scalable metal canister and pack in well insulated styrofoam shipping carton with sufficient solid CO2 to keep samples frozen during transit. Ship by most rapid means possible. Upon receipt, transfer samples to ultra-temp. freezer at −68°C or replenish solid CO2 in shipping carton to maintain temp. at ca. −56°C until samples can be examined. Thaw samples and proceed as for (b) without delay.

(b) For analysis.—Using aseptic technic, weigh 50 g food sample into sterile blender jar. Add 450 mL peptone dill H2O and homogenize 2 min at low speed (13,000 rpm). Use this 1:10 diln to prep. serial dilns from 10^-2 to 10^-4 by transferring 10 mL of 1:10 diln to 90 mL dill blank, mixing well with gentle shaking, and continuing until 10^-4 diln is reached.

46.096 Plate Count Technic

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 diln of homogenate in duplicate onto agar surface in center of dish. Pour addnl 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 diln over previously poured plates of TSC agar contg egg yolk emulsion.

Let plates absorb inoculum 5–10 min; then overlay with 10 mL TSC agar without egg yolk. (TSC agar contg egg yolk is preferred for foods which may also contain other sulfite-reducing Clostridium sp.)

When agar has solidified, place plates in upright position in anaerobic jar. Produce anaerobic conditions, and incubate jar 20 hr at 35°C for TSC agar without egg yolk and 24 hr 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 estd 20–200 black colonies. Using Quebec colony counter with piece of white tissue paper over counting area, count black colonies and calc. number of Clostridium sp. In TSC agar. C. perfringens colonies in medium contg egg yolk are black and usually surrounded by 2–4 mm zone of white pft due to lecinthinase activity.

However, since a few strains are weak or neg. for lecinthinase, count any black colonies suspected to be C. perfringens and confirm identity as in 46.097.

46.097 Confirmation Technic

Select 10 characteristic colonies from countable plates (20–200 colonies), inoculate each into tube of fluid thioglycollate medium, and incubate 18–24 hr at 35°C. Make Gram-stained smear of fluid thioglycollate cultures and check for purity and presence of short, thick, Gram-pos. bacillus characteristic of C. perfringens. Streak contaminated cultures on TSC agar contg egg yolk and incubate plates anaerobically 24 hr 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 hr 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 diffusion 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 which develops within 15 min indicates presence of nitrites. If no color develops, add few grains of powd Zn metal, and let stand 10 min. No color change after addn of Zn indicates that nitrates are completely reduced; change to orange 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 hr at 5°C and check for gelatin liquefaction. If medium solidifies, reincubate addn 24 hr 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-pos. bacilli which produce black colonies in TSC agar, reduce nitrates to nitrites, produce acid and gas from lactose, and liquefy gelatin within 48 hr 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 hr at 35°C, make Gram-stained smear, and check for purity. Inoculate 1 tube of PY medium, (g), contg 1% salcin and 1 tube contg 1% raffinose with 0.1 mL fluid thioglycollate culture. Incubate media 24 hr at 35°C and check PY-salcin for acid and gas. Transfer 1.0 mL culture to test tube and add 1–2 drops 0.04% phenol red. Yellow indicates acid is produced from salcin. (Salcin usually is not fermented by C. perfringens but is rapidly fermented with production of acid and gas by closely related species.) Reincubate media addn 48 hr 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*.

Calc. number of *C. perfringens* in sample on basis of % colonies tested that are confirmed as *C. perfringens*. (Example: If av. plate count of 10^4 diln was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*, number of *C. perfringens* in food is 85 x (8/10) x 10,000 = 680,000.) (Note: Diln factor with plates conig egg yolk is 10-fold higher than diln plated.)

Ref.: JAOC 59, 606(1976).

**Clostridium perfringens** in Foods

**alpha-Toxin Estimation Method**

**Final Action**

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

46.098 **Apparatus**

(a) **Centrifuge.**—High-speed, preferably refrigerated, with 250 mL bottles.

(b) **Seitz filter.**—100-250 mL with sterilizing filter pads.

(c) **High-speed blender.**—Waring Blender or Omni-Mixer homogenizer (DuPont Co., Sorval Operations, Newtown, CT 06470), with blending vessels.

(d) **Vacuum flask.**—Sidearm 1 L erlenmeyer fitted with 1-hole rubber stopper to receive 200 mm glass tubing with 125 cm of 6 mm od (3 mm id) rubber tubing attached.

(e) **Tubing.**—Stainless steel thin wall (No. 9 surgical), 3 (od) x 180 mm (Tubesales, 175 Tubeway St, Forrest Park, GA 30092), with 1 inch rubber tubing attached.

(f) **Delivery tubing.**—1.21' flat width (Fisher Scientific Co., No. 8667C).

46.099 **Reagents**

(a) **N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) buffer solution.**—Dissolve 6.0 g HEPES (Calbiochem) and 11.7 g NaCl in 500 mL H2O. Adjust to pH 8.0 with 3N NaOH and store at 4°.

(b) **Lecithovitellin solution.**—Mix 1 egg yolk with 250 mL saline solution, (e), and clarify by centrifugation 20 min at 14,000 X g at 4°. Filter-sterilize supernatant with Seitz filter and store at 4°.

(c) **Saline agar base.**—Add 15.0 g purified agar (Difco Laboratories) and 8.5 g NaCl to 1 L H2O. Adjust to pH 7.0, heat to dissolve agar, dispense in 100 mL portions, and autoclave 15 min at 121°.

(d) **Washed red blood cells.**—Wash packed human red blood cells 3 times by mixing with 4 vols saline solution, (e), centrifuging 10 min at low speed (2500 rpm) to sediment cells. Remove supernatant with vac. flask. Resuspend cells in addnl saline solution and repeat these steps twice. After final wash, mix cells with equal vol. saline solution. Use sterile precautions.

(e) **Sterile saline solution.**—Dissolve 8.5 g NaCl in 1 L H2O. Adjust to pH 7.0, dispense 250 mL portions into Pyrex containers, and autoclave 15 min at 121°.

(f) **Polyethylene glycol solution.**—30%. Dissolve 120 g polyethylene glycol (Carbowax Compound 20M, Union Carbide Corp., PO Box 8361, S Charleston, WV 25303) in 400 mL H2O.

(g) **Antiserum.**—Clostridium perfringens Type A diagnostic serum (Wellcome Animal Health Products, 520 W 21st St, Kansas City, MO 64101).

46.100 **Preparation of Hemolysin Plates**

Melt 100 mL saline agar base, (e), cool to 50°, and add 11 mL washed red cells, (d). Mix thoroughly and dispense 7 mL into 15 x 100 mm sterile plastic petri dishes. Dry plates overnight at room temp. and store at 4°. Just before use, cut test wells by applying vac. to sterile stainless steel tube, (e), and plunging tube into agar.

Using template, space 9 test wells 3 cm apart and 2 cm from edge, and place 2 addnl wells 3 cm apart near center of plate. 46.101 **Toxin Extraction**

Homogenize 25 g food (do not include fat) in 100 mL HEPES buffer soln, (a), 1 min in high-speed blender. Centrif. homogenate 20 min at 14,000-20,000 X g at 5° Filter supernatant thru Whatman No. 31 paper, or equiv., to remove fat (chill ext centrif. without refrigeration 1 hr at 4° before filtering). Discard solids. Rinse Seitz filter pad with 15 mL saline soln. Discard saline soln and filter-stereilize ext, rinsing filter pad with 10 mL saline soln.

46.102 **Concentration**

Soak 90 cm dialysis tubing 1 hr in H2O. Tie one end and fill with saline soln. Check for leaks and rinse out twice with saline soln. Transfer sterile ext to dialysis sack and concc. to <10 mL by dialyzing 4-5 hr against 400 mL 50% polyethylene glycol, (f), at 4° Rinse outside of sack with tap H2O and collect concd ext in sterile tube.

46.103 **Toxin Testing**

Adjust vol. of concd ext to 10-0.5 mL with saline soln. Set up 10 sterile 13 x 100 mm test tubes and add 0.5 mL saline soln to all tubes except first and last. Add 0.5 mL ext to first and second tubes. Mix ext and saline soln in second tube and transfer 0.5 mL to third tube, etc., to serial dil. ext from 0 to 1 + 255. Change pipet after 3 dilns to prevent excessive carry-over. Mix 0.25 mL ext, 0.25 mL saline soln, and 0.1 mL antiserum, (g), in last tube. Fill 1 peripheral well of duplicate hemolysis plates with each diln of ext, using fine-tipped Pasteur pipet. Fill 1 center well of each plate with ext-antiserum mixt. and the other with saline soln. Add 0.5 mL lecithovitellin soln, (b), to remainder of diln in each tube, including ext-antiserum mixt. Mix well, and incubate tubes and plates (in plastic bag) 24 hr at 35°.

46.104 **alpha-Toxin Titer**

After incubation, refrigerate plates 2 hr at 4°. Measure hemolytic zone (width from edge of well in mm). Last 3 dilns before end point should exhibit ca 1 mm reduction in width for each 2-fold diln. If not, repeat a-toxin test. Hemolytic zone 1 mm in width is end point of titr.

Examine ext-lecithovitellin mixt. in tubes for lecinhinase activity and record results. Max. reaction (+ + + +) is white pellicle 4-5 mm thick over clear liq. Activity decreases with diln to (--) reaction (opaque soln with no pellicle). This diln is end point of lecithovitellin test. Hemolytic and lecinhinase activities neutralized by antiserum are due to a-toxin.

46.105 **Population Estimate**

Compare titer of a-toxin present in ext with data in Table 46.03 to est. population of *C. perfringens*. Hemolysin (H) plate titer is preferred for this because lecithovitellin (LV) test is less sensitive with some food exts.


**Bacillus cereus** in Foods

**Enumeration and Confirmation Microbiological Methods**

**Final Action**

46.106 **Apparatus**

(a) **Pipets.**—1.0 mL with 0.1 mL graduations; also 5.0 mL and 10.0 mL with 1.0 mL graduations.
Enumeration and Confirmation of Clostridium perfringens

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ABSTRACT

A number of media have been proposed for the enumeration and confirmation of Clostridium perfringens in food and water. Most of these employ sulfite and iron together with selective antibiotics. This report discusses these various media and conditions for their use.

During the past few years Clostridium perfringens has established itself as one of the leading causes of human food poisoning. For example, in the United States in 1979 Salmonella, Staphylococcus aureus and C. perfringens accounted for 37, 29 and 17%, respectively, of the confirmed foodborne disease outbreaks of bacterial etiology. Significantly, five deaths were associated with the C. perfringens outbreaks. These occurred in a large outbreak involving debilitated, institutionalized patients. This paper gives an overview of the media which have been proposed for enumeration and confirmation of this organism. Readers interested in specific laboratory procedures related to C. perfringens should consult either the Compendium of Methods for the Microbiological Examination of Foods (12) or Microorganisms in Foods 1, Their Significance and Enumeration (33).

PLATING MEDIUM

Cameron first reported on the use of a differential medium for enumeration of clostridia in foods, specifically sugar (7). He used sulfite-iron agar which had been developed by Wilson and Blair (47) for analysis of water for sulfite-reducing bacteria. Other investigators soon reported on the use of this medium for detection of C. perfringens from clinical specimens (45). In Wilson-Blair medium, the clostridia reduce sulfite which is precipitated as iron sulfide, resulting in black colonies. This principle is still employed today in media for enumeration of C. perfringens. Mossel et al. (39) modified Wilson-Blair medium for use with clostridia by reducing the sulfite concentration and by omitting the glucose to prevent "blowing" of the medium, an occurrence that is a problem even today when a medium, such as brain heart infusion, is employed to enumerate C. perfringens (Labbe and Norris, unpublished observations). Interestingly, Mossel noted that the fact that Salmonella spp. may also form black colonies in this medium would not pose a problem because "wholesome foods are free from these bacteria". Mossel (38) further modified the iron-sulfite agar medium by including 10 ppm polymyxin B sulfate. This medium yielded quantitative recovery of pure cultures of several Clostridium spp. in Miller-Prickett tubes. However, the growth of enterococci, staphylococci and aerobic bacilli was not prevented, and black colony formation by Proteus as well as by Salmonella occurred. To minimize the latter, Angelotti et al. (1) incorporated 0.12 mg of sulfadiazine per ml of media. In addition, they used conventional plate count techniques to avoid the inconvenience associated with isolating colonies from Miller-Prickett tube cultures. The resulting sulfite-polymyxin-sulfadiazine medium (SPS) selectively inhibited sulfite-reducing non-Clostridium spp. and resulted in the quantitative recovery of C. perfringens from various foods after anaerobic incubation at 37°C for 24 h, even in the presence of large numbers of other organisms. However, the problem of differentiating among black colonies produced by C. perfringens and other sulfite-reducing Clostridium spp. remained and required a confirmatory test. They proposed inoculation of nitrate-motility agar (NM) with a representative number of black colonies. In addition, the ability of the isolates to sporulate was determined by inoculation of a sporulation broth. C. perfringens is a sporeformer that is non-motile and reduces nitrate and nitrite. Of course, should C. perfringens comprise a small proportion of the total sulfite-reducing clostralid colonies, it might remain undetected because of the dilution factor.

The persistent problem of non-C. perfringens black colonies on SPS agar was partially solved by the introduction of tryptone-sulfite-neomycin agar (TSN) by Marshall et al. (36).

To the basic iron-sulfite medium, these workers included a final concentration of 20 μg/ml of polymyxin B sulfate and 50 μg/ml of neomycin sulfate. Incubation was at 46°C. The medium was effective in inhibiting Clostridium bifurcations.
mentans. Its effect on Clostridium spp. other than C. bifermens and C. perfringens was not investigated.

In 1969, Fuze and Csukas (15) reported that blood agar containing 800 μg of D-cycloserine per ml was a very selective medium for isolation of C. perfringens because it inhibited many facultative anaerobes. Soon afterwards another medium, Shahidi-Ferguson-perfringens (SFP) was proposed (43). It contained kanamycin and polymyxin B as selective agents, and an emulsion of egg yolk. An agar overlay without egg yolk was also suggested. The egg yolk was included to detect lecithinase production by C. perfringens. Also suggested was the elimination of demonstrating sporulation as part of the confirmatory test. This was due to the difficulty in obtaining sporulation by this organism (17).

Harmon et al. (24) compared SPS, TSN and SFP for their ability to recover pure cultures of C. perfringens and their selective ability. SFP agar was found to be superior in recovery of vegetative cells of C. perfringens, but was least selective of the media, allowing growth to some extent of nearly all of the facultative anaerobes tested. TSN was the best performer in this regard, but, nevertheless, allowed growth of several Clostridium spp. This prompted them to modify SFP by substituting 400 μg of D-cycloserine per ml for the antibiotics recommended by Shahidi and Ferguson (25). This medium, tryptose-sulfite-cycloserine agar (TSC), inhibited practically all facultative anaerobes. C. bifermens was the only other Clostridium spp. tested which grew well on TSC agar after 24 h of incubation at 35°C.

The mid-1970s saw a flurry of activity regarding plating media for C. perfringens. Handford (18) proposed yet another plating medium. It differed from SFP medium in that oleandomycin phosphate and sulfadiazine replaced kanamycin, and egg yolk was omitted. According to the author, this medium, oleandomycin-polymyxin-sulfadiazine-perfringens agar (OPSMA), gave higher counts of C. perfringens and fewer false-positives than SPS, TSN, SFP and TSC media.

Lack of a positive lecithinase reaction by 8 of 21 strains of C. perfringens on SFP and TSC agars prompted Hauschild and Hilsheimer to omit egg yolk from TSC medium (EY-free TSC agar, 29). The modified medium allowed quantitative recoveries of each of 71 C. perfringens strains and eliminated false-negative reactions associated with the use of egg yolk. The NM confirmatory test was also modified to include galactose and glycerol. The same authors then compared SFP, TSC, OPSMA and EY-free TSC agar for their suitability to enumerate C. perfringens in naturally contaminated foods (30). Essentially complete recoveries were obtained in each of the four media, but only TSC and EY-free TSC agars were sufficiently selective to ensure subsequent confirmatory tests without interference from facultative anaerobes. In a separate study and in a further attempt to eliminate false-positives, Hauschild et al. (31) also proposed the use of lactose gelatin (LG) in conjunction with NM agar as confirmatory tests.

Orth (40) compared SPS with EY-free TSC. He found that, although EY-free TSC gave only a modest improvement over SPS for the recovery of spores, it was superior to SPS for recovering actively growing, heat-stressed, cold-shocked, and frozen C. perfringens cultures. He also noted that no increase in counts was obtained by increasing the incubation time from 24 to 48 h.

At the request of the International Commission on Microbiological Specifications for Foods, Hauschild et al. (27, 28) evaluated the following media as part of an international comparative study for the enumeration of C. perfringens from feces and food: SFP, TSC, EY-free TSC and neomycin blood agar. The latter is widely used in Great Britain in investigations of suspected C. perfringens outbreaks, largely because blood agar plates are readily available in public health and hospital laboratories. In overall performance, TSC and EY-free TSC were superior to SFP and blood agar. Because of simplicity, most of the participants preferred EY-free TSC.

In a collaborative study in the United States, TSC medium with and without egg yolk was compared to SPS medium (20). SPS was clearly inferior. Most of the participants preferred the egg yolk-free modification. TSC agar, with or without egg yolk, together with confirmatory media (see below) has been adopted by the Association of Official Analytical Chemists (AOAC) as official first action (2). The composition of commercially available plating media for enumeration of C. perfringens is shown in Table 1.

SPORES

Compared to vegetative cells, the recovery of spores of C. perfringens has received little attention. We found that TSN and SPS media gave higher counts of six strains of heat-activated spores than non-selective media (34). For heat-injured spores, wide variation in recovery was obtained depending on the strain and medium.

Barach et al. (4) reported that SPS, TSN, SFP, TSC and TSN without antibiotics (TSN BASE) were about equally effective for the enumeration of heat-activated spores. TSC and SFP were superior for recovery of spores surviving heat treatment of ultra-high temperatures. This was attributed to germination of injured spores by the lysozyme present in the egg yolk emulsion used in these media. Lysozyme had been previously reported to promote recovery of heat-injured spores (9, 13). The addition of lysozyme to SPS and TSN did not improve the percentage of heat-injured spores recovered because the selective agents in these media interfered with the action of lysozyme on injured spores. TSC yielded higher counts of heat-injured spores plated in the presence of meat broth than TSN BASE. Further research which would clarify the effectiveness of TSC or EY-free TSC (plus lysozyme) for the recovery of heat-injured spores from food seems warranted.

In their collaborative evaluation of media for enumeration of C. perfringens in feces and food, Hauschild et al. (27) made an interesting observation regarding the enumeration of C. perfringens spores from feces. They con-
TABLE 1. Commercially available plating media for C. perfringens.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>SPS</th>
<th>Ingredient</th>
<th>TSN</th>
<th>Ingredient</th>
<th>SFP</th>
<th>Ingredient</th>
<th>TSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Difco)</td>
<td>1.5 g</td>
<td>Tryptone</td>
<td>1.5 g</td>
<td>Tryptone (Difco)</td>
<td>1.5 g</td>
<td>Tryptone</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0 g</td>
<td>Yeast</td>
<td>1.0 g</td>
<td>Yeast extract</td>
<td>0.5 g</td>
<td>Yeast</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Iron citrate</td>
<td>0.05 g</td>
<td>Iron</td>
<td>0.05 g</td>
<td>Soytone (Difco)</td>
<td>0.5 g</td>
<td>Soytone</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5 g</td>
<td>Agar</td>
<td>1.5 g</td>
<td>Ferric ammonium citrate</td>
<td>0.1 g</td>
<td>Ferric</td>
<td>0.1 g</td>
</tr>
<tr>
<td></td>
<td>0.1 g</td>
<td>Sodium</td>
<td>0.1 g</td>
<td>Agar</td>
<td>2.0 g</td>
<td>Sodium</td>
<td>0.1 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sulfate</td>
<td></td>
<td></td>
<td></td>
<td>sulfate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>0.05 g</td>
<td>Polymyxin</td>
<td>2.0 mg</td>
<td>Sodium metabisulfite</td>
<td>0.1 g</td>
<td>Agar</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Polymyxin B sulfate</td>
<td>1.0 mg</td>
<td>B sulfate</td>
<td></td>
<td></td>
<td></td>
<td>pH adjusted to 7.6</td>
<td></td>
</tr>
<tr>
<td>Sodium sulfadiazine</td>
<td>12.0 mg</td>
<td>Neomycin</td>
<td>5.0 mg</td>
<td>Polymyxin B sulfate</td>
<td>3000 units</td>
<td>Cyclodextrine</td>
<td>0.04 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sulfate</td>
<td></td>
<td>Kanamycin sulfate</td>
<td>1.2 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH adjusted to 7.6</td>
<td></td>
<td>Egg yolk emulsion (50%)%</td>
<td>10 ml</td>
<td>Egg yolk emulsion (50%)%</td>
<td>8 ml</td>
</tr>
</tbody>
</table>

*aAvailable as agar base.

*bUse SFP agar base.

*cOmitted in overlay agar and EY-free TSC (30).

TABLE 2. Confirmatory media for C. perfringens.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Motility-nitrate medium g/100 ml</th>
<th>Lactose gelatin medium g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>0.3</td>
<td>12.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Agar</td>
<td>0.3</td>
<td>pH adjusted to 7.5</td>
</tr>
<tr>
<td>pH adjusted to 7.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
cluded that, in investigations of food poisoning outbreaks, spore enumeration alone is adequate and preferable to the total *C. perfringens* count because small differences between the two were observed.

**CONFIRMATORY MEDIA**

Presumptive black colonies are typically stab-inoculated into tubes of motility-nitrate agar and lactose-gelatin medium. The composition of these media is shown in Table 2. *C. perfringens* reduces nitrate to nitrite, is nonmotile, ferments lactose and liquefies gelatin within 48 h at 37°C. It is worth noting that α-naphthol is recommended in place of α-naphthylamine for nitrite detection because α-naphthylamine is potentially carcinogenic.

Mead et al. (37) evaluated the API ZYM system (Analytab Products, Plainview, NY) as a possible confirmatory step. This system is a micro-method which permits rapid, simple assay of 19 enzymes. The test distinguished *C. perfringens* from several *Clostridium* spp., but not from *Clostridium absonum*, *Clostridium paraperfringens* or *Clostridium sardiniensis*.

**TIME AND TEMPERATURE**

Typical conditions for incubation for vegetative cells and spores are 24 h at 37°C. Exceptions are associated with the use of TSN medium, where 46°C is recommended. An inconvenience associated with EY-free TSC is excessive blackening of the medium which occasionally occurs if incubation is continued beyond 18–20 h (20,40). For spores which have been heat-injured, we have found that a 48-h incubation period is necessary (Labbe et al., unpublished observations).

**ANAEROBIC SYSTEMS**

It is important to obtain anaerobic conditions when culturing plates of *C. perfringens* spores or vegetative cells. This is usually achieved by the catalytic removal of oxygen or by gas evacuation-replacement procedures. In the first case, commercially available systems (BBL Microbiology Systems or Oxoid USA, Inc.) are commonly used. This involves the chemical generation of 90% H₂:10% CO₂ in the presence of an activated palladium catalyst to remove oxygen. Alternatively, the ambient atmosphere can be evacuated and replaced with 100% N₂ or 90% N₂:10% CO₂.

Labbe and Norris (34) compared counts obtained when plates were incubated under 100% N₂ vs. incubation in a Gas-Pak container (BBL Microbiology Systems). No appreciable difference in counts of six strains of heat-activated *C. perfringens* spores was obtained using a non-selective plating medium. No comparisons were made using vegetative cells or selective media.

A disadvantage associated with the commercial systems is the cost of the hydrogen-carbon dioxide generator envelopes (currently greater than a dollar each - enough for 12 petri plates). Therefore, for large-scale or recurrent plating of *C. perfringens*, the evacuation-replacement procedure should be considered.

**TRANSPORT**

Vegetative cells of *C. perfringens* are extremely sensitive to storage at low temperatures. Losses of 3–4 log cycles in plate counts can occur following storage for only a few days at low temperatures (8,21,30). Thus, food or fecal specimens that are associated with *C. perfringens* foodborne illness and are held at low temperature before bacteriological examination, may yield low numbers of the organism. This is important because proper bacteriological confirmation of *C. perfringens* outbreaks consists mainly of demonstrating the presence of large numbers of this organism.

To minimize the loss of viability during transport and storage of incriminated food samples, Hauschild and Hilsheimer (30) suggested that samples be mixed 1:1 (wt/vol) with 20% glycerol and stored or transported in a dry-ice container or in a freezer at -60°C. A collaborative study reported by Harmon and Placencia (26) confirmed that these treatments minimize loss in viability.

**LECITHINASE**

The rapid loss in viability of *C. perfringens* mentioned above has resulted in a search for an index of growth of this organism in food. Lecithinase (alpha toxin or phospholipase C) has been proposed as such an indicator. Using hemolysin indicator plates or the lecithovitellin test, lecithinase concentration is expressed as a titer from which the extent of previous growth of the organism is determined by use of a standard table (21). A population of at least 4 to 10×10⁵ is required to produce detectable levels of lecithinase (22,41). *C. perfringens* diagnostic antiserum is available (Burroughs Wellcome Animal Health Division, Kansas City, MO) to confirm the presence of alpha toxin. The Centers for Disease Control (3) lists as one criterion for confirmation of *C. perfringens* as the etiological agent in a foodborne illness, a population of “≥10⁵ organisms per gram... provided specimen properly handled”. Therefore, the lower detection limit of the lecithinase test makes it suitable as a possible alternative test for detecting levels of *C. perfringens* associated with foodborne illness.

There are shortcomings to this test, however. Harmon and Kautter (23) stated that the type of food associated with the different *C. perfringens* food poisoning outbreaks had little effect on population estimates based on the amount of lecithinase. However, Park and Milofajcik (41) found that the amount of lecithinase produced in a food sample can be influenced by the nature of the substrate as well as the time and temperature at which it is held. For example, in thiglycollate medium, maximum lecithinase production occurred at 35°C and was 40 times greater than that observed at 45°C. However, generation time and maximum population were approximately the same at 35°C and 45°C.
Furthermore, Skjelkvale et al. (44) reported that five outbreaks of C. perfringens food poisoning were investigated where the causative organism failed to produce lecinthinase. Also, other Clostridium spp. produce lecinthinases. Nevertheless, the assay for lecinthinase levels can be a valuable method of determining the extent of growth of C. perfringens in food samples which have been frozen or refrigerated for a long time and for which low numbers of viable cells are expected. For such cases, the lecinthinase (α-toxin) method has been adopted as an AOAC first action method.

OTHER TESTS

A need for the ability to detect small numbers of C. perfringens in foods led Debevere (11) to develop an enrichment medium composed of fluid thioglycollate medium without dextrose and containing 400 μg of D-cycloserine/ml with incubation at 46°C. Isolation on iron sulfite agar was followed by various confirmatory tests.

A most probable number (MPN) method for estimating low numbers of C. perfringens was proposed by Erickson and Deibel (14). The procedure employed fortified litmus milk (termed rapid perfringens medium, RPM) in which the organism produces a characteristic stormy fermentation, followed by isolation and confirmation. Although more laborious, the method was significantly more sensitive than SPS or TSC in its ability to detect, and, compared to SPS, to enumerate low numbers of the organism.

The CAMP (initials of original authors) test has been used for the presumptive identification of group B streptococci. The procedure is based on the synergistic hemolysis (arrowhead-shaped) pattern produced by blood agar when C. perfringens or a β-toxin-producing Staphylococcus is streaked at right angles to Streptococcus agalactiae (10,16). This procedure identified 96.8% of the C. perfringens cultures tested within 24 h, with less than 1% false-negatives (19). Forty-four of 45 non-C. perfringens clostridial strains tested negative. Significantly, three of these were lecinthinase-positive species (C. bifermens, C. sordellii, C. novyi). Unfortunately, the performance of lecinthinase-negative strains of C. perfringens was not tested. Nevertheless, the CAMP test seems deserving of further evaluation as a possible confirmatory test.

C. PERFRINGENS IN WATER

Sulfite-reducing anaerobes have been used as indicator bacteria in the examination of water and wastewater for sometime, especially in Europe (fecal coliforms are the indicator organisms of choice in the United States). Since 95% of the flora of sulfite-reducing anaerobes in sewage is C. perfringens (6), some effort has been made to develop a membrane filtration test specific for this organism. Bissen and Cabelli (5) developed a solid, presumptive medium which incorporated D-cycloserine and polymyxin B sulfate together with in situ tests for sucrose fermentation, acid phosphatase production and β-D-glucosidase activity. The verification of typical colonies was 93%.

Hirn and Raevuori (32) evaluated TSC agar as a membrane filtration method for C. perfringens in natural and artificial water samples. The method yielded counts similar to those obtained by a tube colony count method (the Danish standard method for the detection of C. perfringens-like organisms). All typical colonies taken for confirmative tests proved to be C. perfringens. In spite of these results, it is not likely that C. perfringens will replace fecal coliforms as the indicator organism in North America. This is because of the well-established role fecal coliforms have assumed in standard methods of water analysis by public health agencies.

REFERENCES


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Evaluation of Plating Media for Recovery of Heated Clostridium perfringens Spores

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ABSTRACT

Four selective and eight non-selective plating media were evaluated for their ability to enumerate six strains of heat-activated and heat-injured spores of Clostridium perfringens. Trypticase-sulfite-neomycin (TSN) agar and sulfite-polymyxin-sulfadiazine (SPS) agar gave higher counts of heat-activated spores than non-selective media. In the case of heat-injured spores, wide variation in recovery was obtained depending on strain and medium. Higher counts of heat-injured spores were obtained by incubating plates at 37°C than at 45°C, although, except for one strain, no significant difference between the two temperatures was observed using heat-activated spores.

A number of solid media have been devised for the enumeration of Clostridium perfringens. Among these are sulfite-polymyxin-sulfadiazine (SPS) agar (1), tryptone-sulfite-neomycin (TSN) agar (13), neomycin blood agar (17), Shahidi Ferguson perfringens (SFP) agar (16), D-cycloserine blood agar (6), oleandomycin-polymyxin-sulfadiazine-perfringens (OPSP) agar (7) tryptose-sulfite-cycloserine (TSC) agar and egg yolk-free tryptose-sulfite-cycloserine (EY-free TSC) agar (9). Hauschile et al. (10) compared SFP, TSC and EY-free TSC for their ability to enumerate fecal spores of C. perfringens. They found that, when these spores comprised at least 20% of the total anaerobe spores, similar counts were obtained on all three media. However, at lower percentages the most accurate counts were obtained in EY-free TSC agar. Barach et al. (2) evaluated SFP, TSC, SPS, TSN and TSC without antibiotics as media for recovery of heated C. perfringens spores. These media were equally efficient for the enumeration of heat-activated spores, but TSC and SFP were superior for heat-injured spores, presumably because of the presence of lysozyme in the egg yolk emulsion used in these media. Lysozyme is known to promote the recovery of heat-injured C. perfringens spores (4).

Our laboratory's continuing need to enumerate spores of several strains of C. perfringens prompted us to investigate the suitability of a number of plating media designed for C. perfringens vegetative cells as well as several general purpose plating media. If the latter were suitable, a considerable savings in cost could be realized.

MATERIALS AND METHODS

Organisms and preparation of spore suspensions

The following strains of C. perfringens Type A were used: NCTC 8238, NCTC 8239, NCTC 8798, NCTC 10239, FD1 and ATCC 3624. All but the latter two are enterotoxin-positive strains. Spores were prepared in Duncan and Strong sporulation medium (3) as previously described, except that in the case of strains NCTC 8798 and NCTC 10239 starch was replaced with 0.4% raffinose (2). After 18 h, spores were free of their sporangia and were collected by centrifugation at 10,000 x g for 20 min. The pellet was washed repeatedly with cold, distilled water until vegetative cells constituted less than 5% of the population. The spores were frozen until used. Spore suspensions (ca. 1.0 x 10⁷/ml, viable count) were prepared by diluting thawed spores in distilled water followed by brief sonication to disrupt clumps.

Heat treatment of spore suspension

For heat activation of spores, 5 ml of a spore suspension were heated at 75°C for 20 min in 16 x 100-mm screw-cap test tubes. Heat-injured spores of strains NCTC 8238, NCTC 8239, NCTC 10239 and NCTC 8798 were prepared by heating 1.2-ml samples in sealed Pyrex tubes (18 mm o.d. x 15 cm) immersed in a constant temperature glycerol bath set at 100°C. Strains ATCC 3624 and FD1 were heated at 90°C in a water bath. In both cases, following heating the spore suspensions were immediately cooled in ice water. In the case of heat-injured spores, heating times selected were those which resulted in survivor levels of between 10⁹ - 10¹⁰/ml when plated on TSN BASE (see below).

Enumeration of spores

 Twelve plating media were evaluated for enumeration of spores. These included four selective media: TSN, SPS, SFP without egg yolk and TSC without egg yolk; the non-selective media were: TSN without differential agents, egg yolk and antibiotics (TSN BASE), nutrient agar (Fisher Scientific), plate count agar (PCA, Difco), TPD agar (J), brain heart infusion agar (Difco), trypticase soy broth (BBL) and trypticase soy broth (BBL) plus 1.5% agar. The latter is similar to trypticase soy agar, but also includes 0.25% dipotassium phosphate and 0.25% dextrose. Media were prepared on the day of use. All experiments were done in duplicate. One experiment consisted of one strain plated on twelve different media at two different temperatures, 37°C and 45°C. Unless otherwise noted, plates were incubated under nitrogen and colonies counted after 48 h.

Statistical analysis

Statistical analysis was done by analysis of variance using the SPSS computer package.
RESULTS

Compared to the other media, TSN and SPS resulted in the highest recovery (P<0.01) of heat-activated spores for all strains except ATCC 10239 (Table 1). This was surprising since TSN and SPS each contain antibiotics (polymyxin and sulfadiazine or polymyxin and neomycin for SPS and TSN, respectively) which may retard growth. Indeed, even the concentration of neomycin found in TSN (50 μg/ml) was found to be inhibitory to some strains of C. perfringens (I3). TPDP and trypsin-case soy broth were clearly inferior. The other media tested gave intermediate results depending on the strain and temperature of incubation of plates. Except in a few cases, incubation at 37°C resulted in higher counts than incubation at 45°C which is near the optimum temperature for growth of this organism. However, this difference was not statistically significant (P>0.05) except for strain NCTC 10239.

These plating media were then evaluated for their ability to recover heat-injured spores. With four of six strains, the highest count (100%) was observed using a non-selective plating medium. However, when the results obtained on selective media (TSN, SPS, SFP, TSC) were compared to non-selective media, there was no statistically significant difference between the two types of media. Incubation of heat-injured spores at 37°C was superior to 45°C for all strains (P<0.05).

We also compared counts obtained when plates were incubated under nitrogen versus incubation in a Gas-Pak container (BBL Microbiology Systems). Although not analyzed statistically, the data showed no appreciable difference in counts of six strains of heat-activated spores using TSN BASE as the plating medium (data not shown).

DISCUSSION

Of the six plating media tested in this study the most suitable for recovery of C. perfringens spores depended on the heat treatment applied to the spores as well as the temperature at which the plates were incubated. The wide variation in counts obtained emphasizes the need to evaluate different recovery conditions and media if one wishes to obtain highest counts when using pure cultures of C. perfringens spores.

Surprisingly, except in one case, selective media yielded greater recovery of heat-activated spores than non-selective media. In an attempt to explain this, we tested the ability of 0.012% sulfadiazine and 0.001% polymyxin (both found in SPS) to promote germination of heat-activated spores of C. perfringens. However, neither these antibiotics nor the differential agents (sodium sulfite and ferric citrate) in SPS promoted germination above the control (data not shown). This suggests that the higher counts obtained in SPS and TSN were not due to germination by a higher percentage of activated spores. Orth (I4) also reported that a selective medium (EY-free TSC) was superior to a non-selective one (TSC BASE) for enumeration of vegetative cells of C. perfringens. Similarly, Foegeding and Busta (5), using Desulfofranculum nigrificans spores, reported that a selective plating medium recovered more survivors than a non-selective, general-use plating medium. On the contrary, Barach et al. (2) found that TSN BASE gave similar results as TSN, SPS and SFP when used for recovery of three strains of heat-activated spores of C. perfringens. They also found that antibiotics were inhibitory to the recovery of ultrahigh temperature (105°C for 9 min) - treated spores of C. perfringens. Our data also indicate that, for some strains of heat-injured spores, the presence of antibiotics results in fewer colonies. Heated spores are known to be more sensitive to inhibitors than unheated spores (5,15).

Although C. perfringens is known to grow well at elevated temperatures (43°C to 45°C), less is known about the temperature requirements for recovery of heated spores. In this study incubation at 37°C was found to be superior to 45°C for highest recovery of heat-injured spores, although no significant difference was observed in this regard for heat-activated spores. The higher counts of heat-injured spores obtained for several strains on non-selective media emphasizes the need to consider omitting inhibitory agents in plating media when the objective is to maximize recovery from thermal injury. In this regard, inclusion of lysozyme in plating media for such spores should be considered (4). Some selective media, such as TSC and SFP may contain lysozyme as a contaminant of egg yolk used in these media (2).

REFERENCES


con't., p. 694
The Iron Milk Most Probable Number Method for Enumeration of *Clostridium perfringens* in the Diet and the Intestine of the Chick

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ABSTRACT Seven experiments were conducted to evaluate the iron milk most probable number method for enumeration of *Clostridium perfringens* in the diet and the intestine of broiler chicks. Levels of 50 ppm of neomycin and 20 ppm of polymyxin improved the iron milk tube method when compared to the TSN (tryptone-sulfite-neomycin) agar plate method. Low numbers of the organism, approximately 5 per gram, were detected in the practical diet fed to the chicks. Vegetative cell numbers of *C. perfringens* increased from 1.7 log<sub>10</sub> in the duodenum of chicks to >9.2 log<sub>10</sub> in the ceca. Spores of the organism were detected in the ileum and ceca. Results of two experiments demonstrated that *C. perfringens* became established in the ileum of chicks early in life, before initiation of feeding at 2 days of age.

(Key words: *Clostridium perfringens*, iron milk most probable number method, intestine, diet, chicks)

INTRODUCTION

*Clostridium perfringens* has been implicated in the depression of growth of monogastric animals since the advent of antibiotic usage in the 1950's. This anaerobic organism has also been shown to be sensitive *in vitro* to nearly all known feed additive antimicrobial agents in use today for poultry and swine. Investigations of the organism *in vivo*, however, have been limited because of convenient and repeatable techniques for enumeration of the organism.

Several plating methods, developed for evaluation of foods, have been utilized to determine the number of clostridia in the intestine of avian species. Wagner and Wostmann (1959) used a modified sulfite iron agar method of Mossel et al. (1956) to determine the number of clostridia in the ileum of White Leghorn chickens. The method was not specific for *C. perfringens*. Smith (1965) determined the numbers of *C. perfringens* in various sections of the intestinal tract of chickens and ducks using the neomycin Nagler agar method of Lowbury and Lilly (1955). Timms (1968) employed the RCM (reinforced clostridial medium) agar method of Hirsch and Grinsted (1954) with added blood and neomycin to determine the numbers of *C. perfringens* in the small intestine and ceca of three age groups of chickens. Naqi et al. (1970) utilized the SPS (sulfite-polymixin-sulfadiazine) agar method of Angelotti et al. (1962) to estimate *Clostridium spp.* in the small and large intestine of turkey pouls at various ages. The TSN (tryptone-sulfite-neomycin) agar plate method of Marshall et al. (1965) was employed by Stutz et al. (1983a,b,c) to evaluate the effects of antibiotics on *C. perfringens* numbers in the ileum of broiler chicks.

Tube methods are generally less time-consuming and less tedious than plating methods and do not require the additional equipment to maintain anaerobic conditions. Smyser et al. (1952) estimated *C. perfringens* numbers in the feces of broiler chickens by determining the most probable number (MPN) using the iron skin milk medium of Spray (1936). Erickson and Deibel (1978) reported on a new MPN tube method for determination of *C. perfringens* in foods using a litmus milk and gelatin-based media with added neomycin and polymyxin. The method was shown to be more sensitive than plating methods, but the method requires considerable time to prepare the complex media. Wekell et al. (1980) and St. John et al. (1982) developed a simple iron milk MPN tube assay for enumeration of *C. perfringens* in the intestinal tract of the rat and in soil, sludge, and water samples. The media contained no antibiotics and positive tubes were determined by stormy fermentation after incubation at 45°C for 16 to 24 hr.
The objectives of the following experiments were to evaluate the iron milk most probable number method for enumeration of *C. perfringens* in the diet, and the intestine of the broiler chick. Investigations were conducted to determine the effects of media and antibiotics on *C. perfringens* numbers, to compare the iron milk tube method with the TSN agar plate method, and to determine numbers of vegetative cells and spores of *C. perfringens* in various sections of the intestinal tract of the chick. The establishment of the organism in the ileum of the chick was also investigated.

**MATERIALS AND METHODS**

The chicks utilized in four of the experiments were controls from growth and feed efficiency studies that consisted of 24 pens of eight chicks each with six replicates of controls per experiment. Two-day-old male broiler-cross chicks (Hubbard × Hubbard) were started on a nonmedicated practical diet (milled Laboratory Chick Chow #5065, Ralston Purina Company, St. Louis, MO). The practical corn and soybean meal-based diet contained not less than 21% protein and 3% fat. For Experiments 1, 3, 4, and 5 the chicks were fed on a weight basis at 3 days of age and fed practical experimental diets for a 6-day period. At termination of these experiments, control chicks were maintained on the practical diet and sacrificed for intestinal contents for the following 4 days (12 to 15 days of age). For Experiment 6, the chicks were on a weight basis at 3 days of age, fed the practical diet, and sacrificed for the ileum and its contents and ileal contents at days 1 to 3 and 5 to 9, respectively (3 to 11 days of age). The chicks were maintained in battery brooders with raised wire floors and subjected to continuous lighting. Feed and water were supplied *ad libitum* in all of the experiments except in Experiments 6 and 7 where two groups of 6 chicks each were sacrificed for the ileum and contents before initiation of feeding and watering at 2 days of age. For Experiment 2, six samples of the practical corn and soybean meal diet fed to the chicks in the other experiments were retained for enumeration of *C. perfringens*.

Procedures used for collection and dilution of samples for determinations of *C. perfringens* numbers were similar to those of Wekell *et al.* (1980). Approximately 1-g samples of intestinal contents or feed were transferred into tared test tubes containing 9 ml of sterile 0.1% peptone (Difco). The peptone solution was steamed within 30 min before use and was heated at 45°C in a water bath. In those experiments where the ileum and contents were utilized, the total weight of the ileum and contents of each chick were homogenized in sterile 0.1% peptone at a ratio of 1:9. All samples were mixed for 5 sec with a Vortex mixer and serially diluted using sterile 0.1% peptone.

The iron milk 3-tube MPN method of Wekell *et al.* (1980) and St. John *et al.* (1982), with minor modifications, was used for enumeration of *C. perfringens* in the samples. Iron milk media was prepared by adding 10 ml of whole milk to 0.2 g of iron powder in 125 × 20-mm screw cap culture tubes. The tubes were steamed for 5 min and maintained in a water bath at 46°C. One milliliter aliquots of the serial dilutions of the samples were immediately inoculated into the iron milk and the tubes were transferred to an incubator. Positive tubes were determined by stormy fermentation after incubation at 46°C for approximately 21 hr. For the antibiotic supplemented iron milk media 50 ppm of neomycin, as neomycin sulfate, and 20 ppm of polymyxin, as polymyxin B sulfate, were added to the whole milk just prior to dispensing. Spore counts were determined by heating diluted samples to 80°C for 5 min and enumerating as for vegetative cells.

The TSN agar plate method of Marshall *et al.* (1965) was utilized to confirm anaerobic growth and sulfite reduction of positive iron milk tubes and to compare the tube method to a plating method for enumeration of *C. perfringens*. Procedures for the TSN agar plate method were similar to those described by Stutz *et al.* (1983a,b,c). One-half milliliter of the serial dilutions or the contents of incubated iron milk tubes were spread on agar plates followed by TSN agar (BBL) overlays. The plates were incubated anaerobically at 46°C for approximately 24 hr using the Gas Pak system (BBL).

Bacteria determined by the methods are reported as organisms per gram and analyzed for significance using the Mann-Whitney U test (Campbell, 1974).

**RESULTS**

The effects of media and antibiotics on the determination of *C. perfringens* in the ileum of broiler chicks did not influence the cells of the c by the agar antibiotics of neomycin similar to media. The milk media of *C. p* that other was present.

Table 1: Antibiotics of *C. perfringens* in the similar to milk media significantly the results also in origin of the intestine of the chick.

A comparison of the agar plate media was presented in the was observed numbers of the *Clostridium* sections of the chick determined by.

**TABLE 1. Effect of Determination Intestinal Media**

<table>
<thead>
<tr>
<th>Method</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron milk tube</td>
<td>TSN agar plate</td>
</tr>
<tr>
<td>Iron milk tube</td>
<td>TSN agar plate</td>
</tr>
<tr>
<td>TSN agar plate</td>
<td>Antibiotics</td>
</tr>
<tr>
<td>TSN agar plate</td>
<td>TSN agar plate</td>
</tr>
</tbody>
</table>

*Values in p ppm of the chicks that were different.*
broiler chicks are presented in Table 1. Addition of the antibiotics neomycin and polymyxin to the iron milk media resulted in numbers of the organism similar to those confirmed by the agar plate method. The levels of the antibiotics used in the iron milk media, 50 ppm of neomycin and 20 ppm of polymyxin, were similar to those present in TSN agar plate media. The absence of antibiotics in the iron milk media resulted in a significant overestimation of C. perfringens numbers and indicated that other competing organisms were likely present.

Table 2 depicts the effects of media and antibiotics on the determination of C. perfringens in the practical chick diet. Results were similar to those in the ileum in that the iron milk media without antibiotics detected significantly higher numbers of organisms. The results also indicate that the diet may be the origin of the competing organisms in the intestine of the chick.

A comparison of the iron milk tube method, supplemented with antibiotics, with the TSN agar plate method for the determination of C. perfringens in the ileum of broiler chicks is presented in Table 3. No significant difference was observed between the two methods in the numbers of the organism detected.

Clostridium perfringens counts in the three sections of the small intestine of broiler chicks, determined by the modified iron milk method, are presented in Table 4. The numbers of the organism significantly increased from the duodenum to the ileum.

Table 5 depicts the numbers of vegetative cells and spores of C. perfringens in the ileum and ceca of broiler chicks determined by the modified iron milk method. The numbers of both vegetative cells and spores were significantly higher in the ceca when compared to the ileum.

The establishment of C. perfringens in the ileum of broiler chicks is presented in Tables 6 and 7.

**TABLE 2. Effects of media and antibiotics on the determination of Clostridium perfringens in the practical chick diet (Experiment 2)**

<table>
<thead>
<tr>
<th>Method</th>
<th>Numbers in diet (log_{10} g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron milk tube</td>
<td>3.2a (1.3 - 4.3)</td>
</tr>
<tr>
<td>TSN agar plate¹</td>
<td>1.7b (0 - 1.1)</td>
</tr>
<tr>
<td>Iron milk tube and antibiotics²</td>
<td>3.4b (0 - 9)</td>
</tr>
</tbody>
</table>

a,b Means with different superscript letters are significantly different (P<0.05).

¹ Positive tubes confirmed by agar plate method.

² Iron milk tube and TSN = Tryptone-sulphite-neomycin.

Values in parentheses represent the ranges for six diet samples for the three methods.

**TABLE 3. Comparison of the iron milk tube method with the tryptone-sulphite-neomycin (TSN) agar plate method for the determination of Clostridium perfringens in the intestine of broiler chicks (Experiment 3).**

<table>
<thead>
<tr>
<th>Method</th>
<th>Numbers in ileal contents (log_{10} g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron milk tube</td>
<td>3.1a (1.6 - 5.4)</td>
</tr>
<tr>
<td>TSN agar plate¹</td>
<td>3.3a (2.2 - 6.8)</td>
</tr>
</tbody>
</table>

a Means with the same superscript letter are not significantly different (P>0.05).

¹ Iron milk tube and TSN = Tryptone-sulphite-neomycin.

Values in parentheses represent the ranges for 12 chicks for the two methods.
TABLE 4. Clostridium perfringens counts in the small intestine of broiler chicks determined by the iron milk tube method with added antibiotics (Experiment 4)

<table>
<thead>
<tr>
<th>Intestinal section</th>
<th>Numbers in sectional contents</th>
<th>(log₁₀/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>1.7 a</td>
<td>(1.4 – 2.6)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>2.4 a</td>
<td>(1.6 – 3.4)</td>
</tr>
<tr>
<td>Ileum</td>
<td>3.0 a</td>
<td>(2.4 – 5.0)</td>
</tr>
</tbody>
</table>

a,b,c Means with different superscript letters are significantly different (P<0.05).

1 Values in parentheses represent the ranges for eight chicks for the three sections of the small intestine.

and 7 (Experiments 6 and 7). Numbers of the organism determined by the iron milk method supplemented with antibiotics, in the ileum and contents of chicks before feeding, indicated that there was no significant difference between the two experiments. In Experiment 6 (Tables 6 and 7), C. perfringens numbers did not significantly change from 1 to 3 days or 5 to 9 days after initiation of feeding (3 to 11 days of age). The results of these experiments demonstrate that the organism becomes established in the intestine of the chick early in life, before initiation of feeding.

DISCUSSION

The results of Experiments 1 and 2 demonstrated that the iron milk tube method, without added antibiotics, overestimated the C. perfringens numbers in both the ileum and the duodenum. One explanation is that other competing organisms were present that produced a stormy fermentation similar to that of C. perfringens. Smyser et al. (1952) isolated a bacillus from positive skim milk tubes that possessed the characteristics of C. perfringens. St. John et al. (1982) reported that both bacilli and coliforms produced reactions similar to stormy fermentation, but that their reactions were slower than for C. perfringens at 45°C. Nearly all media for the enumeration of C. perfringens contain antibiotics to selectively inhibit competing facultative organisms. The addition of neomycin and polymyxin to the iron milk media significantly improved the method, especially for determinations of the organism in the intestine and the diet of the chick as described here. A comparison of the modified tube method with the TSN agar plate method of Marshall et al. (1965) indicated excellent agreement for determination of C. perfringens in the ileum of chicks. These values were similar to those previously reported by Stutz et al. (1983b) for chicks fed a practical diet and the TSN agar plate method was employed.

Clostridium perfringens numbers increased from the duodenum to the ceca and spores of the organism were detected in the ileum and ceca of chicks fed the practical diet (Exper-

TABLE 5. Vegetative cells and spores of Clostridium perfringens in the intestine of broiler chicks determined by the iron milk tube method with added antibiotics (Experiment 5)

<table>
<thead>
<tr>
<th>Section</th>
<th>Vegetative cells</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileum</td>
<td>2.8 b (2.4 – 4.0)</td>
<td>&lt;1.7 b (&lt;1.5 – 2.0)</td>
</tr>
<tr>
<td>Ceca</td>
<td>&gt;9.2 a (8.4 – 9.4)</td>
<td>3.3 b (1.6 – 4.3)</td>
</tr>
</tbody>
</table>

a,b Means within a column with different superscript letters are significantly different (P<0.01).

1 Values in parentheses represent the ranges for six chicks for the two intestinal sections.

TABLE 6. Establishment of Clostridium perfringens in the intestine of broiler chicks from before feeding to 3 days after feeding

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Numbers in ileum and contents</th>
<th>(log₁₀/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.8 b</td>
<td>(2.5 – 4.7)</td>
</tr>
<tr>
<td>1</td>
<td>4.0 b</td>
<td>(2.4 – 5.2)</td>
</tr>
<tr>
<td>2</td>
<td>4.6 b</td>
<td>(4.0 – 5.4)</td>
</tr>
<tr>
<td>3</td>
<td>3.5 b</td>
<td>(2.3 – 5.3)</td>
</tr>
<tr>
<td>Experiment 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.9 a</td>
<td>(2.3 – 3.3)</td>
</tr>
</tbody>
</table>

a,b Means with different superscript letters are significantly different (P<0.05).

1 Day 0 represents before initiation of feeding at 2 days of age.

2 Values in parentheses represent the ranges for six chicks for each day.
estimated the C. perfringens count in the ileum and the intestine at 9 different sites that were present in a similar test of C. perfringens prepared by using test tubes. The enumeration of C. perfringens was done using the continuous method. The p value was determined for C. perfringens in the ceca in the ceca of chickens fed a practical diet. The method, however, was not specific for C. perfringens, and other species of clostridia may have been detected. Our results agree with those of Lev et al. (1974) and Barnes et al. (1975) that high numbers of C. perfringens are found in the ceca of chickens fed a practical diet. Lev et al. (1957) also detected spores of the organism in the ceca of chickens. Surprisingly few reports exist in the literature that relate to the number of C. perfringens in the intestine of chickens.

The results of two experiments demonstrated that C. perfringens became established in the intestine of the chick early in life, before initiation of feeding at 2 days of age. Lev et al. (1957) and Naqi et al. (1970) reported that C. perfringens and Clostridium spp. became established in the intestine of chicks and turkey pouls, respectively, 1 day after feeding. Barnes et al. (1970) observed high numbers of microorganisms, including Clostridium spp., in the ceca of newly hatched chicks from commercial hatcheries before they were fed. They suggested that the chicks may have developed the flora while in the hatchers or from the boxes in which they were transported. Further research is needed to determine the origin and the establishment of the organism C. perfringens in the intestine of the newly hatched chick.

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REFERENCES


