

CHAPTER 13. EXAMINATION OF MEAT AND POULTRY PRODUCTS FOR
CLOSTRIDIUM PERFRINGENS

Ann Marie McNamara and Charles P. Lattuada

13.1 Introduction

Clostridium perfringens is a spore-forming, anaerobic bacterium that is widespread in soil, water, foods, spices, and the intestinal tract of humans and animals. Viable, sporulating strains that produce typical foodborne illness belong to Type A and produce an enterotoxin that causes typical symptoms of acute abdominal pain and diarrhea. Symptoms of nausea, vomiting and fever are rare. Symptoms usually appear 8-12 (range 6-24) hours after ingestion of a contaminated food, usually cooked meat or poultry. The infectious dose for humans is high, generally considered to be 10^6 - 10^7 cells/g. In foodborne disease outbreaks, findings of hundreds of thousands or more organisms per gram of food supports a diagnosis of *C. perfringens* foodborne illness when appropriate clinical and epidemiological evidence exists. There are four other types of *C. perfringens*: types B, C, D and E. Some strains of type C produce an enterotoxin that causes a rare form of necrotic enteritis that is often fatal and rarely seen outside of New Guinea.

This method for isolating and identifying *C. perfringens* in foods is a modification of the *C. perfringens* method found in the Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition (Labbe & Harmon, 1992).

For use in the FSIS Nationwide Microbiological Baseline Data Collection Programs and product surveys, the following "presumptive" isolation and enumeration method will suffice. This method is considered to be a "presumptive" method because other species of *Clostridia* besides *perfringens* can reduce sulfite and produce black colonies which are egg-yolk positive in TSC and EY-free TSC agar (Labbe and Harmon, 1992). Additionally, some strains of *C. perfringens* may not produce a halo surrounding their black colonies, so all black colonies should be counted whether a halo is present or not (Labbe and Harmon, 1992). For outbreak investigations or investigation of epidemiologically-linked cases, the more lengthy and time-consuming confirmation method should be used.

All samples should be shipped as refrigerated samples (0 - 10°C); this is particularly important with outbreak samples. Samples should be analyzed promptly upon laboratory receipt (Labbe and Harmon, 1992). *C. perfringens* in foods stored for prolonged periods of time or frozen many lose viability. If frozen samples must be shipped, food samples should be treated with buffered glycerol salt solution to give a 10% final concentration of glycerol. Samples should be shipped on dry ice and be stored frozen at -55°C to -60°C until the samples are analyzed.

13.2 Equipment, Reagents and Media

13.21 Equipment

- a. Incubator at 35 ± 1°C
- b. Anaerobic containers
- c. Anaerobic gas mixture consisting of 90% N₂ + 10% CO₂
- d. Colony counter with a piece of white tissue paper over the counting background area to facilitate counting black colonies
- e. Stomacher™ 400 and sterile stomacher bags or Blender and sterile blender jars
- f. Vortex mixer
- g. Water bath 46 ± 1°C
- h. Sterile, bent, glass rods ("hockey sticks")

13.22 Reagents

- a. Nitrate reduction reagents (Method 1)
- b. 0.1% peptone water diluent
- c. Phosphate-buffered saline (PBS)
- d. Physiological saline (0.85% sodium chloride)
- e. Butterfield's Phosphate Diluent
- f. Buffered Glycerol Salt Solution (for frozen samples)

13.23 Media

- a. Tryptose Sulfite Cycloserine (TSC) agar
- b. EY-free TSC agar
- c. Trypticase Peptone Glucose Yeast Extract Broth (buffered)
- d. Fluid Thioglycollate Medium
- e. Motility-Nitrate Medium (buffered)
- f. Lactose Gelatin Medium

- g. Spray's Fermentation Medium (1% salicin, or 1% raffinose)

13.3 Presumptive Test

13.31 Sample Preparation

- a. Meat Samples:
 - i. Label a sterile stomacher bag so that it corresponds to the label on the sample bag.
 - ii. Aseptically remove portions of the sample at random to obtain 25 grams. Place these portions in the sterile stomacher bag.
 - iii. Add 225 ml Butterfield's Phosphate Diluent (BPD) to the stomacher bag of each sample taken.
 - iv. Stomach for 2 minutes. Prepare serial dilutions of 10^{-2} to 10^{-6} .
- b. Poultry Samples:
 - i. Prepare serial dilutions of 10^{-1} to 10^{-3} of the whole bird rinse.

13.32 Enrichment and Plating

- a. Make duplicate spread plates on thin (6-7 ml) TSC with egg yolk agar base, using 0.1 ml/plate of undiluted sample rinse/extract as well as each dilution.
- b. Equally distribute the inoculum using sterile "hockey sticks". Use a new sterile "hockey stick" for each dilution.
- c. After the inoculum has dried slightly, overlay the surface with approximately 10 ml or more of egg yolk free TSC agar. Allow the plates to solidify before placing them, lid side up, in an anaerobic jar. Flush jar 3 or 4 times with 90% N_2 + 10% CO_2 leaving this atmosphere in after the last flush, or alternatively use a system which catalytically removes oxygen.
- d. Incubate all plates for 24 h at 35°C.

13.33 Examination of Plates

- a. After incubation, count the number of presumptive *C. perfringens* colonies. These colonies will be black and usually surrounded by a 2-4 mm opaque zone (halo).
- b. Multiply the number of colonies counted by 10 (since only 0.1 ml used) and then multiply by the appropriate dilution factor to obtain your total count.

13.4 Confirmatory Procedure (for epidemiologically linked cases)

13.41 Colony Selection

- a. Select 10 representative black colonies from each TSC agar plate counted and inoculate each into a freshly boiled (deaerated) and cooled tube of fluid thioglycollate broth.
- b. Incubate for 4 h in a water bath at 46°C or overnight at 35°C. After incubation prepare a Gram stain from each tube and examine microscopically. *C. perfringens* organisms are short, fat Gram positive rods. Endospores are rarely produced in fluid thioglycollate medium.
- c. If contaminants are observed, re-streak the contaminated culture onto the surface of a TSC (with egg yolk) agar plate (do not overlay) and incubate anaerobically before proceeding with any confirmatory tests. Surface colonies will appear as yellowish-grey colonies measuring approximately 2 mm in diameter. If re-streaking was done, it is necessary to repeat a. and b. of Section 13.41 (above).

13.42 Confirmatory Tests

- a. Motility - nitrate reduction test
 - i. Stab inoculate each tube of motility-nitrate medium with two, 2 mm loopfuls of the fluid thioglycollate medium culture.
 - ii. The medium contains 0.5% each of glycerol and galactose to improve the consistency of the nitrate

reduction reaction with different strains of the organism.

- iii. Incubate the inoculated medium at 35°C for 24 h and check motility. Since *C. perfringens* is non-motile, growth should occur only along the line of inoculum and not diffuse from the stab line.
 - iv. Test for reduction of nitrate to nitrite. A red or orange color indicates reduction of nitrate to nitrite. If no color develops, test fluid thioglycollate for residual nitrate by addition of powdered zinc.
- b. Lactose gelatin medium
- i. Stab inoculate each tube of lactose gelatin medium with two, 2 mm loopfuls of the fluid thioglycollate medium culture.
 - ii. Incubate at 35°C for 24 to 48 h. Lactose fermentation is indicated by gas bubbles and a change in color of the medium from red to yellow. Gelatin usually is liquefied by *C. perfringens* within 24 to 48 h.
- c. Carbohydrate fermentation
- i. Inoculate 0.15 ml of the fluid thioglycollate broth culture into 1 tube of freshly deaerated Spray's fermentation medium containing 1% salicin, 1 tube containing 1% raffinose, and 1 tube of medium without carbohydrate for each isolate.
 - ii. Incubate these three media at 35°C for 24 h and then check for production of acid. To test for acid, transfer 1 ml of culture to a test tube or spot plate and add 2 drops of 0.04% bromthymol blue. A yellow color indicates that acid has been produced.
 - iii. Reincubate negative raffinose tubes for an additional 48 h and retest for the production of acid.

- iv. Salicin is rapidly fermented with the production of acid by culturally similar species such as *C. paraperfringens*, *C. baratii*, *C. sardiniense*, *C. absonum*, and *C. celatum*, but usually not by *C. perfringens*.
- v. Acid is produced from raffinose within 3 days by *C. perfringens* but is not produced by culturally similar species.

13.43 Quantitation of *C. perfringens* Populations Based on Confirmed Anaerobic Plate Counts

- a. Cultures obtained from presumptive *C. perfringens* black colonies on selective, differential TSC or EY-free TSC medium are confirmed as *C. perfringens* if they are:
 - i. nonmotile
 - ii. reduce nitrate
 - iii. ferment lactose
 - iv. liquefy gelatin within 48 h
 - v. produce acid from raffinose.
- b. Calculate the number of confirmed *C. perfringens* per gram of food sample as follows:
 - i. Average the paired plates counted, then adjust the average presumptive plate count to 1.0 ml by multiplying by 10.
 - ii. Multiply the adjusted presumptive plate count by the reciprocal of the dilution plated to arrive at the total of presumptive *C. perfringens* colonies.
 - iii. The confirmed colony count is then determined by using the ratio of the colonies confirmed as *C. perfringens* to the total colonies tested.

13.5 Quality Control

- a. The following authentic, reference cultures can be used as control organisms in the above procedures:

C. perfringens ATCC 13124
C. absonum ATCC 27555

- b. The expected reactions produced by these control organisms are as shown in the following table:

Organism	Motility	H ₂ S	Gelatin liq.	Nitrate reduct.	Lactose ferm.	Salicin ferm.	Raffinose ferm.
<i>C. perfringens</i> ATCC 13124	-	+	+	±	+	-	d
<i>C. absonum</i> ATCC 27555	±*	+	d	+	+	+w	-

* usually + in young cultures; d = delayed; w = weak

3.6 Selected References

Granum, E. 1990. *Clostridium perfringens* toxins involved in food poisoning. Intl. J. Food Micro. 10:101-112.

Jay, J. M. 1996. Food poisoning caused by Gram-positive sporeforming bacteria, p. 451-458. In Modern Food Microbiology, 5th Edition. Chapman and Hall, New York, NY 10003

Labbe, R. G., and S. M. Harmon. 1992. *Clostridium perfringens*, p. 623-635. In C. Vanderzant and D. F. Splittstoesser (ed.), Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition. Amer. Publ. Hlth. Assoc., Washington, D.C. 20005.