CHAPTER 14. METHODS FOR THE DETECTION OF *CLOSTRIDIUM BOTULINUM* TOXINS IN MEAT AND POULTRY PRODUCTS

L. Victor Cook, Wei Hwa Lee, Charles P. Lattuada and Gerri M. Ransom

14.1 Introduction

Botulinum toxin (botox) types A-G are produced by heterogeneous strains of *Clostridium botulinum*. In recent years, other strains of *Clostridium spp*. have been shown to produce botulinum toxin (e.g. F-toxin *C. barati*). Botox types A, B, E and F have caused serious, and sometimes fatal, cases of foodborne illness in humans. The vast majority of botulism outbreaks in red meat and poultry products have involved either toxin A or B.

Botulinum toxin exists naturally as a stable complex of 2 protein molecules called "progenitor" or "bimolecular" toxin. The component proteins are the neurotoxin and non-toxic proteins, typically hemagglutinins.

The current botulinum toxin test method used by FSIS (Section 14.4) is the mouse bioassay procedure, published as Official Method 977.26 in Official Methods of Analysis of AOAC International, 16th Edition, 1995.

14.2 Supplies and Equipment

a. Microcentrifuge
b. Refrigerated centrifuge
c. Microfuge tubes, 1.5 ml, PGC Scientific 505-120 or equivalent
d. Sterile 50 ml stainless steel centrifuge tubes, Sorvall 00579 or equivalent
e. Polycarbonate screw cap centrifuge tubes, 30 ml size
f. Sterile Stomacher™ bags
g. White laboratory mice, 15-18 g
h. Hypodermic syringes, 1 or 3 ml size, fitted with 25 gage 5/8 inch needles.

14.21 Media and Reagents

a. Botulinum toxin typing antisera, monovalent types A through F, or polyvalent A-F (CDC, Atlanta, GA, *C. botulinum* laboratory).

b. Citric-Phosphate Buffer, pH 5.5; combine the following:

0.1 M Citric acid	21.6 ml
0.2 M Na_2HPO_4	28.4 ml

c. Sterile Glycerol

Glycerol is sterilized at 121°C for 30 min. It is used in the first 1:2 dilution (50% v/v) of neat (1X) antisera or toxic botulinum culture supernatant fluids prior to storage at -20°C.

d. Botox Stabilizing Diluent:

This diluent is used to stabilize botox culture supernatants. Combine the following:

pH 5.5 citric-phosphate buffer	25 ml
Sterile fetal bovine serum	25 ml
Sterile glycerol	50 ml

Store at -20°C

e. Modified Cooked Meat Medium (MCMM)

f. Gelatin Phosphate Buffer

Gelatin	2 g
Na_2HPO_4	4 g
Distilled Water	1 l

Dissolve the ingredients by heating gently in 800 ml of distilled water. Adjust the pH to 6.2 with HCl and bring the volume to 1 liter. Autoclave for 20 minutes at 121°C.

g. Trypsin 1:250 (Difco or equivalent), 10% in saline

h. Physiological saline, 0.85% NaCl in distilled water

14.3 Preparation of Culture Fluids for Toxin Analysis

a. Select well isolated, typical, *C. botulinum* colonies from the resulting growth on appropriate solid plating medium incubated under anaerobic conditions for 48 h at 35°C and inoculate steamed, cooled tubes of Modified

Cooked Meat Medium (MCMM). Incubate MCMM tubes at 35^{0}C for 5 days.

b. Remove 10 ml of the MCMM culture fluid and place it in sterile 50 ml stainless steel or 30 ml polycarbonate screw cap centrifuge tubes placed inside stainless steel centrifuge cups. Clarify by centrifugation at 15,000 X G for 15 minutes at 5^{0}C

c. Remove clarified culture fluid from centrifuge tube and place it in another sterile tube. Make a 1:10 dilution of this culture fluid in Gelatin Phosphate Buffer.

d. Proceed to test the culture fluid at undilute and 1:10 for the presence of toxins by the mouse bioassay procedure described below (Section 14.41).

14.31 Preparation of Solid Food Samples for Direct Toxin Analysis

a. Following the procedures given in Chapter 10.4, the entire contents, if possible, of a canned product (e.g. corned beef or canned chicken) should be transferred to a sterile stomacher™ bag.

b. Place the sample bag inside a second stomacher™ bag to minimize the possibility of uncontained leakage. Hand mix by gently squeezing the outside of the bags (knead) for 2 minutes to distribute any pockets of *C. botulinum* toxins prior to removing the food sample for testing. Remove at least 50 g for analysis.

c. The aliquot of the food sample removed should be placed in a sterile metal blender jar with a screw cap and blended with an equal volume of Gelatin Phosphate Buffer, pH 6.2., until thoroughly homogenized.

d. Ten ml of the food extract should be clarified by centrifugation at 15,000 X G for 15 minutes at 5°C in sterile 50 ml stainless steel or 30 ml polycarbonate screw cap centrifuge tubes placed inside stainless steel centrifuge cups.

e. An aliquot should be cultured following Chapter 10.45 a., the remainder held in reserve at 4°C.

f. Clarified food supernatants should be tested using the mouse bioassay procedure (Section 14.41) undiluted (1:2 of food) and at 1:5 (1:10 of food) dilution. Dilution is made in Gelatin Phosphate Buffer.

14.32 Sample Reserves

a. Samples reserves may be held at 4°C for 1-2 weeks.

b. For long term storage, culture supernatants should be stabilized with 50% glycerol and stored at -20°C.

14.4 Mouse Bioassay Screening Test for *C. botulinum* Toxins in Foods and Cultures: Introduction

The current FSIS botulinum toxin testing procedure is the mouse bioassay published in Official Methods of Analysis of AOAC International as Official Method 977.26 G.

In years of testing, the vast majority of gas-forming anaerobe cultures isolated by FSIS from meat and poultry product samples were botulinum toxin negative. Therefore FSIS found it feasible to slightly abbreviate the AOAC official mouse bioassay test for botulinum toxins as follows:

14.41 Bioassay Procedure

14.411 Non-treated Preparation

a. Remove approximately 2 ml of each food sample extract or culture fluid (at specified dilutions) being tested and place it in an appropriately labeled sterile container suitable for syringe and needle aspiration.

14.412 Trypsinization

Trypsin treatment potentiates nonproteolytic strains of types B and E that otherwise could escape detection.

a. Remove 3.6 ml of each food sample extract or culture fluid (at specified dilutions) being tested and adjust the pH to 6.0 - 6.2 with HCl.

b. Add 0.4 ml of the 10% Trypsin to 3.6 ml of the above aliquot. Incubate 1 h at 35-37°C with occasional gentle agitation.

c. Place the trypsinized material in an appropriately labeled sterile container suitable for syringe and needle aspiration.

14.413 Boiled Control

Botulinum toxin is destroyed by heating to 80°C for 10 minutes.

a. Transfer 2 ml of each food sample extract or culture fluid (at specified dilutions) being tested to an appropriately labeled sterile container suitable for syringe and needle aspiration.

b. Heat in boiling water for 10 minutes. Allow to cool prior to injection.

14.414 Test

a. Using a 1 or 3 ml syringe fitted with a 25 gage needle, inject two mice intraperitoneally (IP) with 0.5 ml each of the original, non-treated supernatant (treatment 14.411).

b. Inject two mice IP with 0.5 ml of the trypsinized supernatant (treatment 14.412).

c. Inject two mice IP with 0.5 ml of the boiled extract (treatment 14.413), these are the negative controls.

d. Observe all mice periodically for 48 h for typical neurological symptoms. Hold mice and observe for onset of neurological symptoms for an additional 2 days. Record symptoms and time of death of mice.

e. The presence of toxin is presumptively indicated by development of typical symptoms and death from treatment 14.411 and/or 14.412 but not treatment 14.413. Typical botulism symptoms in mice sequentially consist of ruffled fur, labored but not rapid breathing, weakness of the limbs progressing to total paralysis, gasping for breath (opening of lower jaw), followed by death due to

respiratory failure. Be aware that tetanus toxin may produce similar symptoms in mice. Death in the absence of neurological symptoms is not an acceptable indication of mouse botulism; death may be non-specifically caused by other microorganisms, chemicals present in test fluids or injection trauma.

14.415 Confirmation and Toxin Typing by Protection Tests

Any food extracts or culture fluids (non-treated and/or trypsin treated preparations) which produce death in mice with typical botulinum toxin symptoms should be subjected to confirmation and typing of the botulinum toxin present by specific mouse protection tests by the current AOAC Official Method 977.26 H published in Official Methods of Analysis of AOAC International, 16th Edition, 1995.

a. Obtain the same toxic preparations demonstrated to have caused death of mice in procedure 14.414.

b. Either the untreated or trypsin treated toxic preparations may be used. If the trypsin treated preparation is to be used, a freshly trypsinized portion of the untreated fluid should be prepared, as the continued action of trypsin in the original preparation may destroy the toxin.

c. Prepare 1:10, 1:100 and 1:1000 dilutions of the toxic preparations in Gelatin Phosphate Buffer.

d. Rehydrate the lyophilized vials of specific botulinum antitoxin obtained from CDC according to their specific instructions.

e. Dilute the monovalent antitoxin types A, B, C, D, E and F respectively in physiological saline to contain 1 international Unit per 0.5 ml. Prepare a sufficient quantity of each diluted antitoxin to inject 0.5 ml into each of two mice for each dilution of each toxic preparation to be tested.

f. Protect six separate groups of mice by injecting each mouse in a group IP with 0.5 ml (1 international Unit) of one of the above antitoxins (types A, B, C, D, E and F). Each protected group corresponds specifically to types A, B, C, etc.

g. Within 30 minutes to 1 h of antitoxin administration, inject protected mice groups IP with 0.5 ml of each toxic preparation at undiluted, 1:10, 1:100 and 1:1000 dilutions (2 mice per dilution) and also inject similarly pairs of unprotected mice with these same toxic preparations.

h. Observe mice periodically over 48 h for typical symptoms of botulism; record all symptoms and time of deaths. Note specifically the comparative protection afforded to each pair of specific antitoxin protected mice (A, B, C, D, E and F) relative to the unprotected control mice for each respective toxic preparation tested.

i. Protection of mice from death and botulism by one of the monovalent botulinum antitoxins, while unprotected mice die, confirms the presence of botulinum toxin and determines the homologous serological type of toxin in the tested preparation.

j. If all antitoxin protected mice should die, in addition to the unprotected mice, this may be an indication of too high a quantity of toxin in the original test preparation, the presence of more than one toxin serotype or the presence of some other non-botulinum toxic substance.

k. To determine which of the above factors may be responsible in such a case, retest the toxic preparations at higher dilutions of the test fluids and/or use mixtures of antitoxin (or polyvalent antiserum) in place of the monovalent antisera for passive protection of mice groups.

14.5 Method Quality Control

a. The quality control procedures are built in to the above procedure. Because of the nature of this test no additional methods control are recommended. However it is very important to adhere to the following safety procedures and waste disposal.

14.51 Safety Procedures and Waste Disposal

Botulinum toxins are among the most potent toxins known. In a 1989 outbreak, consumption of hazelnut yogurt (125 g) contaminated with only about 200 pg/ml of toxin B caused 28 cases of botulism and one death in England. Therefore, the following precautions must be strictly observed at all times:

a. **MOUTH PIPETTING SHOULD NEVER BE USED IN THIS ASSAY.**

b. **At the completion of an assay, all of the equipment and reagents that have been in contact with the culture or food extract must be autoclaved. Laboratory bench surfaces and non-autoclavable materials and equipment must be treated with 1% sodium hypochlorite, pH 11.0, (1:4 dilution of household bleach in water) to destroy any toxin. Accidental spills can be detoxified using the chlorine bleach solution. A supply of fresh bleach solution must ALWAYS BE AT HAND for emergency situations.**

c. **The work area should be sequestered from the work area used for other analyses if at all possible and should be prominently posted with biohazard signs.**

d. **A Class II or better Biological Containment Hood should be used whenever possible. Botulinum toxin analyses are considered Biosafety Level II analyses.**

e. **When centrifuging toxic materials, use a tightly closed centrifuge equipped with safety cups.**

f. **Botox analysts should be immunized to a protective level with a toxoid vaccine (CDC) before working with this microorganism or its' toxins in order to minimize the possibility of accidental intoxication.**

g. **Emergency phone numbers should be posted prominently in the lab.**

14.6 Selected References

Cunniff, P. (ed.). 1995. Official Methods of Analysis of AOAC International, 16th Edition. Chapter 17, p. 46-48. AOAC International, Inc., Gaithersburg, MD 20877

Kautter, D. A., H. M. Solomon, D. E. Lake, D. T. Bernard, and D. C. Mills. 1992. *Clostridium botulinum* and its toxins, p. 605-621. *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.

Smith, D. S. L. 1977. Botulism: The Organism, its Toxins, the Disease. Charles C. Thomas Publisher, Springfield, IL.