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Testing Protocol

SAM 204

Supplemental Assay Method for Potency Testing *Clostridium perfringens*
Type D Epsilon Antitoxins

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Contact: Janet M. Wilson, (515) 337-7245

Approvals: /s/Larry R. Ludemann Date: 18Dec17
Larry R. Ludemann, Section Leader
Bacteriology

/s/Paul J. Hauer Date: 22Dec17
Paul J. Hauer, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type D Epsilon Antitoxins

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Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type D Epsilon Antitoxins

1. Introduction

This Supplemental Assay Method (SAM) describes the method used to determine the epsilon antitoxin content of *Clostridium perfringens* type D antitoxin as prescribed by title 9, *Code of Federal Regulations* (9 CFR), section 113.455. The epsilon antitoxin is titrated by a toxin-antitoxin neutralization test, using mice as an indicator.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

- 2.1.1 Vortex mixer
- 2.1.2 Autoclave
- 2.1.3 Freezer, -70°C
- 2.1.4 Refrigerator, 2°- 7°C
- 2.1.5 Micropipettes, 100-µL and 1000-µL

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

- 2.2.1 *C. perfringens* type D (epsilon) standard antitoxin IRP 249
- 2.2.2 *C. perfringens* type D (epsilon) standard toxin IRP 632
- 2.2.3 Peptone diluent
- 2.2.4 Glass screw-top tubes, 13 x 100-mm with caps
- 2.2.5 Pipettes, 1-mL, 5-mL, 10-mL, 25-mL
- 2.2.6 Syringes, 1-cc
- 2.2.7 Needles, 25- to 27-gauge x 1/2- to 1 1/4-inch
- 2.2.8 Screw-top Erlenmeyer flask, 500-mL with cap

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2.2.9 Glass dilution bottles, 160-mL

2.2.10 Polypropylene conical screw-cap tubes, 50-mL

2.2.11 Polystyrene snap-top tubes, 17 x 100-mm with caps

2.2.12 Water, distilled or deionized, or water of equivalent purity

2.2.13 Tips for micropipettes

2.3 Test animals

White Swiss non-pregnant female mice, 16-20 g (Five mice are required for each toxin-antitoxin mixture.)

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware; and have specific training and experience in the safe handling of clostridial toxins. Personnel should also have specific training in the care and handling of laboratory mice.

3.2 Preparation of equipment

All equipment is operated according to the manufacturer's instructions.

3.3 Preparation of reagents

3.3.1 Peptone diluent

Peptone (Difco)	8 g
NaCl, reagent grade	2 g
Water q.s. to	800 mL

Dissolve peptone and sodium chloride in water. Adjust pH to 7.2 with 1N sodium hydroxide. Fill a 500-mL Erlenmeyer flask no more than 3/4 full with diluent.

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Autoclave with caps loosened at $\geq 121^{\circ}\text{C}$ for 25 to 30 minutes. Cool flasks in ice water and tighten caps. Store at 2° - 7°C for up to 3 months.

3.3.2 Preparation of *C. perfringens* type D standard epsilon antitoxin

1. *C. perfringens* type D (epsilon) antitoxin IRP 249 contains 50 International Units of antitoxin per mL (AU/mL) and has been standardized against the World Health Organization *C. perfringens* (*C. welchii*) type D International antitoxin. Each vial contains 3.4 mL of antitoxin.

2. Prepare a dilution of antitoxin that contains 1.0 AU epsilon antitoxin per mL by adding 2.0 mL of IRP 249 to 98.0 mL of peptone diluent in a 160-mL glass dilution bottle. Mix well. Dispense in 2.5-mL amounts into 13 x 100-mm tubes. Store at $-70^{\circ}\pm 5^{\circ}\text{C}$ until used.

3.3.3 Preparation of *C. perfringens* type D standard epsilon toxin

1. Prepare a 1:10 dilution of *C. perfringens* type D epsilon toxin by adding 0.5 mL of IRP 632 to 4.5 mL of peptone diluent in a screw-cap tube. Mix well. Dispense diluted epsilon toxin in 1.5-mL amounts into 13 x 100-mm tubes. IRP 632, diluted 1:10, is stable when stored at -60°C or lower.

2. Further dilute the epsilon toxin to 1:150 by adding 1.0 mL of diluted (1:10) epsilon toxin to 14.0 mL of peptone diluent in a 17 x 100-mm snap-top tube. For the purpose of this test, the 1:150 dilution of IRP 632 is referred to as the standard epsilon toxin.

Note: A volume of 0.6 mL of standard epsilon toxin and 0.4 mL of peptone diluent represents 10 L_o doses. A volume of 0.8 mL of the standard epsilon toxin and 0.2 mL of peptone diluent represents 10 L₊ doses (see Sections 4.1.1 and 4.1.2). For the purposes of this SAM, 10 L_o dose is defined as the greatest amount of toxin that, when mixed with 1.0 AU, results in 100% survival of all mice inoculated intravenously (IV) with 0.2 mL of this mixture. The 10 L₊ dose is defined as the least amount of toxin that, when mixed with 1.0 AU, results in the death of 80%-100% of all mice inoculated IV with 0.2 mL of this mixture.

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4. Performance of the Test

4.1 Toxin neutralization

4.1.1 Product and standard epsilon toxin

1. Mix a sufficient volume of standard epsilon toxin and peptone diluent (0.6 mL of standard toxin and 0.4 ml peptone diluent (10 L_o doses)) for each product antitoxin dilution and the L_o control.
2. Mix each product sample (*C. perfringens* type D antitoxin or *C. perfringens* types C & D antitoxin) by shaking thoroughly.
3. Dilute each product sample according to the table below using 50-mL conical tubes. Add 1 mL of each antitoxin dilution to 1 mL of the standard epsilon toxin-peptone diluent mixture (10 L_o doses) in 17 x 100-mm snap-top tubes. Mix each tube with a vortex-type mixer.

Int'l AU tested	Unknown Antitoxin	10 L _o doses	
		Std Toxin	Diluent
34	1.0 mL diluted 1:34 (1 mL product + 33 mL diluent)	0.6 mL	0.4 mL
39	1.0 mL diluted 1:39 (1 mL product + 38 mL diluent)	0.6 mL	0.4 mL

4. Let the mixtures sit at 22°- 26°C (room temperature) for 1 hour before placing tubes in ice.

4.1.2 Standard epsilon toxin and standard epsilon antitoxin controls

1. Add 1.0 mL of standard epsilon antitoxin containing 1.0 AU/mL to 1 mL of the standard epsilon toxin-peptone diluent mixture (10 L_o doses) in a 17 x 100-mm snap-top tube. Mix well with a vortex mixer.
2. Add 1.0 mL of standard epsilon antitoxin containing 1.0 AU/mL to a 17 x 100-mm snap-top tube containing 0.8 mL of epsilon toxin and 0.2 mL of diluent (10 L₊ doses). Mix well with a vortex mixer.
3. Let the mixtures stand at 22°- 26°C (room temperature) for 1 hour.
4. Place tubes in ice.

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4.2 Inoculation of mice

4.2.1 Inject 0.2 mL of each standard epsilon toxin-product antitoxin mixture into each of 5 mice.

4.2.2 Inject 0.2 mL of each standard epsilon toxin-standard epsilon antitoxin mixture into each of 5 mice.

4.2.3 Inoculate all mice intravenously into a lateral tail vein. Use 1-cc syringes fitted with 25- to 27-gauge x 1/2- to 1 1/4-inch.

4.2.4 Always inoculate the mice receiving the standard epsilon toxin-standard epsilon antitoxin mixtures (controls) **last**.

4.2.5 Mouse inoculations should be completed within 1 hour of placing the toxin-antitoxin mixtures in the ice.

4.2.6 The test is concluded 24 hours after the mice are inoculated.

5. Interpretation of the Test Results

5.1 Criteria for a valid test

5.1.1 All 5 mice inoculated with the standard 10 L₀/1.0 AU control mixture must survive.

5.1.2 At least 4 out of 5 mice inoculated with the standard 10 L₊/1.0 AU control mixture must die.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

5.2 Interpretation of serial results

5.2.1 The product contains at least 34 International Units of epsilon antitoxin per ml if 5 out of 5 mice inoculated with the 1:34 dilution of product-standard epsilon toxin mixture survive.

5.2.2 The product contains at least 39 International Units of epsilon antitoxin per mL if 5 out of 5 mice inoculated with the 1:39 dilution of product-standard epsilon toxin mixture survive.

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5.2.3 The product is considered satisfactory if it contains at least 34 International Units of epsilon antitoxin per mL.

5.2.4 The product is considered unsatisfactory if it contains less than 34 International Units of epsilon antitoxin per mL. (If any mice inoculated with the 1:34 dilution and 10 L_o doses of standard epsilon toxin die, the product is considered to contain less than 34 International Units/mL.)

6. Report of Test Results

Report results of the test(s) as described by the standard operating procedures.

7. References

7.1 Title 9, *Code of Federal Regulations*, section 113.455, U.S. Government Printing Office, Washington, DC.

7.2 History of toxin: *C. perfringens* type D (epsilon) culture CN3688, used to produce IRP 632, was obtained from Coopers Animal Health, Inc., 1201 Douglas Avenue, Kansas City, Kansas 66103-1438, on January 5, 1976. The number of passages is unknown.

7.3 History of antitoxin: *C. perfringens* type D (epsilon) antitoxin, IRP 249, was produced in 1981 at the Center for Veterinary Biologics (CVB) (then part of the National Veterinary Services Laboratories (NVSL)), Ames, Iowa. The antitoxin is of equine origin.

8. Summary of Revisions

Version .06

- Toxin lot IRP 632 replaces IRP 450 throughout the document.
- The Director was updated on the cover page.

Version .05

- The Bacteriology Section Leader was updated.
- Minor word changes for clarification of procedures.

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Version .04

- The Contact information has been updated.

Version .03

- The document number has been changed from BBSAM0204 to SAM 204.
- **5.2:** Clarification of a satisfactory product has been added.

Version .02

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- IRP 410 has changed to IRP 450 throughout the document.
- **4.1** The format and content have been modified to clarify the L₀ and L₊ levels of the Toxin Neutralization process.
- Humane endpoint language has been added.
- Dilution/holding vessel sizes have been added for clarification.
- The contact person has been changed to Janet M. Wilson.