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Center for Veterinary Biologics
Testing Protocol

SAM 212

Supplemental Assay Method for Potency Testing *Clostridium sordellii* Antigen

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

Approvals:

/s/Larry R. Ludemann Date: 26Dec17
Larry R. Ludemann, Section Leader
Bacteriology

/s/Paul J. Hauer Date: 27Dec17
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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Entered into CVB Quality Management System
by: /s/Linda S. Snavely 28Dec17
Linda S. Snavely Date
Quality Management Program Assistant

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Supplemental Assay Method for Potency Testing *Clostridium sordellii* Antigen

Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) describes the method used to determine whether biological products containing *Clostridium sordellii* antigen can stimulate the production of satisfactory immunity as prescribed by title 9, *Code of Federal Regulations* (9 CFR), section 113.109. For products that require 2 vaccinations, rabbits are vaccinated twice 20 to 23 days apart and bled 14 to 17 days following the second vaccination. For products that require a single vaccination, rabbits are vaccinated and bled 34 to 40 days later. The serum is titrated by a toxin-antitoxin neutralization test, using mice as an indicator.

2. Materials

2.1 Equipment/instrumentation

2.1.1 Centrifuge

2.1.2 Vortex mixer

2.1.3 Autoclave

2.1.4 Freezers, -20°C and -70°C

2.1.5 Refrigerator, 4°- 8°C

2.1.6 Micropipettes, 100-µL and 1000-µL

2.2 Reagents/supplies

2.2.1 *C. sordellii* standard antitoxin IRP 501 (04)

2.2.2 *C. sordellii* standard toxin IRP 604

2.2.3 Peptone diluent

2.2.4 Xylazine, 20 mg/mL solution

2.2.5 Ketamine hydrochloride, 100 mg/mL solution

2.2.6 Glass screw-top tubes, 13 x 100-mm, with caps

2.2.7 Polypropylene screw-cap tubes, 50-mL

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

2.2.9 Polystyrene screw-cap 17 x 120-mm conical tubes

2.2.10 Serum separation tubes, 12.5-mL

2.2.11 Pipettes, 2-mL, 5-mL, 10-mL, 25-mL

2.2.12 Tips for micropipettes

2.2.13 Syringes (needle-locking), 1-cc, 10-cc, 20-cc, 30-cc

2.2.14 Needles, 25- and 27-gauge x 1/2- to 1 1/4-inch, 20-gauge x 1-inch

2.2.15 Vacutainer® needles, 20-gauge x 1 1/2-inch

2.2.16 Screw-top Erlenmeyer flask, 500-mL, with cap

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

2.3 Test animals

2.3.1 New Zealand White rabbits, non-pregnant females, 4-8 lb. (At least 8 rabbits are required per serial to be tested.)

2.3.2 White Swiss mice, non-pregnant females, 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 to have working knowledge of the use of general laboratory chemicals, equipment, and glassware; and must have specific training and experience in the safe handling of clostridial toxins. Personnel need specific training in the care and handling of laboratory rabbits and mice.

3.2 Preparation of equipment/instrumentation

All equipment is operated according to manufacturers' instructions.

3.3 Preparation of reagents/control procedures

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 1 N sodium hydroxide. Fill a 500-mL Erlenmeyer flask no more than 3/4 full with diluent. Autoclave with caps loose at ≥121°C for 25-30 minutes following manufacturer's recommendations. Cool flasks in ice water and tighten caps. Store at 2°- 7°C for up to 3 months.

3.3.2 Preparation of *C. sordellii* standard antitoxin

1. *C. sordellii* antitoxin IRP 501 (04) contains 170 antitoxin units per mL (AU/mL), and has been standardized against the World Health Organization (WHO) gas-gangrene (*C. sordellii*) international antitoxin, equine origin. Each vial contains 1.3 mL of antitoxin.

2. Prepare a solution of *C. sordellii* antitoxin that contains 17 AU/mL by adding 1.0 mL of IRP 501 (04) to 9.0 mL of peptone diluent in a 17 x 120-mm screw-cap tube. Dispense 1.5-mL amounts into 13 x 100-mm tubes. IRP 501 (04), diluted 1:10, is stable when stored at -70°± 5° C.

3.3.3 Preparation of *C. sordellii* standard toxin

Each vial of *C. sordellii* standard toxin IRP 604 contains 1.3 mL of toxin. Store the toxin at -70°± 5°C until used.

4. Performance of the Test

4.1 Vaccination of rabbits

4.1.1 Thoroughly shake each bottle of product and wipe the top with alcohol before filling the syringe.

4.1.2 Vaccinate each rabbit subcutaneously in the shoulder region with not more than half of the recommended cattle dose. (If the product is recommended only for sheep, use half of the recommended sheep dose.) Use 10-, 20- or 30-cc syringes fitted with 20-gauge x 1-inch needles to vaccinate the rabbits.

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4.1.3 For products that require 2 vaccinations, give the second vaccination 20 to 23 days after the first.

4.2 Collection and preparation of rabbit serum

4.2.1 Collect blood from the test rabbits 34 to 40 days after vaccination (or 14 to 17 days after the second vaccination for products that require 2 vaccinations).

4.2.2 Anesthetize rabbits for bleeding with a mixture of 1.32 mg/kg of xylazine and 8.8 mg/kg of ketamine hydrochloride. Give the anesthetic mixture by intramuscular injection.

4.2.3 Use a 12.5-mL Vacutainer® serum separation tube fitted with a 20-gauge x 1 1/2-inch Vacutainer® needle to collect blood from the heart. Collect approximately 12.5 mL of blood from each rabbit. Gently invert tubes 5 times. Let the tubes of blood sit at 22°- 26°C (room temperature) for 30 to 60 minutes.

4.2.4 Centrifuge blood at 1000 X g for 10 to 20 minutes at room temperature.

4.3 Preparation of pooled serum

4.3.1 Prepare a pooled sample using an equal volume of serum from at least 7 rabbits per vaccinated group (provided that, if more than 7 rabbits are bled per vaccinated group, then equal volumes from each rabbit are used for the serum pool). If less than 7 rabbits are bled, the test is invalid and should be repeated.

The pooled sample may be held at 2°- 7°C for up to 7 days. If testing will not be completed within 7 days, store the pooled sample at -20°C or lower.

4.4 Toxin neutralization

4.4.1 Preparation of standard test toxin

1. Add 1.0 mL of well-mixed *C. sordellii* toxin IRP 604 to 18.0 mL of diluent in a 50-mL screw-cap tube. (This represents a 1:19 dilution of toxin and will be referred to as the standard test toxin.)

2. A volume of 0.5 mL and 0.8 mL of the standard test toxin mixed with 1.0 AU represents 1 L_o and 1 L_+ dose, respectively.

3. For the purposes of this SAM, 1 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 1 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.

4.4.2 Preparation of standard antitoxin

Add 1.0 mL of the dilution containing 17 AU/mL (see **Section 3.3.2**) to 16.0 mL of diluent in a 50-mL screw-cap tube. (This dilution contains 1 AU/mL and is referred to as the standard antitoxin.)

4.4.3 Product and standard test toxin

1. Mix a sufficient volume of standard toxin and peptone diluent (0.5 mL of standard toxin and 0.5 mL peptone diluent [1 L_o dose]) in a 17 x 120-mm screw-cap tube for each serum pool and the L_o control.

2. Add 1 mL of each of the serum dilutions (examples listed in the table below) to 1 mL of the standard toxin-peptone diluent L_o mixture in 17 x 100-mm snap-top tubes. Mix each tube with a vortex-type mixer.

Int'l AU tested	Serum	1 L_o dose	
		Std Toxin	Diluent
1.0	1.0 mL undiluted	0.5 mL	0.5 mL
2.0	1.0 mL diluted 1:2	0.5 mL	0.5 mL
5.0	1.0 mL diluted 1:5	0.5 mL	0.5 mL
10.0	1.0 mL diluted 1:10	0.5 mL	0.5 mL

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

4.4.4 Standard test toxin and standard antitoxin controls

1. Add 1.0 mL of standard antitoxin containing 1.0 AU/mL to 1.0 mL of the standard toxin-peptone diluent L_o mixture in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.

2. Add 1.0 mL of standard antitoxin containing 1.0 AU/mL to a 17 x 100-mm snap-top tube containing 0.8 mL of standard test toxin and 0.2 mL of diluent (1 L_+ dose). Mix well with a vortex-type mixer.

3. Let the mixtures stand at room temperature for 1 hour before placing tubes in ice.

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4.5 Inoculation of mice

4.5.1 Inject 0.2 mL of each standard test toxin-product antitoxin mixture into each of 5 mice.

4.5.2 Inject 0.2 mL of each standard test toxin-standard antitoxin mixture into each of 5 mice.

4.5.3 Inoculate all mice intravenously into 1 of the lateral tail veins. Use 1-cc syringes fitted with 25- or 27-gauge x 1/2-inch to 1 1/4-inch needles.

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

4.5.5 Mouse inoculations need to be completed within 1 hour of placing the toxin-antitoxin mixtures in the ice.

4.5.6 The test is concluded 72 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 1 L_o/1.0 AU control mixture must survive.

5.1.2 At least 4 of the 5 mice inoculated with the standard 1 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 If 5 of the 5 mice inoculated with the undiluted serum-toxin mixture survive, the serum contains at least 1.0 AU/mL of *C. sordellii* antitoxin and the product is satisfactory.

5.2.2 If 5 of the 5 mice inoculated with the diluted serum-toxin mixture survive, the serum contains 1.0 AU/mL times the reciprocal of the dilution and the product is satisfactory.

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5.2.3 The product is considered unsatisfactory if the serum pool from at least 7 rabbits contains less than 1.0 AU/mL of *C. sordellii* antitoxin.

6. Reporting of Test Results

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

7. References

7.1 Title 9, *Code of Federal Regulations*, section 113.109, U.S. Government Printing Office, Washington, DC.

7.2 History of antitoxin: *C. sordellii* antitoxin [IRP 501(04)] was produced in goats at the Center for Veterinary Biologics (CVB), Ames, Iowa, in August 2004.

7.3 History of toxin: *C. sordellii* toxin (IRP 604) was produced at the CVB, Ames, Iowa, in September 2011. The toxin was made from *C. sordellii* culture No. 7502, which was obtained from Montana State University, Bozeman, Montana, on September 16, 1968. The number of passages is unknown.

8. Summary of Revisions

Version .06

- The Director was updated on the cover page.

Version .05

- The Bacteriology Section Leader was updated.
- The standard toxin was changed from IRP 497 to IRP 604 throughout the document.
- Minor word changes for clarification of procedures.

Version .04

- The Contact information has been updated.

Version .03

- One of the signatories for this document has been changed due to personnel changes.

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Supplemental Assay Method for Potency Testing *Clostridium sordellii* Antigen

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:

- The standard toxin IRP 334 was changed to IRP 497 throughout the document.
- The standard antitoxin IRP 333 was changed to IRP 501(04) throughout the document.
- Humane endpoint language was added.
- Dilution/holding vessel sizes were added for clarification.
- The contact person was changed to Janet M. Wilson.

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