United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 206

Supplemental Assay Method for Potency Testing Tetanus Antitoxins

Date: **June 12, 2014**

Number: SAM 206.04

Supersedes: SAM 206.03, June 28, 2011

Standard Requirement: 9 CFR, Part 113.451

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

Approvals:

/s/Larry R. Ludemann Date: 18Jun14
Larry R. Ludemann, Section Leader
Bacteriology

/s/Byron E. Rippke Date: 30Jun14
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 30Jan14
Rebecca L.W. Hyde, Section Leader
Quality Management
Center for Veterinary Biologics

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

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

UNCONTROLLED COPY

Supplemental Assay Method for Potency Testing Tetanus Antitoxins

Table of Contents

1. Introduction

2. Materials

   2.1 Equipment/instrumentation
   2.2 Reagents/supplies
   2.3 Test Animals

3. Preparation for the Test

   3.1 Personnel qualifications/training
   3.2 Preparation of supplies
   3.3 Preparation of diluent buffer

4. Performance of the Test

5. Interpretation of the Test Results

6. Report of Test Results

7. References

8. Summary of Revisions

UNCONTROLLED COPY

Supplemental Assay Method for Potency Testing Tetanus Antitoxins

1. Introduction

This Supplemental Assay Method (SAM) describes a method used to determine the antibody content of tetanus (*Clostridium tetani*) antitoxin, as required in the title 9, *Code of Federal Regulations* (9 CFR), part 113.451. The antitoxin is titrated by a comparative toxin-antitoxin neutralization test using guinea pigs 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 Mixer, vortex-type

2.1.2 Freezers, -70°C or lower

2.1.3 Refrigerator, 2°- 7°C

2.2 Reagents/supplies

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

2.2.1 Standard equine tetanus antitoxin containing 500 antitoxin units (AU)/mL. This standard antitoxin is provided by the Center for Veterinary Biologics (CVB).

2.2.2 Standard *C. tetani* toxin. This is provided by the CVB.

2.2.3 1/15 M phosphate buffered physiological saline with 0.2% gelatin

2.2.4 Class A graduated cylinders, various sizes

2.2.5 Serum vials, 20-mL, with caps and seals

2.2.6 Glass pipettes, various sizes

2.2.7 Syringes, 10-mL

2.2.8 Needles, 22-gauge x 1 1/2-inch

UNCONTROLLED COPY

2.3 Test Animals

Guinea pigs, 340-380 g. These animals should be healthy, not pregnant, free of external parasites, and have no signs of ringworm infection. Each serial of test product requires 6 guinea pigs. Two additional control guinea pigs are required for each testing session. Although the 9 CFR does not specify a specific guinea pig source and strain, the CVB uses Charles River Hartley guinea pigs.

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel need to have a working knowledge of the use of general laboratory chemicals, equipment, and glassware; and have specific training and experience in the safe handling of *Clostridium* toxins. Personnel need specific training in the care and handling of laboratory guinea pigs. Personnel need to have been vaccinated against tetanus within the past 10 years.

3.2 Preparation of supplies

3.2.1 Operate all equipment according to the manufacturers' instructions.

3.2.2 Use only sterile supplies (e.g., needles, syringes, pipettes, etc.).

3.3 Preparation of diluent buffer

Solution A: Stock solution of M/15 KH_2PO_4
Dissolve 9.072 grams of KH_2PO_4 in one liter of deionized water.

Solution B: Stock solution of M/15 Na_2HPO_4
Dissolve 9.465 grams of Na_2HPO_4 in one liter of deionized water.

M/15 phosphate buffer

Solution A	192.0 mL
Solution B	808.0 mL

Adjust to pH 7.4, if needed.

1/15M phosphate-buffered saline with 0.2% gelatin (Diluent Buffer)

NaCl	8.5 g
Gelatin	2.0 g
M/15 phosphate buffer, q.s to	1000.0 mL

UNCONTROLLED COPY

Autoclave at ≥ 121°C for 30 minutes following manufacturer's recommendations. Store at 2°- 7°C for no longer than 6 months.

4. Performance of the Test

4.1 Preparation of Tetanus Antitoxin to be tested

For each serial of product being tested:

4.1.1 Measure the volume of antitoxin in a single container of test product by pouring the entire contents into a graduated cylinder. Use an appropriately sized cylinder that will accurately measure the volume, and read the volume at the bottom of the meniscus.

4.1.2 Determine the expected total number of antitoxin units per container which is specified on the label of the test product. Serials at release must have a certain amount of excess antitoxin beyond that which is reported on the label. If this potency test is being done to support serial release:

1. If the product has 1-year dating (per Section VI.D. of the Outline of Production), the container must have at least 10% excess antitoxin beyond the labeled amount. Multiply the AU dose on the label by 1.10.

2. If the product has 3-year dating (per Section VI.D. of the Outline of Production), the container must have at least 20% excess antitoxin. Multiply the AU dose on the label by 1.20.

4.1.3 Divide the labeled number of antitoxin units by the volume determined in **Section 4.1.1** to calculate the expected number of AU/mL (corrected for overage, if appropriate).

4.1.4 Assuming that the expected potency (AU/mL) is the actual potency, dilute the test antitoxin to 0.1 AU/mL. This may require stepwise/serial dilutions. Each individual dilution should not exceed 1:10 and the transfer volume should not be less than 1.0 mL.

4.1.5 Place 3.0 mL of the final dilution of test antitoxin in a labeled 20-mL serum bottle. Hold the samples at 20°- 25°C for 30 ± 5 minutes before mixing with a test dose of toxin.

UNCONTROLLED COPY

4.2 Standard Equine tetanus antitoxin preparation

4.2.1 Add 1.0 mL of equine tetanus antitoxin (standard) to a 50-mL graduated cylinder. Add 49.0 mL of diluent buffer. Mix thoroughly.

4.2.2 Make two serial tenfold dilutions (1.0 mL antitoxin into 9.0 mL diluent buffer), starting with the dilution from **Section 4.2.1**, to prepare a solution containing 0.1 AU/mL.

4.2.3 Add 3.0 mL of the diluted antitoxin to a 20-mL serum bottle. Allow the antitoxin to stand at 20°- 25°C for 30 ± 5 minutes before mixing with the test dose of toxin.

4.3 Preparation of Standard *Clostridium tetani* toxin

4.3.1 The test dose of toxin is defined as the amount of toxin (in a 2-mL dose volume) which, when allowed to neutralize with 0.1 AU of standard antitoxin for one hour at 20°- 25°C and then inoculated subcutaneously into a 340 to 380 gram guinea pig, will result in the death of the animal in 60 to 120 hours, with clinical signs of tetanus. The working dilution of toxin is lot specific and indicated on the Reagent Data Sheet for the lot being used.

4.3.2 Dilute the toxin to the specified working dilution. If necessary, prepare stepwise/serial dilutions to obtain the final working dilution. Each individual dilution should not exceed 1:20 and the transfer volume should not be less than 1.0 mL.

4.4 Mixing standard toxin (test dose) with antitoxin dilutions

4.4.1 Add 6.0 mL of standard toxin (diluted as in **Section 4.3.2**) to each of the serum bottles containing diluted antitoxin (**Sections 4.1.5 and 4.2.3**).

4.4.2 Cap and seal the serum bottles.

4.4.3 Thoroughly mix the toxin-antitoxin mixtures and hold at 20°- 25°C for 1 hour.

4.5 Inoculation of test animals

4.5.1 Inoculate the toxin-antitoxin mixture from each serum bottle into two guinea pigs. Administer 3.0 mL subcutaneously in the ventral flank region with a 10-mL syringe fitted with a 22-gauge x 1 1/2-inch disposable needle.

UNCONTROLLED COPY

4.5.2 In making the inoculation, insert the needle through the skin on one side of the flank and pass it subcutaneously across the abdomen and linea alba so that the mixture is deposited in the opposite flank region. Care must be taken not to puncture the peritoneum, or the skin along the subcutaneous route, as any consequent leakage may interfere with test results. A distinct subcutaneous bleb is present when the injection is properly made.

4.5.3 All of the guinea pigs in a single test session must be inoculated within a 1 hour period to ensure that the toxin does not deteriorate before it is injected into the animal. Inoculate animals in the order in which the toxin-antitoxin mixtures were prepared. Inoculate the control animals last so that these animals can also provide confirmation that the toxin retained its activity throughout the inoculation period.

5. Interpretation of the Test Results

5.1 Observations

5.1.1 Observe the animals daily 24 to 48 hours postinoculation, and 3 to 6 times between 60 and 96 hours postinoculation; this is the time the animals are most likely to become paralyzed or die due to non-neutralized tetanus toxin. Continue observations up to 120 hours postinoculation or until the two control animals die or become moribund (i.e., unable to rise or stand under their own power). Moribund animals will be euthanized and considered as deaths. Record the clinical signs* for each animal observed and the time elapsed from inoculation.

*The clinical signs to be observed are increased muscle tonus; curvature of the spine; asymmetry of the body outline when the resting animal is viewed from above; generalized spastic paralysis, particularly of the extensor muscles; inability to rise from a smooth surface when the animal is placed on its side; or any combination of these signs.

5.1.2 Once the control animals have succumbed, the test may be terminated and the rest of the test animals euthanized. Observe and record each animal's condition prior to euthanasia.

5.1.3 Average the survival times for the two guinea pigs injected with the same toxin-antitoxin mixture.

5.2 Validity Requirements

Control guinea pigs must die (or become moribund) within 24 hours of each other and within an overall time of 60 to 120 hours postinoculation; otherwise, the assay is invalid.

UNCONTROLLED COPY

5.3 Criteria for a satisfactory test

This test, as described in this SAM, is a qualitative test, indicating whether the test product has greater, or fewer, antitoxin units than the expected amount determined in **Section 4.1.2**. The test product is considered satisfactory if, in a valid test, it contains *at least* the expected amount of antitoxin.

5.3.1 The test product is considered to have the expected amount of antitoxin if the test animals progress with tetanus symptoms and have the same average time to death as the control animals.

5.3.2 The test product is considered to have less than the expected amount of antitoxin if the test animals develop symptoms of tetanus and have a shorter average time to death than the control animals.

5.3.3 The test product is considered to have more than the expected amount of antitoxin if the test animals have a longer average time to death than the control animals (or are still healthy at the time the test is terminated).

6. Report of Test Results

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

7. References

Title 9, *Code of Federal Regulations*, part 113.451, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

<u>Version .04</u>

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

<u>Version .03</u>

- The Contact information has been updated.
- **2.3:** Clarification on the type of guinea pigs CVB uses for this test has been added.

UNCONTROLLED COPY

Version .02

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

- **5.1.1** Humane endpoint language has been added.
- The contact, Janet M. Wilson, has been added.

UNCONTROLLED COPY