United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 612

Supplemental Assay Method for Bacterial Plate Count of *Erysipelothrix rhusiopathiae* Vaccines

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1. Introduction

This Supplemental Assay Method (SAM) establishes the titration method for the analysis of *Erysipelothrix rhusiopathiae* vaccine, live culture, to determine the colony-forming units (CFU) in final container samples, as prescribed in title 9, *Code of Federal Regulations* (9 CFR), part 113.67. This method uses 5% bovine blood agar for determining CFUs and 1% peptone saline as a diluent.

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 Colony counter

2.1.3 Pipetting aid

2.1.4 35°± 2°C incubator

2.1.5 Laminar-flow Class II biosafety cabinet (BSC)

2.2 Reagents/supplies

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

2.2.1 1% Peptone-saline solution (**Appendix I**) - National Centers for Animal Health (NCAH) Media #10138

2.2.2 Blood agar with 5% bovine blood (**Appendix II**) - NCAH Media #10006 or as stated in the Outline of Production (OP) from the biologics manufacturer

2.2.3 *E. rhusiopathiae* reference culture (American Type Culture Collection (ATCC) #19414**)**

2.2.4 70% ethanol

2.2.5 Sterile water in serum vials

2.2.6 Inoculum spreader

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2.2.7 Disposable syringes and needles

2.2.8 Sterile disposable pipettes

2.2.9 Sterile screw-capped culture tubes

2.2.10 Gloves and lab coat

2.2.11 4 x 4-inch sterile gauze pads

2.2.12 Test tube rack

2.2.13 Sharps container

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Operate all equipment and instrumentation according to the manufacturer's instructions and maintain according to standard operating procedures (SOPs).

3.2.2 Monitor temperature of incubators according to SOPs.

3.2.3 Turn on the biosafety cabinet at least 30 minutes before starting work.

3.2.4 Label all plates with sample number or name, vial number, and dilution series. Label 3 plates per dilution series for each serial.

3.3 Preparation of reagents/control procedures

3.3.1 Warm the samples and reference culture to room temperature before rehydrating to the appropriate volume.

3.3.2 *E. rhusiopathiae* reference stock culture is prepared according to the manufacturer's instructions.

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3.3.3 Negative and Positive Controls: Incubate 1 uninoculated plate of 5% bovine blood agar and 1 plate inoculated with sterile diluent along with the test sample plates as negative control plates. *E. rhusiopathiae* reference culture (positive control) is diluted the same as the test samples, but plated depending on the titer found in **Section 3.3.2.**

3.3.4 Store plates used for making counts at refrigerator temperature. Place plates to be used for counts in a 35°± 2°C incubator overnight prior to use or dry in a BSC before use. At the time of use, plates are no more than 3 months old.

3.4 Preparation of the sample

Samples are *E. rhusiopathiae* vaccines and/or combination products containing this fraction. Sterile purified water in serum vials in volumes specified on the product label or in the firm's OP is used for rehydrating samples that are not accompanied by diluent.

4. Performance of the Test

4.1 Remove 2 vials (or the number of vials stated in the OP for testing) of product to be tested and 1 vial of *E. rhusiopathiae* reference stock culture from the freezer or cooler storage and allow to warm to room temperature.

4.2 Disinfect the cap with 70% ethanol. Rehydrate the vials and allow the contents to reconstitute for at least 5 minutes. Shake the vials by inversion until thoroughly mixed.

4.3 Prepare a tenfold dilution series of the product by setting up a rack of 20 x 150-mm screw-capped tubes and pipetting 9.0 mL of 1% peptone saline solution into each tube using a 10-mL pipette. Label the tubes 10^{-1} to 10^{-x} as needed.

4.4 Transfer 1.0 mL of the first sample from **Section 4.2** into the first tube of 1% peptone saline solution by using a pipette. Cap the tube and vortex. The dilution series is continued by using a new pipette to transfer a 1.0 mL sample from this tube to the tube labeled 10^{-2}. Repeat this method using a sterile pipette for each transfer until the required number of serial tenfold dilutions (refer to the firm's OP) is attained.

4.5 Deposit 0.1 mL of the sample from the last 3 dilution points of the dilution series for the product onto the surface of media in **Section 2.2.2** using a sterile pipette.

4.6 Use a sterile inoculum spreader to evenly distribute the inoculum over the surface of the agar medium.

4.7 Repeat **Sections 4.4 through 4.6** with the second vial of product.

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4.8 Prepare 3 plates of media as in **Sections 4.5** through **4.6** from each of 3 reference control dilutions as determined from **Section 3.3.2**.

4.9 Invert all plates and incubate at 35°± 2°C for up to 72 hours. After incubation, count plates from each series that contains 30 to 300 CFUs. Determine the mean CFU per dose for the number of vials tested using the calculation listed below.

$$\frac{\text{(Average Count) x (mL used to rehydrate)}}{\text{(Dilution used) x (mL plated) x (Number of doses)}} = \text{CFU/dose}$$

5. Interpretation of the Test Results

5.1 If on the initial test the CFU per dose is equal to or exceeds the required minimum as written in the firm's OP, the serial or subserial is satisfactory (SAT) for bacterial count without additional testing.

5.2 If on the initial test the CFU per dose is less than the required minimum as written in the firm's OP, the serial or subserial may be retested using double the amount of new vaccine samples used in the initial test, provided that if the retest is not done, the serial or subserial is unsatisfactory (UNSAT). Compare the firm's OP method to this SAM when retesting with the new vaccine samples. If on the RT the average count of the new vaccine samples with the firm's OP method is less than the required minimum, the serial or subserial is UNSAT.

5.3 If on the retest with the new vaccine samples, the average using the firm's OP method count is equal to or exceeds the required minimum, the serial is SAT.

5.4 If on the initial test the reference culture or positive control culture is not within the titer range determined in **Section 3.3.2**, but the serial being tested has a SAT result, the serial or subserial is a no test (NT) for bacterial count without additional testing, and the product is released on the results of the firm's tests. If the reference culture is not within its titer range and the serial being tested is below its minimum release titer, the serial is retested without bias using the same number of new vaccine samples as the initial test. If on the initial test there is growth on the negative control plates, the serial or subserial is a NT for bacterial count without additional testing.

6. Record and Report of Test Results

Record and report results of the test(s) according to SOPs.

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7. References

Title 9, *Code of Federal Regulations*, part 113.67, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .04

- The Bacteriology Section Leader has been updated.
- **1-6:** These Sections have been clarified and updated to reflect current practices.
- **Appendices:** Media storage conditions have been updated.

Version .03

- The Contact information has been updated.
- References to NVSL have been changed to NCAH throughout the document.
- **Appendices:** Media storage conditions have been added.

Version .02

This document was revised to clarify 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:

- Amanda Byersdorfer has been added as a Contact.
- **2.1:** The Bunsen burner has been removed from the equipment list.
- **4.8:** The use of 1 plate inoculated with sterile diluent as a negative control to run sterility check on diluent used in testing has been added.
- **4.9:** The calculation to determine CFU/dose has been added.

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Appendix I

NCAH Media #10138

Peptone solution 1% + 0.5% NaCl

Bacto peptone	10.0 g
NaCl	5.0 g
QH_2O	1000 mL

Autoclave for 20 minutes at 121°C. Media is stored at 2°- 5°C for up to 3 months.

Appendix II

NCAH Media #10006

Blood agar base with 5% bovine blood

Blood agar base (Difco)	40.0 g
H_2O	950 mL

Autoclave for 20 minutes at 121°C.

Cool to 47°C and add:

Defibrinated bovine blood	50 mL

Media is stored at 2°- 5°C for up to 3 months.

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