United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 918

Supplemental Assay Method for Bacterial Count of *Pasteurella multocida,* Avian Isolates, Vaccines

Date: **October 18, 2007**

Number: SAM 918.02

Supersedes: STSAM0918.01, November 10, 1999

Standard Requirement: 9 CFR Part 113.70

Contact: Sophia G. Campbell, (515) 663-7489

Approvals: /s/Geetha B. Srinivas Date: 31Oct07
Geetha B. Srinivas, Section Leader
Bacteriology

/s/Byron E. Rippke Date: 17Dec07
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 17Dec07
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

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1. Introduction

This is a Supplemental Assay Method (SAM) for the titration analysis of *Pasteurella multocida*, avian isolates, vaccine, live culture. It determines the colony-forming units (CFU) in final container samples as prescribed by the Code of Federal Regulations, Title 9 (9 CFR) Part 113.70. This method uses tryptose agar with 5% bovine blood and tryptose broth 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 Inoculum spreader

2.1.4 Disposable syringes and needles--appropriate sizes

2.1.5 Sterile disposable pipettes--appropriate sizes

2.1.6 Sterile screw-capped culture tubes, 20 x 150-mm

2.1.7 Pipetting aid

2.1.8 35° ± 2°C incubator

2.1.9 Biosafety cabinet

2.1.10 Gloves and lab coat

2.1.11 4 x 4-inch sterile gauze pads

2.1.12 Test tube rack

2.2 Reagents/supplies

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

2.2.1 Tryptose broth (**Appendix I**)

2.2.2 Tryptose agar with 5% bovine blood (**Appendix II**) or as stated in the biologics manufacturer's Outline of Production.

2.2.3 *P. multocida* reference culture (American Type Culture Collection 11039)

2.2.4 70% ethyl alcohol

2.2.5 Sterile water in serum vials--volumes determined by referring to the biologics manufacturer's Outline of Production or as stated on the vaccine vial

3. Preparation for the Test

3.1 Personnel qualifications/training

The 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. The personnel must also have knowledge of safe operating procedures and policies and guidelines of the Center for Veterinary Biologics (CVB) or equivalent, 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 Turn on the biosafety cabinet 30 minutes before use and turn off after use.

3.2.2 Monitor the incubator, freezers, and coolers daily for temperature.

3.2.3 Label all plates with the 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 Prepare *P. multocida* reference control samples according to the manufacturers' instructions.

3.3.3 Negative and Positive Controls: Incubate 2 plates of tryptose agar with 5% bovine blood inoculated with sterile diluent used in testing with test sample plates as negative control plates. *P. multocida* 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. Plates to be used for counts are placed in a 35°± 2°C incubator overnight prior to use or allowed to dry in a biosafety cabinet before use. At the time of use, plates are no more than 14 days old.

3.4 Preparation of the sample

Samples are *P. multocida* vaccines**.**

4. Performance of the Test

4.1 Remove 2 vials of product to be tested and 1 vial of *P. multocida* reference control sample from the freezer or cooler storage and allow to warm to room temperature.

4.2 Disinfect the caps with 70% ethyl alcohol. If needed, rehydrate the vials with the accompanying diluent, sterile water as stated in the Outline of Production or on the vaccine vial. Allow the contents of the vials to reconstitute for at least 5 minutes. Shake the vials by inversion until thoroughly mixed.

4.3 Prepare a tenfold dilution series of the first vial of the product by setting up a rack of 20 x 150-mm screw-capped tubes and pipetting 9 mL of tryptose broth into each tube using a 10-mL pipette. Label the tubes 10^{-1} to 10^{-x} as needed.

4.4 Transfer 1 mL of sample from **Section 4.2** into the first tube of tryptose broth using a pipette. Cap the tube and vortex. Continue the dilution series by using a pipette to transfer a 1-mL sample to the next tube, labeled 10^{-2}. Repeat this method using a sterile pipette for each transfer until the required number of serial tenfold dilutions (as determined by the release titer in the firm's Outline of Production) 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 pipette.

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

4.7 Repeat **Sections 4.3 through 4.6** with the second vial of product.

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.** Two plates of media inoculated with sterile diluent are used as negative controls to check for sterility.

4.9 Invert all plates and incubate at 35° ± 2°C for 24 hours. After incubation, count plates from each series that contain 30 to 300 CFU. Determine the mean CFU per dose for the number of vials tested by 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 Outline of Production, 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 release titer as written in the firm's Outline of Production, the serial or subserial may be retested using 4 new vaccine samples. A comparison of the firm's Outline of Production method to this SAM shall be done. If the retest (RT) is not done, the serial or subserial is unsatisfactory (UNSAT). If on the RT, the average count of the 4 vaccine samples is less than the required minimum, the serial or subserial is UNSAT.

5.3 If on the RT of the 4 new vials of vaccine, the average count is equal to or greater than the required minimum release titer, the serial or subserial 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. If the reference culture is not within the titer range and the serial being tested is below the minimum release titer, the serial is retested using 2 new vaccine samples. 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. Report of Test Results

Record the CFU per dose along with the final conclusion for the product tested after calculating the CFU per dose and interpreting the results.

7. References

Code of Federal Regulations, Title 9, Part 113.70, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

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:

- **2.1:** The Bunsen burner has been removed from the list of equipment that is needed for the test.

- **4.9:** The calculation for CFU/dose has been added.

Appendices

Appendix I

Tryptose broth--National Veterinary Services Laboratories (NVSL) Media #10404

Tryptose broth	26.0 g
H_2O	1000.0 mL

Autoclave 20 minutes at 121°C.

Appendix II

Tryptose agar with 5% bovine blood (defibrinated)--NVSL Media #10218

Tryptose agar	41.0 g
QH_2O	950.0 mL

Autoclave 25 minutes at 121°C. Cool in waterbath at 56°C and add 50.0 mL defibrinated bovine blood.