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

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1. Introduction

This Supplemental Assay Method (SAM) establishes the titration method for the analysis of *Pasteurella multocida*, avian isolates, vaccines to determine the colony-forming units (CFU) in final container samples, per title 9, *Code of Federal Regulations* (9 CFR), part 113.70(c)(2). This method uses Tryptose Broth (TB) as a diluent and Tryptose Agar (TA) plates with 5% bovine blood for determining CFUs.

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 HandyStep® electronic pipette

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 Tryptose broth (TB) (**Appendix I**), National Centers for Animal Health (NCAH) Media #10404

2.2.2 Tryptose agar (TA) with 5% bovine blood (**Appendix II**), NCAH Media #10218 or as stated in the Outline of Production (OP) from the biologics manufacturer.

2.2.3 *P. multocida* reference culture (American Type Culture Collection [ATCC] #11039)

2.2.4 70% ethanol

2.2.5 Sterile water in serum vials
2.2.6 Inoculum spreader

2.2.7 Sterile syringes and needles

2.2.8 Sterile pipettes, individually packaged

2.2.9 Sterile culture tubes

2.2.10 Lab coat and gloves

2.2.11 4 x 4-inch sterile gauze pads

2.2.12 Test tube rack

2.2.13 Sharps container

2.2.14 Pipetting aid

2.2.15 Micropipettors, 100-µL to 1.0-mL

2.2.16 Pipette tips, 100-µL to 1-mL

2.2.17 BRAND PD-Tip™ Syringe Tips

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, freezers, and coolers according to SOPs.

3.2.3 Turn on BSC at least 30 minutes prior to testing.

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 20°- 25°C before rehydrating to the appropriate volume.

3.3.2 *P. multocida* reference stock culture is prepared according to the manufacturers' instructions.

3.3.3 Negative and Positive Controls: Incubate 1 uninoculated plate of TA with 5% bovine blood and 1 plate 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 BSC before use. At the time of use, plates are no more than 6 months old.

3.4 Preparation of the sample

Samples are *P. multocida* 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, are used for samples that are not accompanied by a 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 *P. multocida* reference stock culture from the freezer or cooler storage and allow sample to warm to room temperature.

4.2 Disinfect the caps 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 sterile culture tubes and pipetting 9.0 mL of tryptose broth into each tube using a HandyStep pipette with a sterile syringe tip. 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 by using a micropipettor with a sterile tip. Cap the tube and vortex. The dilution series is continued by using a new sterile tip to transfer a 1.0 mL sample from this tube to the tube labeled 10^{-2}. Repeat this method using a new sterile tip 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 micropipettor with a sterile tip.

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.

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 24 ± 4 hours. After incubation, count plates from each series that contain 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 results are inconclusive (INC) and may be retested using double the amount of new vaccine samples used in the initial test, provided that if the retest (RT) is not conducted within 21 days, the serial or subserial is unsatisfactory (UNSAT) by the results determined in the first test. Compare the firm's OP method to this SAM when retesting the vials. If on the RT, the average count of the doubled 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 RT, the average using the firm's OP method count is equal to or exceeds the required minimum, 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, 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 using double the amount of 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. Record and Report of Test Results

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

7. References

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

8. Summary of Revisions

Version .05

- The Bacteriology Section Leader, the CVB-PEL Director, and page 1 were updated.
- **1-5:** These Sections have been updated and rewritten for clarification.
- **Appendices:** Media storage conditions have been updated.

Version .04

- **Sections 1-6** have been updated to reflect current practices.
- **Appendices:** Media storage conditions have been updated.

Version .03

- The Contact information has been updated.
- **6:** This section has been rewritten for clarification.
- **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:

- **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 Centers for Animal Health (NCAH) Media #10404

Tryptose broth	26.0 g
H_2O	1000.0 mL

Autoclave 20 minutes at 121°C. Media is stored at 2°- 5°C for up to 6 months.

Appendix II

Tryptose Agar with 5% Bovine Blood (defibrinated) – NCAH 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. Media is stored at 2°- 5°C for up to 6 months.