United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 603

Supplemental Assay Method for Potency Testing of Fowl Cholera (*Pasteurella multocida*) Bacterins, Type 3

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Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing avian *Pasteurella multocida* type 3, as prescribed title 9, *Code of Federal Regulation*s (9 CFR), part 113.118. Turkeys are vaccinated twice, 21 days apart, and challenged with a standard dose of virulent *P. multocida,* type 3, 14 days after the second vaccination. This is a 2-stage test in which the second stage is applied when 7 or 8 vaccinated turkeys die in the first stage.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Spectrophotometer, Spectronic 20D+ (Spectronic Instruments)

2.1.2 Sterile inoculating loop

2.1.3 Bunsen burner or Bacti-Cinerator® (if non-sterile wire loop is used)

2.1.4 Incubator, 35°- 37°C

2.1.5 Micropipettors, 20- to 200-µL and 200- to 1000-µL

2.1.6 Test tube mixer, vortex-type

2.1.7 Crimper for aluminum rings on serum vials

2.1.8 Biological safety cabinet

2.2 Reagents/supplies

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

2.2.1 *P. multocida*, type 3, strain P-1059. This culture must be obtained from the United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Center for Veterinary Biologics (CVB). Refer to the current reagent data sheet for details.

2.2.2 Test bacterin(s) containing *P. multocida,* type 3

2.2.3 Syringes, Luer-lock, 3-mL or 5-mL

2.2.4 Needles, 18-gauge x 1 1/2-inch

2.2.5 Glass serum bottle, 20- to 100-mL

2.2.6 Rubber stopper, 13 x 20-mm, and aluminum cap for serum bottle

2.2.7 Screw-top glass tubes, 13 x 100-mm, with caps

2.2.8 Pipettes, 5-mL, 10-mL, 25-mL

2.2.9 Micropipette tips, up to 1000-µL capacity

2.2.10 Bovine blood agar plates

2.2.11 Tryptose broth

2.2.12 Sterile cotton swabs

2.2.13 Poultry leg bands (size 11) or livestock spray paint, 1 color per treatment group, for animal identification

2.3 Animals

Turkeys, broad-breasted white, at least 6 weeks of age. Twenty turkeys are required for each serial to be tested. Ten additional turkeys are required as controls. All birds must be from the same source and hatch. The birds must be from flocks with no history of fowl cholera. Birds must not be previously vaccinated with any products containing *P. multocida.*

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel need working knowledge of the use of general laboratory chemicals, equipment, and glassware and need to have specific training and experience in sterile technique, the handling of live bacterial cultures, and the handling of poultry.

3.2 Selection and handling of test birds

3.2.1 Turkeys of either sex may be used.

3.2.3 It is permissible to house vaccinates and controls in the same enclosure, provided that space allocation is sufficient to meet requirements set forth by the

CVB/National Veterinary Services Laboratories (NVSL) Animal Care and Use Committee.

3.2.4 Positively identify each bird by treatment group. Identification may be by means of leg bands or livestock body paint.

1. If leg bands are used, band each leg in case 1 band is lost.

2. If body paint is used, freshen it at least every 3 weeks.

3.2.5 If any turkeys die after vaccination for suspected vaccine related causes, but prior to challenge with live *P. multocida*, these birds must be necropsied to determine cause of death. If cause of death is unrelated to vaccination, the pathologist's report is filed with the test records and no additional action is taken. If death is attributable to the test bacterin, the death must be reported immediately to CVB-Inspection and Compliance, which may request further safety testing of the bacterin.

3.2.6 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the birds and to sanitize contaminated rooms.

3.3 Preparation of supplies/equipment

3.3.1 Sterilize all glassware before use.

3.3.2 Use only sterile bacteriological supplies (pipettes, syringes, needles, rubber stoppers, saline, etc.).

3.3.3 Operate and maintain all equipment according to manufacturers' recommendations and applicable standard operating procedures.

3.4 Preparation of reagents

3.4.1 *P. multocida*, type 3 (Lyon and Little classification), strain P-1059 challenge culture. Refer to the current reagent data sheet for details on storage and preparation.

3.4.2 Tryptose broth – National Centers for Animal Health (NCAH) Media #10404

Tryptose broth powder	26 g
Deionized water	q.s. 1 L

Autoclave 15 minutes at ≥ 121°C. Cool before using. Store at 20°- 25°C for no more than 6 months.

3.4.3 Bovine blood agar – NCAH Media #10006

Blood agar base powder	40 g
Deionized water	q.s. 950 mL

Autoclave 20 minutes at ≥ 121°C. Cool to 45°- 47°C.

Add:

Defibrinated bovine blood	50 mL

Pour into sterile petri dishes. Allow to cool to 20°- 25°C. Store at 2°- 7°C for no more than 6 months.

4. Performance of the Test

4.1 Vaccination of test animals

4.1.1 Check the label on each product and/or Section VI of the current Outline of Production to confirm identity, recommended field dose, and route of injection.

4.1.2 Thoroughly mix product by inverting end-to-end at least 10 times before the syringes are filled. Use 3- or 5-mL syringes, fitted with 18-gauge x 1 1/2-inch needles.

4.1.3 Vaccinate separate groups of not more than 21 turkeys with each of the test bacterins. Use the dose volume and injection route recommended on the product label for each bacterin. Unless otherwise specified on the product label and/or Section VI of the current Outline of Production, subcutaneous injections are given in the unfeathered, loose skin on the back of the lower neck.

4.1.4 Revaccinate the turkeys in a similar manner 21 days after the first vaccination.

4.1.5 Retain not more than 11 turkeys as nonvaccinated controls.

4.2 Preparation of challenge in a biological safety cabinet

4.2.1 Reconstitute a vial of challenge culture in 2 mL tryptose broth.

4.2.2 Inoculate 2 blood agar plates with 100 µL of reconstituted culture and streak for isolation.

4.2.3 Incubate the inoculated blood agar plates at 35°- 37°C for 16 to 19 hours.

4.2.4 Use plates that have pure growth by visual inspection to prepare the challenge inoculum.

4.2.5 Scrape several bacterial colonies from the surface of the blood agar plates using a sterile cotton swab and suspend in tryptose broth in a 13 x 100-mm tube. Add bacterial growth until the suspension measures 65-69%T at 630 nm using a Spectronic 20D+ spectrophotometer or equivalent. Use sterile tryptose broth in a 13 x 100-mm tube as a blank for the spectrophotometer.

4.2.6 Prepare a 10^{-6} dilution of the standardized culture in tryptose broth. **This is the inoculum used to challenge the turkeys.** Dispense challenge liquid into a serum vial and seal with a rubber stopper and aluminum ring.

4.2.7 Prepare one additional tenfold dilution (10^{-7}) for post-inoculation plate counts or alternatively, save an aliquot of the challenge inoculum in a separate vial and prepare this additional dilution later (see **Section 4.4**).

4.2.8 Place vial(s) of challenge inoculum and dilution tubes on ice. Keep on ice through challenge procedure and until added to plates for post-inoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge 20 vaccinates per serial of product 14 to 18 days after the second vaccination. Euthanize any additional vaccinated birds at this time.

4.3.2 Challenge nonvaccinated controls at the same time as the vaccinates. Euthanize any additional control birds at this time.

4.3.3 Inoculate each turkey with 0.5 mL of challenge inoculum (10^{-6} dilution of standardized culture, see **Section 4.2.6**) intramuscularly in the breast muscle, using a 3-mL or 5-mL syringe and 18-gauge x 1 1/2-inch needle.

4.4 Postinoculation plate count in a biological safety cabinet

4.4.1 After birds are challenged, prepare the 10^{-7} dilution using tryptose broth as the diluent (if not already completed, see **Section 4.2.7**).

4.4.2 All bacterial suspensions must be mixed well prior to placing an aliquot on an agar plate. Plate each dilution (10^{-5}, 10^{-6}, and 10^{-7}) in triplicate using 0.1 mL on bovine blood agar. Inoculum must be spread evenly on the surface of the agar plates and not allowed to pool around the edges. Complete all plate inoculations within 1 hour of challenge.

4.4.3 Incubate the plates aerobically at 35°- 37°C for 18 to 30 hours.

4.4.4 Using the dilution yielding 30-300 colonies per plate, calculate the colony forming units (CFU)/challenge dose according to the following formula:

$$\frac{\text{Colony count sum}}{\text{Number of plates}} \times \frac{1}{\text{Dilution factor plated}} \times \frac{1}{\text{Plated volume (mL)}} \times \frac{\text{Challenge dilution}}{1} \times \frac{\text{Challenge vol. (mL)}}{\text{Dose}} = \frac{\text{CFU}}{\text{Dose}}$$

4.5 Observation of turkeys after challenge

4.5.1 Observe the turkeys up to twice daily for 14 days after challenge. Record deaths and euthanize any moribund birds as recommended by the Institutional Animal Care and Use Committee.

4.5.2 If deaths occurring after challenge are suspected to be due to causes other than fowl cholera, such turkeys are necropsied to determine cause of death. If cause of death is unrelated to vaccination and/or challenge, the deaths are not included in the total deaths for the test.

5. Interpretation of the Test Results

Interpret the test as prescribed in 9 CFR 113.118.

5.1 For a valid test, at least 8 of 10 control turkeys must die during the 14-day post-challenge period, and the plate count of the challenge dose must be at least 150 cfu/dose.

Stage	Number of vaccinates	Cumulative number of vaccinates	Cumulative number of dead vaccinates for...	
			Satisfactory serial	Unsatisfactory serial
1	20	20	6 or less	9 or more
2	20	40	15 or less	16 or more

5.2 The second stage may be conducted when 7 or 8 vaccinates die in the first stage of a valid test. The serial is Unsatisfactory if the test is not repeated. The second-stage test is performed in a manner identical to the first-stage test and evaluated according to the Table in **Section 5.1**.

5.3 If fewer than 8 of 10 control turkeys die during the post-challenge period, or if the challenge dose is less than 150 CFU, the test is considered invalid due to insufficient challenge and is reported as Inconclusive. The test may be repeated without prejudice, and the repeat test is considered to be a first-stage test.

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 113.118, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .05

- Updated coversheet and contact information.

Version .04

- The Section Leader and Director information has been updated.

Version .03

- The Contact information has been updated.
- **2.1.3:** This section has been updated to reflect current practices.
- **2.2.12/4.2.5:** Sterile cotton swabs have been added.
- **4.1.3/4.1.5:** Bird group numbers have been updated to reflect 9 CFR 113.118.
- **4.2.7/4.4.4/4.4.2:** These sections have been updated to reflect new plate count dilutions.

- **4.3.1/4.3.2:** Instructions on euthanizing extra birds have been added.

- **4.5.1:** This section has been updated to reflect current practices.

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:

- **2.1.8** A biological safety cabinet has been added to the equipment needed to conduct this test.

- **2.2.4** The size of needles used to conduct the test has been modified.

- **2.2.12** The size of leg band used has been indicated.

- **3.4.2** The autoclave operation parameters have been changed.

- **4.1.3** Details regarding the location of the subcutaneous injection have been added.

- **4.2.1** The amount of tryptose broth used to reconstitute the reagent has been updated.

- Information regarding the challenge culture has been modified to indicate the current reagent data sheet throughout the document.
- Plate count dilution details have been added.

- References to internal CVB SOP's have been replaced with summarized information throughout the document.

- The contact person has been changed to Janet Wilson.