

**Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol**

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

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Supplemental Assay Method for Potency Testing Fowl Cholera
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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 in the Code of Federal Regulations, Title 9 (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

2.1.1 Spectrophotometer, Spectronic 70™ (Bausch and Lomb, Rochester, New York), or equivalent

2.1.2 Wire inoculating loop

2.1.3 Bunsen burner

2.1.4 Incubator, 37°C

2.1.5 Micropipettors, 20-200 µl and 200-1000 µl

2.1.6 Test tube mixer, vortex-type

2.1.7 Crimper for aluminum rings on serum vials

2.2 Reagents/supplies

2.2.1 *P. multocida*, type 3, strain P-1059 (IRP P3C, serial 1). This culture must be obtained from the United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Center for Veterinary Biologics-Laboratory (CVB-L).

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- 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, 20 ga x 1 in
- 2.2.5 Glass serum bottle, 20-100 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- μ l capacity
- 2.2.10 Bovine blood agar plates
- 2.2.11 Tryptose broth
- 2.2.12 Poultry leg bands OR livestock spray paint, 1 color per treatment group, for animal identification
- 2.2.13 Water, distilled or deionized, or water of equivalent quality

2.3 Animals

Turkeys, broad-breasted white, at least 6 wk 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*.

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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.

3.2.2 House and feed all turkeys in a similar manner.

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 National Veterinary Services Laboratories (NVSL)/CVB-L 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 wk.

3.2.5 If any turkeys die after vaccination, but prior to challenge with live *P. multocida*, these birds must be necropsied by the Pathobiology Laboratory (PL), NVSL, 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 Inspection and Compliance, CVB, which may request further safety testing of the bacterin.

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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 All equipment must be operated according to manufacturers' instructions. Maintain and calibrate equipment, as applicable, according to current CVB-L Standard Operating Procedures (SOPs).

3.4 Preparation of reagents

3.4.1 *P. multocida*, type 3 (Lyon and Little classification), strain P-1059 challenge culture. The challenge culture, IRP P3C Serial 1, is lyophilized in 0.5-ml amounts. Store vials of lyophilized culture at 4°C or colder.

3.4.2 Tryptose broth (NVSL media 10404)

Tryptose broth powder	26 g
Water	q.s. 1 L

Autoclave 20 min at 121°C. Cool before using.
Store at room temperature no more than 6 mo.

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3.4.3 Bovine blood agar (NVSL media 10006)

Blood agar base powder	40 g
Water	q.s. 950 ml

Autoclave 20 min at 121°C. Cool to 47°C.

Add:

Defibrinated bovine blood	50 ml
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Pour into sterile petri dishes. Allow to cool to room temperature. Store at 4°C for no more than 6 mo.

4. Performance of the test

4.1 Vaccination of test animals

4.1.1 Check the label on each product 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 20-ga x 1-in needles.

4.1.3 Vaccinate separate groups of 20 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, subcutaneous injections must be given in the unfeathered, loose skin of the lower neck.

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

4.1.5 Retain 10 turkeys as nonvaccinated controls.

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4.2 Preparation of challenge

4.2.1 Reconstitute a vial of challenge culture in 1 ml tryptose broth.

4.2.2 Inoculate 2 blood agar plates with a loopful of reconstituted culture and streak for isolation.

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

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 and suspend in tryptose broth in a 13 x 100-mm tube. Add bacterial growth until the suspension measures $67\% \pm 2\%$ T at 630 nm using a spectrophotometer.

1. Use sterile tryptose broth in a 13 x 100-mm tube as a blank for the spectrophotometer.

4.2.6 Prepare a 10^{-5} dilution of the standardized culture in tryptose broth. **This is the inoculum used to challenge the turkeys.** Place in a serum vial and seal with a rubber stopper and aluminum ring. Save aliquot(s) of this inoculum in a separate vial; retain vial(s) as sample(s) for postchallenge plate counts.

4.2.7 Place vials of challenge inoculum on ice to transport to barn. Keep on ice through challenge procedure and until added to plates for postinoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge 20 vaccinates per serial of product 14-18 days after the second vaccination.

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4.3.2 Challenge nonvaccinated controls at the same time as the vaccinates.

4.3.3 Inoculate each turkey with 0.5 ml of challenge inoculum (10^{-5} dilution of standardized culture, see **Section 4.2.6**) intramuscularly in the breast muscle, using a 3-ml or 5-ml syringe and 20-ga x 1-in needle.

4.4 Postinoculation plate count

4.4.1 After birds are challenged, perform a plate count on the challenge inoculum according to the current version of BBSOP0019.

1. Use tryptose broth as the diluent for the plate count and plate on bovine blood agar. Incubate plates aerobically at 37°C for 18-30 hr.

2. Calculate the cfu/challenge dose according to the following formula:

Average count in 0.1 ml x 5 x dilution factor (see table below)=cfu/0.5 ml dose of challenge

If plates used for avg. count were inoculated with:	Dilution factor
challenge inoculum, as given to turkeys	1
10^{-1} dilution of challenge inoculum	10
10^{-2} dilution of challenge inoculum	100

4.5 Observation of turkeys after challenge

4.5.1 Observe the turkeys daily for 14 days after challenge. Record deaths.

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4.5.2 If deaths occurring after challenge are suspected to be due to causes other than fowl cholera, such turkeys are necropsied by the PL, NVSL, 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

5.1 Interpret the test as prescribed in 9 CFR, Part 113.118.

5.1.1 For a valid test, at least 8 of 10 control turkeys must die during the 14-day postchallenge 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

5.1.2 The second stage is required only when 7 or 8 vaccinates die in the first stage of a valid test. The second-stage test is performed in a manner identical to the first-stage test.

5.1.3 If fewer than 8 of 10 control turkeys die during the postchallenge period, or if the challenge dose is less than 150 cfu, the test is considered invalid due to insufficient challenge and is reported as a No Test. The test may be repeated without prejudice, and the repeat test is considered to be a first-stage test.

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6. Report of test results

Report results as described by the current version of BBSOP0020.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.118,
U.S. Government Printing Office, Washington, DC, 1998.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.