United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

SAM 634

Supplemental Assay Method for Potency Testing of *Pasteurella multocida* Bacterins of Bovine Origin

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Supplemental Assay Method for Potency Testing of *Pasteurella multocida* Bacterins of Bovine Origin

Table of Contents

1. Introduction

2. Materials

- **2.1 Equipment/instrumentation**
- **2.2 Reagents/supplies**
- **2.3 Animals**

3. Preparation for the Test

- **3.1 Personnel qualifications/training**
- **3.2 Selection and handling of test mice**
- **3.3 Preparation of supplies/equipment**
- **3.4 Preparation of reagents**

4. Performance of the Test

- **4.1 Vaccination of test animals**
- **4.2 Preparation of challenge in a biological safety cabinet**
- **4.3 Timing and administration of challenge**
- **4.4 Postinoculation plate count in a biological safety cabinet**
- **4.5 Observation of mice after challenge**

5. Interpretation of the Test Results

6. Report of Test Results

7. References

8. Summary of Revisions

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Supplemental Assay Method for Potency Testing of *Pasteurella multocida* Bacterins of Bovine Origin

1. Introduction

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing *Pasteurella multocida* of bovine origin, as prescribed in title 9, *Code of Federal Regulations* (9 CFR), part 113.121. Mice are vaccinated twice, 14 days apart, and challenged with a standard dose of virulent *P. multocida* 10 to 12 days after the second vaccination.

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 used)

2.1.4 Incubator**,** 35°- 37°C

2.1.5 Micropipettors, 20- to 1000-µL

2.1.6 Test tube mixer, vortex-type

2.1.7 Crimper for aluminum caps 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* challenge culture, Strain P-1062 Type A, available from the Center for Veterinary Biologics (CVB). Refer to the current reagent data sheet for additional information.

2.2.2 Test bacterin(s) containing *P. multocida*

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2.2.3 *P. multocida* reference bacterin, available from the CVB. Refer to the current reagent data sheet for additional information.

2.2.4 Syringes, 1-mL

2.2.5 Needles, 26-gauge, 3/8-inch

2.2.6 Glass-serum bottle, 20- to 100-mL

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

2.2.8 Glass screw-cap tubes, 13 x 100-mm

2.2.9 Pipettes, 5-mL, 10-mL, 25-mL

2.2.10 Micropipette tips, up to 1000-µL capacity

2.2.11 Bovine blood agar plates

2.2.12 Tryptose broth

2.2.13 Phosphate-buffered saline (PBS)

2.2.14 Sterile cotton swabs

2.3 Animals

2.3.1 Mice, 16-22 g. Although the 9 CFR does not specify a specific mouse type, the CVB uses CF-1 mice.

2.3.2 Sixty mice are required for each bacterin to be tested (20 mice/dilution; 3 dilutions/bacterin). Sixty additional mice are required for the reference bacterin. Thirty mice are required to determine the LD_{50} of the challenge inoculum. All mice must be from the same source colony and of similar weight and/or age.

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel must have a working knowledge of the use of general laboratory chemicals, equipment, and glassware; and must have specific training and experience in sterile technique, the handling of live bacterial cultures, and the handling of mice.

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3.2 Selection and handling of test mice

3.2.1 Mice of either sex may be used, but females are recommended.

3.2.2 All mice must be housed and fed in a similar manner.

3.2.3 Identify each cage of mice by treatment group.

3.2.4 If any mice die after vaccination, but prior to challenge with live *P. multocida*, perform a necropsy on these mice to determine cause of death if the cause of death is not outwardly apparent. If the cause of death is unrelated to vaccination, file the necropsy report with the test records, and no additional action is needed. If death is attributable to the test bacterin, report the death immediately to the CVB-Inspection and Compliance, which may request further safety testing of the bacterin.

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

3.3 Preparation of supplies/equipment

3.3.1 Use only sterile supplies.

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

3.4 Preparation of reagents

3.4.1 *P. multocida* Reference Bacterin. Refer to the current reagent data sheet for details.

3.4.2 *P. multocida* challenge culture, Strain P-1062 Type A. Refer to the current reagent data sheet for details.

3.4.3 Phosphate-buffered saline--National Centers for Animal Health (NCAH) Media #10559

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Sodium phosphate, dibasic	1.15 g
Potassium phosphate, monobasic	0.2 g
Deionized water	q.s. to 1000 mL

Adjust pH to 7.2 ± 0.1. Autoclave 20-30 minutes at ≥ 121°C following manufacturer's recommendations. Store at 20°- 25°C for no longer than 1 year.

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3.4.4 Tryptose broth--NCAH Media #10404

Tryptose broth powder	26 g
Deionized water	q.s. 1000 mL

Autoclave 15-30 minutes at ≥ 121°C following manufacturer's recommendations. Cool before using. Store at 20°- 25°C for no longer than 1 year.

3.4.5 5% bovine blood agar--NCAH Media #10006

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

Autoclave 20-30 minutes at ≥ 121°C following manufacturer's recommendations. Cool to 45°- 47°C.

Add:

Defibrinated bovine blood	50 mL

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

4. Performance of the Test

4.1 Vaccination of test animals

4.1.1 Check the label on each product and Section VI of the current Outline of Production to confirm identity and dose volume.

4.1.2 Test each test bacterin and the reference bacterin at 3 fivefold dilutions. Typically, test the bacterins undiluted, 1:5 and 1:25. Refer to the current reagent data sheet for any starting dilutions of the reference bacterin. It is permissible to make fivefold dilutions other than those described as long as the reference and test bacterins are tested at the same dilutions. For viscous bacterins, it is advisable to start at 1:2 or 1:3 and make fivefold dilutions from this starting point to increase injectability of the product at the low dilution.

4.1.3 Thoroughly mix product by inverting end-to-end at least 10 times. Make the appropriate fivefold dilutions of the reference bacterin in PBS. Make identical fivefold dilutions of the test bacterin(s) in the diluent approved in the specific Outline of Production for that product. (Some oil-adjuvanted products require oil-based diluents.) Place each dilution in a separate sterile serum bottle. Prepare dilutions immediately prior to use; do not store in diluted form.

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4.1.4 Vaccinate separate groups of 20 mice with each of the test bacterin dilutions and reference bacterin dilutions. For reference bacterin groups, inject each mouse with 0.1 mL intraperitoneally. Inject test bacterins intraperitoneally at a dose volume that corresponds to 1/20 of the lowest dose recommended on the product label or Outline of Production. This volume must not be < 0.1 mL

Note: **It is permissible to vaccinate a few extra mice in each group to compensate for any potential deaths that may occur prior to challenge and are not related to vaccination. However, if extra mice are vaccinated, all surviving at the time of challenge must be challenged with live *P. multocida* and included in data calculations.**

4.1.5 Revaccinate the mice in a similar manner 14 days after the first vaccination.

4.1.6 Retain 30 non-vaccinated mice to determine LD_{50} of the challenge.

4.2 Preparation of challenge in a biological safety cabinet

4.2.1 Reconstitute a vial of challenge 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 35°- 37°C for 16 to 18 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 76-80% 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^{-5} dilution of the standardized culture in tryptose broth. **This is the inoculum used to challenge the mice.** Dispense challenge liquid into a serum vial and seal with a rubber stopper and aluminum ring.

4.2.7 Prepare additional tenfold dilutions for challenge LD_{50} determination (10^{-7} to 10^{-9}) and postinoculation plate counts (10^{-5} to 10^{-7}). Dispense an aliquot of each LD_{50} dilution in a separate serum vial and seal.

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4.2.8 Place the vials of challenge inoculum and additional dilution tubes on ice. Keep on ice through challenge procedure and until culture is added to plates for postinoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge all vaccinates 10 to 12 days after the second vaccination.

4.3.2 Challenge non-vaccinated LD_{50} controls at the same time as the vaccinates.

4.3.3 Inoculate each vaccinated mouse with 0.2 mL of challenge inoculum intraperitoneally, using a 1-mL syringe and 26-gauge, 3/8-inch needle.

4.3.4 Inoculate separate groups of 10 non-vaccinated control mice intraperitoneally with 0.2 mL of each of the LD_{50} dilutions.

4.4 Postinoculation plate count in a biological safety cabinet

4.4.1 All bacterial suspensions must be mixed well prior to placing an aliquot on an agar plate. Plate each dilution (10^{-5} to 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.

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

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

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

4.4.4 Record the plate count (CFU/dose) of the challenge on the test result form. This information is for informational purposes to track trends and to troubleshoot problem tests. The 9 CFR does not specify a minimum or maximum CFU/dose for this test.

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4.5 Observation of mice after challenge

4.5.1 Observe the mice up to twice daily for 10 days after challenge. Record deaths and euthanize any moribund mice 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 pasteurellosis, perform a necropsy on such mice to determine the cause of death. If cause of death is unrelated to vaccination and/or challenge, do not include the deaths in the total deaths for the test.

5. Interpretation of the Test Results

Interpret the test as prescribed in 9 CFR 113.121.

5.1 Calculate the LD_{50} (theoretical dose/dilution at which the challenge would be lethal to 50% of the control mice) of the challenge inoculum using the Reed-Muench or Spearman-Kärber method of estimation. A valid test must have an LD_{50} between 100 and 10,000.

5.2 Calculate the PD_{50} of the reference bacterin and each test bacterin (theoretical dose/dilution at which the bacterin would protect 50% of the mice) using the Reed-Muench or Spearman-Kärber method of estimation.

5.3 If the PD_{50} of the reference bacterin cannot be calculated because the lowest dilution protects < 50% of the mice or the highest dilution protects > 50% of the mice, the test is invalid. The reference bacterin also must protect > 0% and < 100% of the mice at 2 or more dilutions in a valid test.

5.4 If the PD_{50} of the test bacterin cannot be calculated because the lowest dilution tested protects < 50% of the mice, the bacterin may be retested, ***provided the following***:

5.4.1 If the bacterin is not retested, it is unsatisfactory.

5.4.2 If the protection provided by the lowest dilution of the reference exceeds that provided by the lowest dilution of the test bacterin by at least 6 mice, the test bacterin is unsatisfactory without additional testing.

5.4.3 If the total number of mice protected by the reference bacterin (sum of survivors in all dilution groups) exceeds the total number protected by the test bacterin by 8 mice or more, the test bacterin is unsatisfactory without additional testing.

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5.5 If the PD_{50} of the test bacterin in a valid test cannot be calculated because the highest dilution protected more than 50% of the mice, the bacterin is satisfactory without further testing.

5.6 Divide the PD_{50} of each test bacterin by the PD_{50} of the reference to calculate the relative potency (RP) for each bacterin.

5.7 If the RP of the test bacterin is ≥ 0.50, the test bacterin is satisfactory.

5.8 If the RP of the test bacterin is < 0.50, the test bacterin is unsatisfactory.

5.9 A test bacterin with an RP < 0.50 may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. Calculate the results of the retests in the following manner:

5.9.1 Average the RP values of the retests.

5.9.2 If the average RP of the retests is < 0.50, the bacterin is unsatisfactory.

5.9.3 If the average RP of the retests is ≥ 0.50 **AND** the RP obtained in the original test is $\leq 1/3$ the average RP of the retests, the test bacterin is satisfactory. Consider the initial test to be the result of test system error.

5.9.4 If the average of the retests is ≥ 0.50 **BUT** the RP of the original test is $> 1/3$ of the average RP of the retests, calculate a new average RP using the RP values obtained in all tests (original plus retests). If the new average RP is ≥ 0.50, the test bacterin is satisfactory. If the new average RP is < 0.50, the test bacterin is unsatisfactory.

6. Report of Test Results

Report results of the test(s) as described by standard operating procedures.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.121, U.S. Government Printing Office, Washington, DC.

7.2 Reed LJ, Muench H, 1938. A simple method of estimating 50% endpoints. *Am J Hygiene*, 27:493-497.

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8. Summary of Revisions

Version .04

- Bacteriology Section Leader updated.
- Clarified media expiration dates.

Version .03

- The Contact information has been updated.
- **2.1.3/4.5.1:** These sections have been updated to reflect current practices.
- **2.2.14/4.2.5:** Sterile cotton swabs 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** A sterile inoculating loop and biological safety cabinet have been added.
- **4.1.1** Reference to Section VI of the current Outline of Production has been added as an additional source of the needed information.
- **4.2.1** The amount of media used to reconstitute has been adjusted.
- **4.2.2** The number of plates used has been updated to reflect current practices.
- References to the current reagent data sheet have been added throughout the document.
- References to internal documents have been replaced with summarized information.
- The autoclave parameters have been adjusted.
- The spectrophotometer parameters currently in use have been updated.
- The contact has been changed to Janet Wilson.

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