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Supplemental Assay Method for Potency Testing of Erysipelas Bacterins in Mice

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1. Introduction

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing *Erysipelothrix rhusiopathiae*, as prescribed in the title 9, *Code of Federal Regulations* (9 CFR), part 113.119. Mice are vaccinated and then challenged with a standard dose of virulent *E. rhusiopathiae* 14 to 21 days after 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 20 D+ (Spectronic Instruments)

2.1.2 Sterile inoculating loop

2.1.3 Bunsen burner (if nonsterile wire loop is used)

2.1.4 Incubator, 35°- 37°C

2.1.5 Crimper for aluminum caps on serum vials

2.1.6 Biological safety cabinet

2.2 Reagents/supplies

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

2.2.1 *E. rhusiopathiae* strain E1-6P challenge culture. This culture is 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 *E. rhusiopathiae*

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

2.2.4 Syringes, 1-mL tuberculin

2.2.5 Needles, 26-gauge x 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 and 25-mL

2.2.10 Erysipelas medium broth

2.2.11 Bovine blood agar plates, 5%

2.2.12 Peptone buffer, 1%

2.2.13 Saline, 0.85%

2.3 Animals

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

2.3.2 Eighty mice are required for each serial to be tested (20 mice/dilution; 4 dilutions/serial). Eighty 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.

Note: Although 9 CFR regulations require only 3 dilutions/serial, the CVB tests an additional dilution to increase the probability of bracketing the PD_{50} dilution.

3. Preparation for the Test

3.1 Personnel qualifications/training

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

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 *E. rhusiopathiae*, necropsy 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 *E. rhusiopathiae* reference bacterin. Refer to the current reagent data sheet for use dilutions.

3.4.2 Test bacterin(s) containing *E. rhusiopathiae*. For each test bacterin, make threefold dilutions in the appropriate diluent (see **Section 4.1.3**) immediately prior to use. Use dilutions identical to the dilutions used for the Reference bacterin (see **Section 3.4.1**). Place each of the dilutions in separate sterile serum bottles.

3.4.3 *E. rhusiopathiae* challenge culture. Refer to the current reagent data sheet for preparation and storage information.

3.4.4 Saline, 0.85% - National Centers for Animal Health (NCAH) Media #30201

Sodium chloride	8.5 g
Deionized water	q.s. to 1000 mL

Autoclave 20 to 40 minutes at ≥ 121°C. Store at 20°- 25°C for no longer than 1 year.

3.4.5 Peptone buffer, 1%--NCAH Media #10522

Peptone	10 g
Sodium phosphate, dibasic	12.01 g
Potassium phosphate, monobasic	2.09 g
Deionized water	q.s. to 1000 mL

Adjust pH to 7.4 ± 0.1. Autoclave 20 to 40 minutes at ≥ 121°C. Cool before using. Store at 20°- 25°C for no longer than 6 months.

3.4.6 Bovine blood agar, 5%--NCAH Media #10006

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

Autoclave 20 to 40 minutes at ≥ 121°C. 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.

3.4.7 Horse Infusion Broth--NCAH Media #40143

Horse meat (no fat)	454 g
Horse liver	18 g
Deionized water	1000 mL

Grind tissue and dispense in hot water in a cooker. Heat to boiling and simmer for approximately 1 hour. Remove from heat and allow to settle at least 2 hours. Skim off fat. Strain through cheese cloth and discard meat. Filter broth through No. 2 Whatman filter paper. Use in **Section 3.4.8** or hold for future use at -20°C or lower.

3.4.8 Erysipelas Medium (broth)--NCAH Media #10133

Horse Infusion Broth (**Section 3.4.7**)	1000 mL
Sodium phosphate, dibasic	11 g
Potassium phosphate, monobasic	1 g
Oxgall® (1 g dissolved in 10 mL deionized water)	10 mL
Peptone	20 g
Gelatin, granulated	5 g

Heat to just below boiling to dissolve the gelatin. Cool to 55°- 57°C. Adjust pH to 7.6 ± 0.1.

Add:

Dextrose	5 g
Horse serum (not heat inactivated)	100 mL

Filter while still warm through a sterile, disposable 0.2-µm filter. Adjust final pH to 7.7 ± 0.1 with filtered acid or base. 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 for identity and dose volume.

4.1.2 Test each test bacterin and the reference bacterin at a minimum of 4 threefold dilutions.

4.1.3 Thoroughly mix product by inverting end-to-end. Make the appropriate threefold dilutions of the reference bacterin in saline. Make identical threefold dilutions of the test bacterin(s) in saline or 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.

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 0.2 mL subcutaneously. Inject test bacterins subcutaneously at a dose volume that corresponds to 1/10 of the smallest dose recommended on the product label or Section VI of the current Outline of Production. This volume must not be < 0.1 mL.

4.1.5 Retain 30 nonvaccinated 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 1.5 mL 1% peptone buffer.

4.2.2 Inoculate 100 mL of erysipelas medium broth with the entire contents of a vial of reconstituted culture.

4.2.3 Incubate the inoculated broth at 35°- 37°C for 18 to 24 hours.

4.2.4 Perform a Gram stain using standard methods. If the Gram stain shows a pure culture of Gram-positive rods, continue with the challenge procedure. If the culture is not pure, begin the preparation of the challenge again (**Section 4.2.1**).

4.2.5 Dilute overnight culture as necessary in sterile erysipelas challenge culture broth to 74-78% T at 600 nm, using a Spectronic 20D+ spectrophotometer. Use sterile erysipelas medium broth as a blank for the spectrophotometer.

4.2.6 Prepare a 10^{-5} dilution of the standardized culture in sterile erysipelas challenge medium broth. This is the inoculum used to challenge the vaccinated mice. Dispense an aliquot of the challenge liquid into a serum vial and seal it with a rubber stopper and aluminum ring.

4.2.7 Continue preparing additional tenfold dilutions (10^{-6} to 10^{-9}) of the standardized culture for postinoculation plate counts and challenge LD_{50} determination. Dispense an aliquot of each LD_{50} dilution (10^{-7} to 10^{-9}) in a separate serum vial and seal.

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 counts.

4.3 Timing and administration of challenge

4.3.1 Challenge all vaccinated mice 14 to 21 days after the vaccination. Inoculate each vaccinated mouse with 0.2 mL of challenge inoculum (see **Section 4.2.6**) subcutaneously, using a 1-mL tuberculin syringe and 26-gauge x 3/8-inch needle.

4.3.2 Inoculate the nonvaccinated control mice (separate groups of 10 mice each) immediately following the challenge of vaccinated mice. Each mouse is inoculated subcutaneously with 0.2 mL of the appropriate LD_{50} dilution (10^{-7} to 10^{-9} prepared in **Section 4.2.7**) using a 1-mL tuberculin syringe and 26-gauge x 3/8-inch needle.

4.4 Postinoculation plate count in a biological safety cabinet

4.4.1 After the mice are challenged, perform a colony count on blood agar plates using the tubes retained for this purpose.

4.4.2 All bacterial suspensions must be mixed well prior to placing an aliquot on an agar plate. Plate each dilution (10^{-5} to 10^{-7} from **Sections 4.2.6 and 4.2.7**) 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 48 to 72 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:

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

4.4.5 Record the plate count (CFU/dose) of the challenge on the test result form 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.

4.5 Observation of mice after challenge

4.5.1 Observe the mice daily for 10 days after challenge. Record deaths.

4.5.2 If deaths occurring after challenge are suspected to be due to causes other than erysipelas, necropsy the 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

5.1 Interpret the test as prescribed in 9 CFR 113.119.

5.1.1 Calculate the LD_{50}/dose (theoretical 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. For a test to be valid, the challenge inoculum must contain at least 100 LD_{50}/0.2 mL dose.

5.1.2 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.

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

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The lowest dilution of the reference shall protect > 50% of the mice. The highest dilution of the reference shall protect < 50% of the mice.

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

1. If the serial is not retested, it is unsatisfactory.

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

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

5.1.5 If the PD_{50} of the test serial in a valid test cannot be calculated because the highest dilution protected > 50% of the mice, the serial is satisfactory without further testing.

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

5.1.7 If the RP of the test bacterin is ≥ 0.6, the test bacterin is satisfactory.

5.1.8 If the RP of the test serial(s) is < 0.6, the test serial is unsatisfactory.

5.1.9 A test serial with an RP < 0.6 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:

1. Average the RP values of the retests.

2. If the average RP of the retests is < 0.6, the serial is unsatisfactory.

3. If the average RP of the retests is ≥ 0.6 **AND** the RP obtained in the original test is ≤ 1/3 of the average RP of the retests, the test bacterin is satisfactory. Consider the initial test to be the result of test system error.

4. If the average of the retests is ≥0.6 **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 ≥ 0.6, the test bacterin is satisfactory. If the new average RP is < 0.6, 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 113.119, 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.

7.3 Cottral, G.E., (Ed.), 1978. *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY. pg. 731.

7.4 Finney, DJ, 1978, *Statistical method in biological assay*. Griffin, London. 3rd edition, pp. 394-401.

8. Summary of Revisions

Version .06

- Updated cover page information.

Version .05

- The Bacteriology Section Leader was updated.
- Minor word changes for clarification of procedures.

Version .04

- The Contact information has been updated.
- **3.4:** Autoclave time ranges have been added.
- References to NVSL have been updated to NCAH throughout the document.

Version .03

- **4.2/4.3:** Clarification changes have been made to these sections.

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 biological safety cabinet has been added to the equipment needed to conduct this test.
- **2.2.1** The strain designation has been corrected.
- **2.2.4** The tuberculin designation of the 1 mL syringe has been added.
- **2.2.5** The larger needle size has been deleted.
- **2.2.10** The media designation has been updated and made consistent throughout the remainder of the document.
- **3.4.7** and **3.4.8** The media recipe has been further clarified.
- **4.1.1** Section VI of the current Outline of Production has been added as an additional source of information
- **4.1.5** Section VI of the current Outline of Production has been added to indicate the route of vaccination.
- **4.2.2** The amount of media has been updated.
- **4.2.7** The dilution scheme relative to the standardized culture has been added for clarity.
- **4.2.8** This Section has been added to allow the option of preparing plate count dilutions at the same time as the dilutions of the challenge inoculums.
- **5.1** The option of using the Reed-Muench or Spearman-Kärber method of estimation has been added.
- Information regarding the challenge culture and reference bacterin has been modified to indicate the current reagent data sheet throughout the document.

- References to internal CVB SOPs have been replaced with summarized information throughout the document.

- The contact person has been changed to Janet Wilson.