Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Testing of Erysipelas Antiserum in Mice

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Supplemental Assay Method for Potency Testing Erysipelas Antiserum in Mice

1. Introduction

This Supplemental Assay Method (SAM) describes procedures for determining potency *Erysipelothrix rhusiopathiae* antiserum, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.452. It is a test designed to use mice, in which the degree of passive protection against a known challenge of *E. rhusiopathiae* is determined.

2. Materials

2.1 Equipment/instrumentation

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

2.1.2 Incubator, 37°C

2.1.3 Automatic pipetting device, or pipette bulb

2.1.4 Crimper for aluminum caps on serum vials

2.2 Reagents/supplies

2.2.1 *E. rhusiopathiae* strain E1-6 challenge culture, IRP ERC, current lot. This culture is available from the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Center for Veterinary Biologics-Labortory, Ames, IA.

2.2.2 Positive control *E. rhusiopathiae* antiserum, IRP ERHU AS, current lot. This control serum is available from the CVB-L.

2.2.3 Test antiserum

2.2.4 Syringes, 1 ml

2.2.5 Needles, 26 ga x δ in

2.2.6 Glass serum bottle, 20-100 ml

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

2.2.8 Glass screw-top tubes, 13 x 100 mm, with caps

2.2.9 Pipettes, 5 ml and 25 ml

2.2.10 Erysipelas challenge culture medium

2.2.11 Bovine blood agar plates

2.2.12 Peptone buffer

2.2.13 Water, distilled or deionized, or water of equivalent purity

2.3 Animals

2.3.1 Swiss mice, 16-20 g. Although the 9 CFR does not specify sex, the CVB-L uses female mice.

2.3.2 Forty mice are required for each lot of antiserum to be tested. Forty additional mice are required for the positive control antiserum, and 10 mice are required as negative controls. Thirty mice are required to determine the LD_{50} of the challenge inoculum. All mice should be from the same source colony. **Note:** Although 9 CFR regulations do not require the use of a positive control antiserum or a determination of LD_{50}, the CVB-L uses these additional mice as checks on test validity.

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 antiserum, report the death immediately to Inspection and Compliance, Center for Veterinary Biologics (CVB), which may request further safety testing of the antiserum.

3.2.5 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the mice 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 supplies (pipettes, syringes, needles, rubber stoppers, diluents, etc.).

3.3.3 All equipment must be operated according to manufacturers' instructions and maintained and calibrated, as applicable, according to current CVB-L Standard Operating Procedures.

3.4 Preparation of reagents

3.4.1 *E. rhusiopathiae* challenge culture. The challenge culture, IRP ERC, is lyophilized in 0.2 ml amounts. Store vials of lyophilized culture at ≤4°C.

3.4.2 Peptone buffer (NVSL media 10522)

Peptone	10 g
Sodium phosphate, dibasic	12.01 g
Potassium phosphate, monobasic	2.09g
Water	q.s. 1000ml

Adjust pH to 7.3-7.5. Autoclave 20 min at 121°C. Cool before using. Store at room temperature no more than 6 mo.

3.4.3 Bovine blood agar (NVSL media 10006)

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

Autoclave 20 min at 121°C. Cool to 47°C. Add 50 ml defibrinated bovine blood. Pour into sterile petri dishes. Cool to room temperature. Store at 4°C for no more than 6 mo.

3.4.4 Erysipelas challenge culture medium (NVSL media 10133)

Horse meat (no fat)	454 g
Horse liver	8 g
Water	1000 ml

Grind tissue and dispense in hot water in a cooker. Heat to boiling and simmer 1 hr. Allow to settle at least 2 hr. Skim off fat and discard meat. Strain through cheese cloth. Filter through No. 2 Whatman filter paper.
Combine 1000 ml of filtrate with:

Sodium phosphate, monobasic	11 g
Potassium phosphate, monobasic	1 g
Bile (fresh frozen fluid)	10 ml
OR Oxgall® (1 g in 10 ml H_2O)	
Peptone	20 g
Gelatin, granulated	5 g

Heat to just below boiling to dissolve the gelatin. Cool to 56°C. Adjust to pH 7.8.

Add:

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

Filter while still hot through a sterile Horman filter. Filter should also be hot. Adjust final pH to 7.6-7.8. Store at 4°C for no longer than 6 mo.

4. Performance of the test

4.1 Injection of test animals with antiserum

4.1.1 Check the label on each product to confirm identity. Thoroughly mix product by inverting end-to-end.

4.1.2 Weigh 5 randomly selected mice immediately prior to injection to assure that the average body weight of the mice is between 16 and 20 g. Record weights.

4.1.3 Inject each of 40 mice subcutaneously with 0.1 ml of the test antiserum and each of 40 additional mice subcutaneously with 0.1 ml of the positive control antiserum.

4.1.4 Retain 10 noninjected mice as negative controls and 30 additional noninjected mice to determine LD_{50} of the challenge.

4.2 Preparation of challenge

4.2.1 Reconstitute a vial of IRP ERC challenge culture in 1.5 ml peptone buffer.

4.2.2 Inoculate 10 ml erysipelas challenge culture medium with entire contents of vial of reconstituted culture.

4.2.3 Incubate the inoculated broth at 37°C for 18-20 hr.

4.2.4 Perform a Gram stain, according to the current version of BBSOP0004, on the overnight cultures. If the Gram stain shows a pure culture of Gram positive rods, continue with the challenge procedure.

4.2.5 Dilute overnight culture, as necessary, in sterile erysipelas challenge culture medium to 70% ± 2% T at 600 nm, using a spectrophotometer.

Note: Use sterile erysipelas challenge culture medium as a blank for the spectrophotometer.

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

4.2.7 Make 3 additional tenfold dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of the challenge inoculum to determine LD_{50} of the challenge. Place each dilution in a separate labeled serum vial and seal.

4.2.8 Place all vials of challenge on ice to transport to animal room. Keep on ice throughout 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 24 hr after injecting antiserum.

4.3.2 Challenge noninjected controls and LD_{50} mice at the same time as the injected mice.

4.3.3 Administer 0.2 ml of challenge inoculum subcutaneously to each mouse that received antiserum and to each of the negative control mice, using a 1-ml syringe with a 26-ga x δ-in needle.

4.3.4 Divide the LD_{50} mice into groups of 10. For each of the LD_{50} dilutions, inoculate each of 10 mice subcutaneously with 0.2 ml of the appropriate preparation.

4.4 Postinoculation plate count

4.4.1 After mice are challenged, perform a colony count on blood agar plates according to current version of BBSOP0019, using the vials retained for this purpose.

1. Use sterile erysipelas challenge culture broth as the diluent for the plate count, and plate on bovine blood agar. Incubate plates aerobically at 37°C for 48-72 hr.

2. Calculate the colony-forming units (CFU) per challenge dose according to the following formula:

Note: Average count in 0.1 ml culture x 2 x dilution factor (see table below)=CFU/0.2 ml dose of challenge culture

If plates used for average count were inoculated with:	Dilution factor
10^{-2} dilution of challenge inoculum	100
10^{-3} dilution of challenge inoculum	1000
10^{-4} dilution of challenge inoculum	10000

4.5 Observation of mice after challenge

4.5.1 Observe the negative control mice and LD_{50} mice daily for 7 days after challenge. Observe the remaining mice (those receiving antiserum) 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 such mice to determine the cause of death. If cause of death is unrelated to antiserum and/or challenge, do not include the deaths in the total deaths for the test.

5. Interpretation of test results

5.1 Interpret the test as prescribed in 9 CFR, Part 113.452.

5.1.1 At least 8 of 10 negative control mice must die within 7 days of challenge for a valid test. At least 34 of 40 mice receiving positive control antiserum must live for 10 days postchallenge for a valid test.

5.1.2 If at least 34 of 40 mice receiving the test antiserum survive 10 days after challenge, the serial is satisfactory without additional testing. If 11 or more mice die, the serial is unsatisfactory without additional testing. If 7-10 mice die, the serial is eligible for second stage testing.

5.1.3 Perform the second-stage test in a manner identical to the original test. Results of both tests

are combined and interpreted according to the following chart:

Stage	Number of mice receiving antiserum	Cumulative number of mice receiving antiserum	Cumulative number of dead mice for....	
			Satisfactory serial	Unsatisfactory serial
1	40	40	6 or less	11 or more
2	40	80	12 or less	13 or more

5.1.4 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 method of estimation. Record 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 an acceptable LD_{50} range for this test.

5.1.5 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 LD_{50} or CFU/dose for this test.

6. Report of test results

Report the results of the test(s) as described by the current version of BBSOP0020.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.452, U.S. Government Printing Office, Washington, DC, 1998.

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

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.