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SAM 303

Supplemental Assay Method for the Titration of Distemper Virus in Embryonated Chicken Eggs

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vivo* method for assaying the viral content of mink distemper virus (MDV) vaccines and that of chicken-embryo-adapted canine distemper virus (CDV) Positive Control. The method uses embryonated chicken eggs as the test system. The distemper viruses (DV) endpoint is determined by quantification of viral plaques on the chorioallantoic membrane (CAM) of inoculated chicken embryos.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Egg incubator, 36°± 2°C, humidified (Jamesway Model No. 252, Midwest Incubators Sales and Service)

2.1.2 Cabinet, laboratory biosafety level-2

2.1.3 Water bath, 36°± 2°C

2.1.4 Vortex mixer (Vortex-2 Genie, G-560, Scientific Industries Inc.)

2.1.5 Colony counter, darkfield Quebec (Model 3330, Reichert Scientific Instruments)

2.1.6 Syringe, 2-mL

2.1.7 Micropipettor, 200-µL, and tips

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 CDV Positive Control, Lederle strain of CDV

2.2.2 Embryonated chicken eggs, 7 days, from specific pathogen free hens, in accordance with title 9, *Code of Federal Regulations* (9 CFR)

2.2.3 Diluent

1. Nutrient broth, 8.0 g

2. Deionized water (DI), 1000 mL

3. Mix until dissolved.

4. Sterilize at 15 ± 1 psi, 121°± 5°C for 30 ± 5 minutes.

5. Store at 2°- 7°C.

2.2.4 70% Ethanol Solution

1. 718 mL ethanol, 95%

2. 282 mL DI

2.2.5 2% Iodine Solution

1. 2.0 g iodine

2. 100 mL 70% Ethanol Solution

2.2.6 Egg-candling light

2.2.7 Etcher/engraver, electric

2.2.8 Needles, 22-gauge x 1-inch and 20-gauge x 1-inch

2.2.9 Syringe, 1-mL tuberculin

2.2.10 Polystyrene tubes, 12 x 75-mm

2.2.11 Graduated cylinders, 25-mL, 50-mL, 100-mL, and 250-mL, sterile

2.2.12 Suction bulb, rubber

2.2.13 Forceps, curved-tip and blunt thumb

2.2.14 Petri dishes, 100 x 15-mm

2.2.15 Pipette-aid

2.2.16 Duco® cement

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel shall have training in the propagation of animal viruses and the quantitation of virus infectivity in embryonated chicken eggs.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set the water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of eggs

1. On the day of test inoculation, using the egg-candling light, check each egg for viability, proper growth of embryo, and integrity of the egg shell. Appropriately dispose of eggs which do not meet these criteria. Candle a sufficient number of eggs for the Test Vaccine, the CDV Positive Control, the Diluent Control, and the Uninoculated Control eggs.

2. Label 5 eggs for each dilution of the Test Vaccine and CDV Positive Control. Also label 5 additional eggs as Diluent Controls and 5 others as Uninoculated Controls. The Diluent Control eggs will be inoculated with the diluent only.

The Uninoculated Controls will not be inoculated (their CAMs will not be dropped). Place labeled eggs in double-layer cardboard flats.

3. Using the egg-candling light, hold the egg with the large end up and slowly turn the egg to locate the embryo. (If the embryo is hard to discern, give the egg a gentle, twisting shake to locate it.) Locate the edge of the air sac on the side opposite the embryo, and mark it with a pencil. The first drill site will be above this marked line by the center of the air sac. The second drill site will be half way between the edge of the air sac and the tip of the narrow end of the egg, on a site clear of large blood vessels.

4. Swab the 2 drill sites with 2% Iodine Solution and allow to air dry.

5. With the etcher/engraver, gently drill a hole through the shell and inner membrane at the mark for the first drill site (on the air sac end).

6. Turn the egg horizontally on the egg flat and carefully drill a small hole through the egg shell at the second drill site, **but not** through the inner shell membrane.

7. Using the egg-candling light for guidance, drop the CAM by a gentle suction with a rubber bulb placed over the first drilled hole on the air sac end. This causes the CAM to pull away from the shell under the second drilled hole, creating a new artificial air sac for the CAM inoculation.

8. After dropping the CAMs, return eggs to the egg flat in a horizontal position and place them in the 36°± 2°C egg incubator until time of inoculation, 2 ± 2 hours.

3.3.2 Diluent for the Diluent Control Eggs

Dispense 2 mL of diluent into a polystyrene tube.

3.3.3 Preparation of CDV Positive Control

1. On the day of test initiation, rapidly thaw a vial of CDV Positive Control in the water bath.

2. Dispense 1.8 mL of Diluent into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the CVB Reagent Data Sheet; appropriately label each tube (e.g., 5 tubes, labeled 10^{-1} through 10^{-5}**,** respectively).

3. With a 200-µL micropipettor, transfer 200 µL of the CDV Positive Control to the tube labeled 10^{-1}; mix by vortexing.

4. Using a new pipette tip, transfer 200 µL from the 10^{-1} labeled tube (**Step 3**) to the 10^{-2} tube; mix by vortexing.

5. Repeat **Step 4** for each of the remaining tubes, transferring 200 µL to the next dilution tube until the tenfold dilution series is completed.

3.4 Preparation of the Test Vaccine

3.4.1 The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). On the day of test inoculation:

1. For a single dose of Test Vaccine, using a sterile 1.0-mL syringe and a 20-gauge x 1-inch needle, rehydrate a vial of the Test Vaccine by transferring 1.0 mL for 1-mL-dose vaccines, 0.5 mL for 1/2-mL-dose vaccines, etc., of the provided diluent into the vial containing the lyophilized Test Vaccine; mix by vortexing.

2. For a multiple-dose Test Vaccine, remove the seal and stopper from the lyophilized Test Vaccine and the provided diluent. Measure the provided diluent into a sterile graduated cylinder according to the number of doses indicated on the manufacturer's instructions (e.g., for a 250-dose container of 1 mL per dose, measure 250 mL of diluent). Rehydrate the Test Vaccine by aseptically pouring the measured diluent into the lyophilized Test Vaccine bottle; mix by vortexing.

3.4.2 Incubate for 15 ± 5 minutes at room temperature.

3.4.3 Using the same method described for diluting the CDV Positive Control in **Section 3.3.3, Steps 2 through 5,** prepare an appropriate number of tenfold serial dilutions of the reconstituted Test Vaccine to bracket its expected titration endpoint titer specified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or special outline.

4. Performance of the Test

4.1 Starting with the highest dilution of the Test Vaccine, mix the dilution tubes by vortexing, and aspirate 0.5 mL with a 1-mL syringe and 22-gauge x 1-inch needle. Holding the syringe at a 45° angle, insert the tip of the needle through the opening of the dropped CAM and deliver 100 µL of the Test Vaccine dilution into each of 5 eggs/dilution. This allows the inoculum to be delivered onto the CAM without causing damage by the needle.

4.2 With the same syringe, and after vortexing, draw up an equal volume of the next lower Test Vaccine dilution and inoculate the dropped CAMs of 5 more eggs.

4.3 Inoculate the remaining dilutions of the Test Vaccine as described in **Section 4.1**. Separate syringes are not necessary between each dilution in a series if inoculating from the most dilute to the most concentrated within that series (e.g., 10^{-3} through 10^{-1}).

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4.4 In a similar manner, inoculate 5 eggs/dilution of the CDV Positive Control.

4.5 Inoculate each of the 5 Diluent Control eggs with 100 µl of the Diluent. To prevent cross-contamination, it is recommended to inoculate these eggs first.

4.6 Seal the inoculation opening and the suction hole of all eggs with Duco® cement and allow to air dry 5 ± 2 minutes.

4.7 Incubate the inoculated eggs and Diluent Controls at 36°± 2°C in the horizontal position on the cardboard egg flat for 6 days.

4.8 Candle inoculated eggs daily. Those chicken embryos dying within the first 24 hours after inoculation are considered to be nonspecific and are discarded.

4.9 Harvest and examine the CAMs.

4.9.1 Using curved-tip forceps, open the egg over the area opposite the inoculation site. Remove and discard the embryo, yolk sac, and fluids, leaving the CAM attached to its shell.

4.9.2 Detach the CAM with blunt-thumb forceps. Place all 5 CAMs from 1 dilution into a Petri dish. CAMs from Diluent Controls are placed in a separate Petri dish.

4.9.3 With the aid of a darkfield Quebec bacterial colony counter, examine CAMs for typical 1- to 4-mm diameter DV plaques, characterized by circumscribed white to grayish-white raised areas.

1. CAMs containing 1 or more DV plaques are considered to be DV positive.

2. Record results as the number of plaque-positive CAMs versus the total number of CAMs examined for each dilution of a Test Vaccine and the CDV Positive Control.

4.9.4 Calculate the DV endpoints of the Test Vaccine and the CDV Positive Control using the method of Spearman-Kärber as modified by Finney. The titers are expressed as $\log_{10}$ 50% chicken embryo infective dose ($CEID_{50}$).

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Example:

10^{-1} dilution of Test Vaccine = 5/5 CAMs plaque positive
10^{-2} dilution of Test Vaccine = 3/4 CAMs plaque positive
10^{-3} dilution of Test Vaccine = 1/5 CAMs plaque positive
10^{-4} dilution of Test Vaccine = 0/5 CAMs plaque positive

Test Vaccine titer = (X - d/2 + [d • S]) where:

X = $\log_{10}$ of the reciprocal of the highest dilution with all CAMs plaque positive (1)
d = $\log_{10}$ of tenfold dilution factor (1)
S = sum of proportion of CAMs plaque positive for all dilutions tested:

$$\frac{5}{5} + \frac{3}{4} + \frac{1}{5} + \frac{0}{5} = \frac{20 + 15 + 4}{20} = \frac{39}{20} = 1.95$$

Test Vaccine titer = (1 - 1/2) + (1 • 1.95) = 2.45

Adjust the titer to the recommended Test Vaccine dose as follows:

A. divide the **Test Vaccine Dose** by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this test MDV vaccine, the recommended dose is 1 mL)

Inoculation Dose = amount of diluted Test Vaccine inoculated onto each CAM of the embryonated chicken eggs (for this test MDV vaccine, the inoculation dose is 0.1 mL)

$$\frac{1 \text{ mL dose}}{0.1 \text{ mL}} = 10$$

B. calculate $\log_{10}$ of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

Log of 10 = 1

Test Vaccine titer = 2.45 + 1 = 3.45

Therefore the titer of the M**DV Test Vaccine** is $10^{3.5}$ $CEID_{50}$/mL.

5. Interpretation of the Test Results

5.1 Valid assay

5.1.1 The calculated titer of the CDV Positive Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established by a minimum of 10 previous titrations.

5.1.2 The lowest inoculated dilution of the CDV Positive Control must induce plaques in 100% of the CAMs (5/5). If an endpoint is not reached (1 or more CAMs are plaque positive at the highest dilution), the titer is expressed as "greater than or equal to" the calculated titer. If an endpoint is critical to testing, the highest (most dilute) must exhibit no CDV plaques (0/5).

5.1.3 The Diluent and Uninoculated Controls must not exhibit any typical DV plaques as described above.

5.1.4 At least 4 of the 5 chicken embryos inoculated with a given test dilution should remain alive throughout the 6-day incubation period.

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.3 In a valid test, if the titer of the Test Vaccine is greater than or equal to the titer specified in the APHIS filed Outline of Production, the Test Vaccine is considered **SATISFACTORY**.

5.4 In a valid test, if the titer of the Test Vaccine is less than the titer specified in the APHIS filed Outline of Production, the Test Vaccine is retested according to 9CFR, Part 113.8.b.

6. Report of Test Results

Report results as $CEID_{50}$ per dose of Test Vaccine.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.306, U.S. Government Printing Office, Washington, DC.

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

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

8. Summary of Revisions

Version .05

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

Version .04

- The Contact information has been updated.

Version .03

- The term "Reference" has been changed to "Positive Control" throughout the document.

Version .02

This document was revised to clarify the 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:

- The "Introduction" has been rewritten to provide clarity.
- **4.9.4** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.
- "Test Serial" was changed to "Test Vaccine" throughout the document.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.