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Center for Veterinary Biologics
Testing Protocol

SAM 302

Supplemental Assay Method for Titration of Canine
Distemper Virus in Primary Cell Culture

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying the viral content of modified-live canine distemper virus (CDV) vaccines. The test is applicable to CDV vaccines when the master seed virus (MSV) is not distinctly cytopathogenic. A direct fluorescent antibody (FA) or indirect fluorescent antibody (IFA) staining method is used, utilizing primary dog kidney (DKp) cell culture.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 36°± 2°C, high humidity, 5% ± 1% CO₂ incubator (Model 3336, Forma Scientific Inc.)

2.1.2 36°± 2°C aerobic incubator (Model 2, Precision Scientific)

2.1.3 36°± 2°C water bath

2.1.4 Ultraviolet (UV) light microscope (Model BH2, Olympus America Inc.)

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

2.1.6 Microscope slide glass staining dish with rack (glass staining dish)

2.1.7 Self-refilling repetitive syringe, 2-ml

2.1.8 Pipette-aid

2.1.9 Pipettor with tips and/or motorized microliter pipette and tips

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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 Reference, Rockborn strain [available from the Center for Veterinary Biologics (CVB)]

2.2.2 Monospecific antisera, free of CDV antibody, that neutralize the non-CDV fractions present in multifraction vaccines, e.g., canine adenovirus (CAV), canine parainfluenza virus (CPI), canine parvovirus (CPV), etc. [available from CVB]

2.2.3 DKp cell culture, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR) [available from CVB]

2.2.4 For FA: Anti-CDV fluorescein isothiocyanate (FITC)-labeled conjugate (Anti-CDV Conjugate) or for IFA: CDV antiserum and anti-dog IgG FITC conjugate (Anti-dog Conjugate).

2.2.5 Minimum essential medium (MEM)

2.2.5.1 9.61 g MEM with Earles salts without bicarbonate

2.2.5.2 1.1 g sodium bicarbonate

2.2.5.3 Dissolve with 900 ml deionized water (DW).

2.2.5.4 Add 5.0 g lactalbumin hydrolysate or edamine to 10 ml DW. Heat to 60°± 2°C until dissolved. Then add to the solution in **Section 2.2.5.3** with constant mixing.

2.2.5.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

2.2.5.6 Sterilize through a 0.22-µm filter.

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2.2.5.7 Aseptically add 50 µg/ml gentamicin sulfate

2.2.5.8 Store at 2°- 7°C.

2.2.6 Growth Medium

2.2.6.1 900 ml MEM

2.2.6.2 Aseptically add:

1. 100 ml gamma-irradiated fetal bovine serum (FBS)

2. 10 ml L-glutamine (200 mM)

2.2.6.3 Store at 2°- 7°C.

2.2.7 Dulbecco's phosphate buffered saline (DPBS)

2.2.7.1 8.0 g sodium chloride (NaCl)

2.2.7.2 0.2 g potassium chloride (KCl)

2.2.7.3 0.2 g potassium phosphate, monobasic, anhydrous (KH₂PO₄)

2.2.7.4 0.1 g magnesium chloride, hexahydrate (MgCl₂•6H₂O)

2.2.7.5 Dissolve reagents in **Section 2.2.7.1 through Section 2.2.7.4** with 900 ml DW.

2.2.7.6 Add 1.03 g sodium phosphate, dibasic, anhydrous (Na₂HPO₄) to 10 ml DW, heat to 60°± 2°C until dissolved, then add to **Section 2.2.7.5** with constant mixing.

2.2.7.7 Dissolve 0.1 g calcium chloride, anhydrous (CaCl₂) with 10 ml DW and add slowly to **Section 2.2.7.6** to avoid precipitation.

2.2.7.8 Q.S. to 1000 ml with DW; adjust pH to 7.0-7.3 with 2N HCl.

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2.2.7.9 Sterilize through a 0.22- μ m filter.

2.2.7.10 Store at 2°- 7°C.

2.2.8 Glass slides, 8-chamber (Lab-Tek® Slides)

2.2.9 Polystyrene tubes, 12 x 75-mm

2.2.10 Acetone, 100%

2.2.11 Pipettes

2.2.12 Disposable transfer pipette, 3.5-ml

2.2.13 Needles, 18-gauge x 1 1/2-inch

2.2.14 Syringe (tuberculin slip tip), 1-ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses and the quantitation of virus infectivity by FA.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of inoculation, set a water bath at 36°± 2°C.

3.2.2 On the day of the FA, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 Pretreated Lab-Tek® Slides

Two days before the day of inoculation, prepare sufficient Lab-Tek® Slides to allow 25 chambers for controls and 20 chambers for each Test Vaccine. Using

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a 2-ml self-refilling repetitive syringe, add 0.4 ml/chamber of Growth Medium to all chambers of the Lab-Tek® Slides; incubate at 37°± 2°C for 48 ± 8 hours.

3.3.2 Preparation of DKp Slides

Cells are prepared from healthy, confluent DKp cell cultures at second or third passage. On the day of test initiation, decant Growth Medium from slides and add 0.4 ml/chamber of approximately $10^{4.9}$ to $10^{5.2}$ cells/ml suspended in Growth Medium into all chambers of the Lab-Tek® Slides. Incubate at 37°± 2°C in a CO₂ incubator and use within 4 hours.

3.3.3 Preparation of CDV Reference Virus Control

3.3.3.1 On the day of inoculation, rapidly thaw a vial of CDV Reference in a 36°± 2°C water bath.

3.3.3.2 Dispense 1.8 ml MEM into sufficient 12 x 75-mm polystyrene tubes to bracket the expected endpoint according to the Center for Veterinary Biologics (CVB) Reagent Data Sheet and label appropriately (e.g., 5 tubes, labeled 10^{-1} through 10^{-5} , respectively).

3.3.3.3 With a 200-µl pipettor, transfer 200 µl of the CDV Reference to the tube labeled 10^{-1} ; mix by vortexing.

3.3.3.4 Using a new pipette tip, transfer 200 µl from the 10^{-1} labeled tube (**Section 3.3.3.3**) to the 10^{-2} tube; mix by vortexing.

3.3.3.5 Repeat **Section 3.3.3.4** for each of the subsequent dilutions, transferring 200 µl from the previous dilution to the next dilution tube.

3.3.4 Preparation of working antisera and/or FA conjugate solutions

For FA staining, dilute the Anti-CDV Conjugate; for IFA staining, dilute CDV antiserum and Anti-dog conjugate. Reagents are diluted in DPBS according to the manufacturer's recommendations.

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3.4 Preparation of the sample

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 inoculation, using a sterile 1.0-ml syringe and an 18-gauge x 1 1/2-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. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 For multifraction CDV vaccines, neutralize the non-CDV fractions with antiserum specific to each virus fraction.

3.4.2.1 Prepare dilutions of each neutralizing non-CDV antiserum according to the CVB Reagent Data Sheet or the manufacturer's instructions.

3.4.2.2 Dispense 200 μ l of each of the required neutralizing antisera into a 12 x 75-mm polystyrene tube, labeled 10^{-1} , and q.s. to 1.8 ml with MEM. For example, to neutralize the 3 non-CDV viral fractions of a CDV/CAV/CPI/CPV vaccine, dispense 200 μ l of each of the diluted CAV, CPI, and CPV antisera into the tube labeled 10^{-1} ; add 1.2 ml of MEM to obtain a final volume of 1.8 ml.

3.4.2.3 Pipette 200 μ l of the reconstituted Test Vaccine to the labeled tube to yield a 10^{-1} dilution; mix by vortexing.

3.4.2.4 Incubate at room temperature for 30 ± 5 minutes.

3.4.3 For Test Vaccines containing only the CDV fraction, the 10^{-1} dilution is prepared by adding 200 μ l of the Test Vaccine to 1.8 ml of MEM in a 12 x 75-mm polystyrene tube, labeled 10^{-1} ; mix by vortexing.

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3.4.4 Vaccine tenfold dilutions

3.4.4.1 Dispense 1.8 ml MEM into each of 3, 12 x 75-mm polystyrene tubes, labeled 10^{-2} through 10^{-4} (or more if the expected CDV endpoint of the Test Vaccine is higher than 10^{-4}).

3.4.4.2 Using a new pipette tip, transfer 200 μ l from the tube labeled 10^{-1} to the next dilution tube; mix by vortexing.

3.4.4.3 Repeat **Section 3.4.4.2** to the remaining tubes, transferring 200 μ l from the previous dilution to the next dilution tube, until the tenfold dilution series is completed.

4. Performance of the test

4.1 Label the DKp Slides and inoculate each of 5 chambers/dilution with 100 μ l of the Test Vaccine. In a similar manner, inoculate 5 chambers/dilution of the CDV Reference Control (with dilutions 10^{-5} through 10^{-2} from **Section 3.3.3**). Change tips between each unique sample (i.e., each Test Vaccine and the CDV Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g., 10^{-5} through 10^{-2}).

4.2 Five uninoculated chambers serve as negative cell controls.

4.3 Incubate slides in a $36^{\circ} \pm 2^{\circ}\text{C}$ CO_2 incubator for 5 days \pm 1 day.

4.4 After incubation, decant the media from the DKp Slides into an autoclavable waste container. Remove the plastic chambers by tipping them away from the slide, leaving the rubber gasket attached to the slide.

4.5 Place the slides in a rack, set in a glass staining dish filled with DPBS, and incubate 15 ± 5 minutes at room temperature.

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4.6 Place the rack in a glass staining dish filled with 100% acetone for 15 ± 5 minutes at room temperature. Remove and allow to air dry.

4.7 For FA: pipette 75 ± 25 μ l of the Working Anti-CDV Conjugate into each chamber of the DKp Slides; for IFA: pipette 75 ± 25 μ l of the Working CDV antiserum into each chamber of the DKp Slides; incubate at $36^\circ \pm 2^\circ\text{C}$ in the high humidity, aerobic incubator for 30 ± 5 minutes.

4.8 Remove the DKp Slides from the incubator, decant and wash per **Section 4.5**.

4.9 For IFA: pipette 75 ± 25 μ l of the Working Anti-Dog conjugate into each chamber of the DKp Slides; incubate at $36^\circ \pm 2^\circ\text{C}$ in the high humidity, aerobic incubator for 30 ± 5 minutes. Wash per **Section 4.5**.

4.10 Rinse by quickly dipping the rack of slides in a glass staining dish filled with DW; allow to air dry.

4.11 Read at 100X or 200X magnification on a UV-light microscope and examine cells for typical apple-green cytoplasmic fluorescence.

4.11.1 Chambers containing 1 or more cells displaying specific fluorescence are considered to be positive for CDV virus.

4.11.2 Record results as the number of FA-positive chambers versus the total number of chambers examined for each dilution of a Test Vaccine and the CDV Reference Control.

Calculate the CDV endpoints of the Test Vaccine and the CDV Reference Control using the Spearman-Kärber method as modified by Finney. The titers are expressed as \log_{10} 50% fluorescent antibody infective dose (FAID₅₀).

Example:

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

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Spearman-Kärber formula:

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

X = log₁₀ of dilution with all chambers FA positive (1)

d = log₁₀ of dilution factor (1)

S = sum of proportions of chambers FA positive for all dilutions tested:

$$\frac{5}{5} + \frac{4}{5} + \frac{2}{5} + \frac{0}{5} = \frac{11}{5} = 2.2$$

Test Vaccine titer = (1 - 1/2) + (1 • 2.2) = 2.7

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

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

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

Inoculation Dose = amount of diluted Test vaccine added to each chamber of the Lab-Tek slide (for this test CDV vaccine, the inoculation dose is 0.1 ml)

$$\frac{\underline{\underline{1 \text{ ml dose}}}}{0.1 \text{ ml inoculum}} = 10$$

B. calculate log₁₀ of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

$$\text{Log of } 10 = 1.0$$

Test Vaccine titer = 2.7 + 1.0 = 3.7.

Therefore, the titer of the CDV **Test Vaccine** is 10^{3.7} FAID₅₀/ml.

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5. Interpretation of the test results

5.1 Valid assay

5.1.1 The calculated titer of the CDV Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The lowest inoculated dilution of the CDV Reference Control must exhibit a 100% positive FA/IFA reaction (5/5). If an endpoint is not reached (1 or more chambers are FA/IFA 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 positive FA/IFA reaction (0/5).

5.1.3 The uninoculated cell controls must not exhibit any cytopathic effect (CPE), specific CDV virus fluorescence, or cloudy media that would indicate contamination.

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

5.1.5 If the validity requirements are met and the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service filed Outline of Production for the product under test, the Test Vaccine is considered **SATISFACTORY**.

5.1.6 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum, the Test Vaccine is retested in accordance with 9 CFR, Part 113.8.

6. Report of test results

Results are reported as FAID₅₀ per dose of Test Vaccine.

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7. References

- 7.1 Code of Federal Regulations, Title 9, Part 113.306, U.S. Government Printing Office, Washington, DC, 2004.
- 7.2 Cottral, GE (Ed.) *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca and London, 1978, pg. 731.
- 7.3 Finney, DJ *Statistical methods in biological assay*. Griffin, London. 3rd ed., 1978, pp. 394-401.

8. Summary of revisions

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:

- 2.2.5.2 The amount of sodium bicarbonate (NaHCO_3) has been changed from 2.2 g to 1.1 g.
- 2.2.5.7 Penicillin and streptomycin have been deleted.
- 4.11.2 Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- 5.1.2 Recording the rate of the FA positive reaction for validity requirements.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.
- The refrigeration temperatures have been changed from $4^\circ \pm 2^\circ\text{C}$ to $2^\circ - 7^\circ\text{C}$. This reflects the parameters established and monitored by the Rees system.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.