United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 301

Supplemental Assay Method for Titration of Neutralizing Antibody - Canine Distemper

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Supplemental Assay Method for Titration of Neutralizing Antibody - Canine Distemper

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1. Introduction

This Supplemental Assay Method (SAM) describes a serum neutralization method for assaying canine distemper antiserum. The method uses seven-day-old chicken embryos in the test system to determine the level of antibody against a measured dose of canine distemper virus.

2. Materials

2.1 Chicken embryos

Seven-day chicken embryos are used in the test system. The embryonated eggs are routinely received from the hatchery on the 6th day of incubation and inoculated on the 7th day. The eggs are candled and all dead or weak embryos are discarded.

2.2 Diluent

Nutrient broth is used to make dilutions of the indicator virus and sera. Difco nutrient broth is mixed according to Difco instructions with 200 units of crystalline penicillin G and 200 mcg of streptomycin per mL added.

2.3 Indicator virus

The Lederle strain of canine distemper virus in the 40th to 45th egg passage is used as an indicator virus. Batches are prepared as needed, stabilized, and stored in the wet state in appropriate aliquots at -80°F.

2.4 Test sera

These are tested at final dilutions of 1:50, 1:150, 1:450, and 1:1350.

3. Performance of the Test

3.1 Dilution of the indicator virus

A geometric mean titer of the indicator virus is determined by at least 3 weekly titrations in embryos. Utilizing this mean titer, the indicator virus is diluted so that 1 mL of diluted virus contains 1,000 to 3,000 $TCID_{50}$. This will provide 100 to 300 $TCID_{50}$ of virus per 0.1 mL, the amount inoculated into each embryo in the serum neutralization (SN) test system. When calculating the dilution factor, aim for 2,000 $TCID_{50}$ per mL in the working virus. Following are two examples for calculating the dilution factor. For these examples, assume that the mean titer of the indicator virus is 10^6 $TCID_{50}$/mL (1,000,000 $TCID_{50}$/mL).

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Example 1

Arithmetic Method - Divide 1,000,000 by 2,000 (the desired $TCID_{50}$/mL) to arrive at the dilution factor or final dilution of the virus.

$$\frac{1{,}000{,}000}{2{,}000} = 500, \text{ the dilution factor}$$

Example 2

Logarithmic Method - Divide 10^6 by $10^{3.3}$ ($10^{3.3} = 2{,}000$, the desired $TCID_{50}$/mL). Division of logarithms is done by subtracting one exponent from the other.

$10^6 - 10^{3.3} = 10^{2.7}$ or 500, the diluent factor

Now that the dilution factor (final dilution of 1:500) has been determined, make serial dilutions as follows:

1 mL virus + 4 mL diluent = 1:5 dilution
1 mL of 1:5 dilution + 9 mL diluent = 1:50 dilution
1 mL of 1:50 dilution + 9 mL diluent = 1:500 dilution

which yields 2,000 $TCID_{50}$ per mL or 200 $TCID_{50}$ per 0.1 mL. This dilution of indicator virus (200 $TCID_{50}$/0.1 mL) is referred to as the working virus.

3.2 Standard antiserum

The standard reference canine distemper antiserum is tested at the same time, in the same cell system, and by exactly the same method as the unknown sera being tested.

3.3 Dilution of test serum

Threefold dilutions are made of the serum to be tested. Final dilutions of 1:50, 1:150, 1:450, and 1:1350 are tested. The addition of an equal part of working virus doubles the dilution; therefore, dilutions of 1:25, 1:75, 1:225, and 1:675 of serum only are made. To arrive at these dilutions, set up 5 diluent blanks and make serial dilutions as follows:

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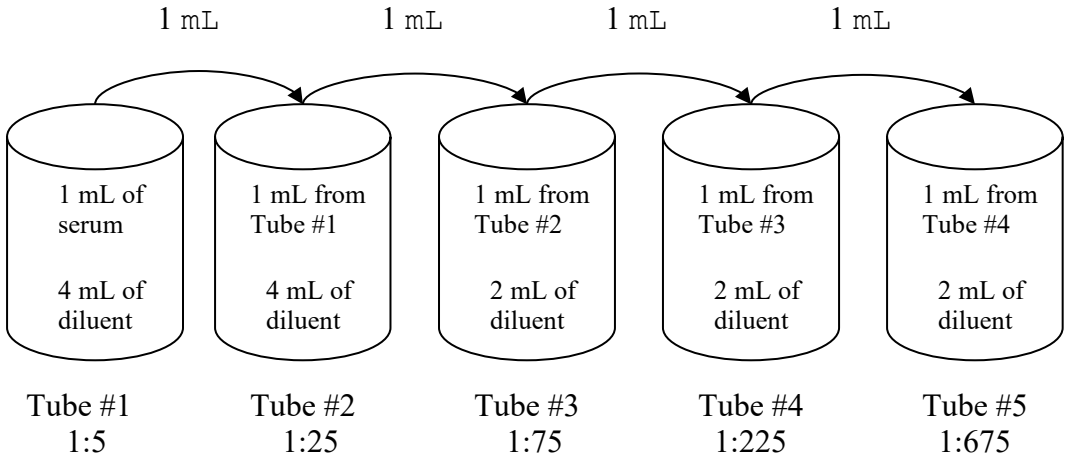
Add 1 mL of Undiluted Serum



Throughout the dilution procedure, transfers are made with a 1 mL disposable pipette. Mixing is done using an eccentric, rotating mixer (Vortex or similar). (Serum concentrates must be diluted to the equivalence of unconcentrated serum before testing.)

3.3.1 To tube No. 1 add 1 mL of test serum. Discard pipette. Mix. This gives a 1:5 dilution of serum.

3.3.2 To tube No. 2 add 1 mL of the mixture from tube No. 1. Discard pipette. Mix. This gives a 1:25 dilution.

3.3.3 To tube No. 3 add 1 mL of the mixture from tube No. 2. Discard pipette. Mix. This gives a 1:75 dilution.

3.3.4 To tube No. 4 add 1 mL of the mixture from tube No. 3. Discard pipette. Mix. This gives a 1:225 dilution.

3.3.5 To tube No. 5 add 1 mL of the mixture from tube No. 4. Discard pipette. Mix. This gives a 1:675 dilution.

3.4 Serum neutralization of working virus

Serum/virus mixtures are made by adding an equal volume of the working virus to each dilution of the serum on test. Set up 4 tubes each containing 1 mL of a test dilution of serum--1:24, 1:75, 1:225, and 1:675. Then to each tube, add 1 mL of working virus.

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Tube	Initial Serum Dilution	plus	Working Virus	equals	Final Serum Dilution	Final Virus Concentration
1	1 mL of 1:25	+	1 mL (2000 $TCID_{50}$)	=	2 mL of 1:50	containing 2000 $TCID_{50}$
2	1 mL of 1:75	+	1 mL (2000 $TCID_{50}$)	=	2 mL of 1:150	containing 2000 $TCID_{50}$
3	1 mL of 1:225	+	1 mL (2000 $TCID_{50}$)	=	2 mL of 1:450	containing 2000 $TCID_{50}$
4	1 mL of 1:675	+	1 mL (2000 $TCID_{50}$)	=	2 mL of 1:1350	containing 2000 $TCID_{50}$

The serum/virus mixtures are incubated for 1 hour at room temperature before inoculating chicken embryos.

3.5 Inoculation of embryos

The Gorham method is used to inoculate the chorioallantoic membrane (CAM) of the embryos. Five embryos are inoculated with each serum/virus mixture (0.2 mL per embryo).

3.6 Controls

3.6.1 The indicator virus is titrated to ascertain if titer is being maintained under storage conditions.

3.6.2 A 1:2 dilution of the working virus is titrated in tenfold dilutions to determine the actual amount of virus used in the SN test. These dilutions are incubated along with serum/virus mixtures before inoculation of eggs.

3.6.3 Five uninoculated embryos are incubated with the test to monitor the cell system.

3.6.4 Titration of the standard reference antiserum constitutes a positive serum control.

3.7 Incubation

Inoculated and uninoculated embryos are incubated at 35°- 37°C.

3.8 Reading

After 6 days incubation, the eggs are opened and the CAMs examined for white to grayish-white plaques usually surrounded by an edematous area. Disregard scar tissue caused by needle trauma when CAM was inoculated.

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To open the egg, pierce the shell about 1/2 to 1 inch from the small end with a small pair of sharp scissors. Cut through the shell and its membrane in a complete circle. This forms a cap of the small end of the egg which when removed exposes the embryo. Discard the embryo, leaving the CAM in place. Tease out the CAM with blunt thumb forceps. Place all 5 CAMs from the same dilution into a petri dish and examine, using a Quebec counter, for typical distemper plaques as described above.

4. Interpretation of the Test Results

The number of CAMs in each dilution showing no plaques is recorded as the number of positive responses and the 50% endpoint of neutralization (ND_{50}) is calculated by the statistical method of Reed and Muench or Spearman-Kärber.

Note: The ND_{50} is based upon the dilutions after working virus is added, not on serum dilutions alone.

A serial of antiserum to be considered satisfactory must meet or exceed minimums as described in Standard Requirement S-28 virus neutralization tests for canine distemper.

5. Report of Test Results

Record all test results on the test record.

6. References

6.1 Robson, D.S.; Hildreth, B.P.; Atkinson, G.F.; Carmichael, L.E.; Barnes, F.D.; Pakkala, B.; and Baker, J.A.; *Standardization of Quantitative Serological Tests*, 65th Annual Proceedings, United States Livestock Sanitary Association, October, 1961. Pages 74-78.

6.2 Carmichael, L.E., *Factors that Influence the Neutralization Test*, 66th Annual Proceedings, United States Livestock Sanitary Association, October, 1962. Pages 59-70.

6.3 Gorham, J.R., *A Simple Technique for the Inoculation of the Chorioallantoic Membrane of Chicken Embryos*, Amer. J. Vet. Res., Vol. 18. Pages 691-692

6.4 Steward, D.L.; Hebert, C.N.; and Davidson, I., International Standard for Anti-Canine Distemper Serum, Bull. Wld. Hlth. Org., Vol. 39 (1968). Pages 917-924.

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6.5 Anonymous: Standard Requirement for Anti-Canine Distemper-Hepatitis-Leptospira Serum and Products Containing any Combination of These Products. S-28; Published by the Veterinary Biologics Division, Agricultural Research Service, USDA, January 2, 1964.

6.6 Anonymous: Report of the ad hoc Committee, Standard Reagents and Test Procedures, Journal of the AVMA, Vol. 149, September 1, 1966, No. 5, Part 2, Canine Distemper Supplement. Pages 717-718.

7. Summary of Revisions

Version .05

- **Coversheet and contact information have been updated.**

Version .04

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

Version .03

- The phrase "available from the Center for Veterinary Biologics/CVB" has been removed from the document as these reagents are no longer supplied by the CVB.

Version .02 (February 11, 2011)

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 change was made to the document:

- The Contact information has been updated.

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