United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 300

Supplemental Assay Method for Titration of Neutralizing Antibody

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Standard Requirement:

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method for infectious canine hepatitis antiserum. The method uses a cell culture system to determine level of antibody against infectious canine hepatitis (ICH) viral antigens in commercial antisera.

2. Materials

2.1 Cell cultures

Use secondary (first subculture) or tertiary (second subculture) dog kidney (DK) cells. They are grown in roller tubes and are used when approximately 70% confluent. They are planted 1.0 mL per tube.

2.2 Growth medium for cell cultures

Gibco's F-15 medium (Eagles Minimum Essential Medium with Earles salts base, L-glutamine, and nonessential amino acids) is used with 5% fetal calf serum (heated to 56°C for 30 minutes), 0.5% lactalbumin hydrolysate, 1.0% sodium pyruvate, and 0.22% (2.2 gms/l) sodium bicarbonate added. Before adding the serum, the complete medium is filtered through a 0.22 micron membrane filter (Millipore Corp.).

Penicillin, 100 IU per mL, and streptomycin, 100 mcg per mL, are used in the medium. Antibiotics to control fungi and mycoplasmata may also be used if desired.

2.3 Maintenance medium for cell cultures

This is growth medium with a different serum content. Fetal calf serum (heated at 56°C for 30 minutes) is added at the rate of 1% to 5% depending upon growth rate of the cell sheet. Two percent is usually optimum.

2.4 Indicator virus

Mirandola strain of infectious canine hepatitis virus (ICH) in the 31st passage in DK cells is used as an indicator virus. Batches are prepared as needed, stabilized, and stored frozen in appropriate aliquots at -80°F.

2.5 Diluent

Maintenance medium is used to make dilutions of virus and sera.

2.6 Standard reference antiserum

This is a standard reference supplied by the Center for Veterinary Biologics (CVB).

2.7 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 DK cells. Utilizing this mean titer, the indicator virus is diluted with maintenance medium 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 tissue culture (TC) tube 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. Assume that the mean titer of the indicator virus is 10^6 $TCID_{50}$/mL (1,000,000 $TCID_{50}$/mL).

Example 1

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

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

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.

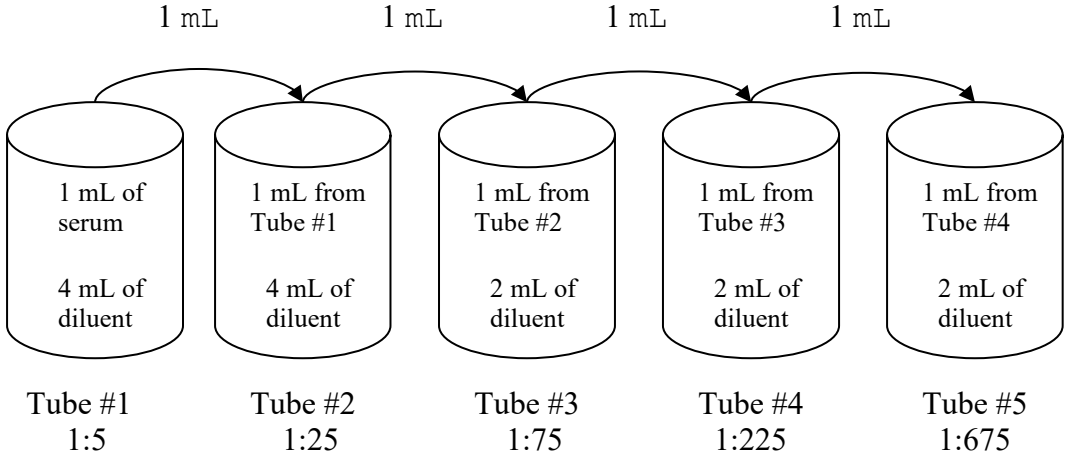
3.2 Standard antiserum

The standard reference infectious canine hepatitis 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:

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 [See Standard Requirement S-28]).

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. To each tube add 1 mL of working virus.

Tube	Initial Serum Dilution		Working Virus		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 tissue culture tubes.

3.5 Inoculation of roller tubes

Before inoculating the dog kidney monolayers, the growth medium is replaced with 1.5 mL of maintenance medium. Five cell culture tubes are inoculated with each serum/virus mixture (0.2 mL per tube).

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 culture tubes.

3.6.3 Five uninoculated cell culture tubes 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 cell culture tubes are incubated at 35°- 37°C. Maintenance medium is changed every 48 hours regardless of pH change in the medium.

3.8 Reading

Tubes are read daily for typical ICH cytopathogenic effect (CPE) beginning on the 4th day and ending on the 10th day postinoculation at which time the test is concluded.

4. Interpretation of the Test Results

The number of tubes in each dilution showing CPE is recorded 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 dilutions of serums alone.

A serial of antiserum to be considered satisfactory must meet minimums as described in Standard Requirement S-28 virus neutralization potency test for ICH antiserum.

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.; Atkinson, G.F.; and Barnes, F.D.; *Conditions Influencing Virus-Neutralization Tests for Infectious Canine Hepatitis Antibody*, The Corneal Veterinarian, Volume LIII, No. 3, July, 1963. Pages 369-388.

6.3 Herbert, C. Nancy; Stewart, Doreen L.; and Davidson, Ian; International Standard for Anti-Canine Hepatitis Serum, Bull. Wld. Hlth. Org., Volume 39, 1968. Pages 909-916.

6.4 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.

6.5 Carmichael, L.E., Factors that Influence the Neutralization Test, 65th Annual Proceedings, United States Livestock Sanitary Association, October, 1962. Pages 59-70.

7. Summary of Revisions

Version .04

- **Update to coversheet and contact information.**

Version .03

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

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.