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Center for Veterinary Biologics
Testing Protocol

SAM 106

Supplemental Assay Method for Titration of Neutralizing Antibody (Constant
Virus-Varying Serum Method)

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Contact: Alethea M. Fry, (515) 337-7200
Peggy A. Patterson

Approvals: /s/Geetha B. Srinivas Date: 31Dec14
Geetha B. Srinivas, Section Leader
Virology

/s/Byron E. Rippke Date: 06Jan15
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 09Jan15
Rebecca L.W. Hyde, Section Leader
Quality Management
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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Supplemental Assay Method for Titration of Neutralizing Antibody (Constant Virus-Varying Serum Method)

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method which employs a cell culture system for determining the antibody titer of serum against Bovine Rhinotracheitis (IBR), Bovine Virus Diarrhea (BVD), and Parainfluenza-3 (PI₃) Viruses.

2. Materials

2.1 Cell cultures

Roller tubes (16 x 150-mm) containing monolayers of primary bovine embryonic kidney (BEK) cells are used for IBR and PI₃ serum neutralization (SN) tests, and Leighton tubes containing monolayer BEK cells on coverslips (10.5 x 35-mm) are used for BVD SN tests. Cells found free from extraneous agents are used in these tests.

2.1.1 Primary BEK cells are grown from trypsinized kidney cortical tissue, frozen and stored at -80°C, and tested for extraneous agents.

2.1.2 Frozen cells are thawed, suspended in growth medium (**Appendix I**), and 1 mL amounts dispensed into Leighton or roller tubes.

2.1.3 The tubes containing the cells are incubated in stationary racks at 36°- 37°C until the monolayer is at least 80% confluent. The growth medium is replaced with maintenance medium (**Appendix II**) just before the tubes are inoculated.

2.2 Indicator viruses

Reference IBR, BVD, or PI₃ viruses are used.

2.3 Diluent

Maintenance medium, without serum, is used to make dilutions of the virus and serum.

2.4 Test serums

2.4.1 Serums to be tested

2.4.2 Negative control serum

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2.5 Conjugate

Conjugated BVD specific immune serum is used in the BVD SN test system.

2.6 Guinea pig red blood cells (RBCs) for the hemadsorption (HAd) test

2.6.1 Blood from healthy guinea pigs is collected aseptically in an equal volume of sterile Alsever's solution (**Appendix III**).

2.6.2 The RBCs are washed 3 times in Alsever's solution and sedimented each time by centrifugation at 1,000 rpm (250 Gs) for 15 minutes.

2.6.3 The RBCs are stored at 5°C as a 50% suspension in Alsever's solution.

2.6.4 For the hemadsorption test, the RBCs are diluted to a 0.5% suspension in phosphate buffered saline (PBS) (**Appendix IV**).

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have training in antibody titration assays, cell culture maintenance, and in the principles of aseptic techniques.

3.2 Preparation of reagents/control procedures

Dilution of indicator virus: The indicator virus is diluted to contain 100 to 500 TCID₅₀/0.1 mL; 0.1 mL is inoculated into each tissue culture tube in the test system. This dilution is determined by previous titrations and is designated as the "stock virus."

Calculation of the dilution factor is as follows: Divide the titer of the indicator virus by the desired titer of stock virus. This equals the dilution factor.

Example:

$$\frac{\text{Indicator virus titer}}{\text{Stock virus titer wanted}} = \text{dilution factor}$$

$$\frac{1,000,000 \text{ TCID}_{50}/0.1 \text{ mL}}{200 \text{ TCID}_{50}/0.1 \text{ mL}} = 5,000$$

The indicator virus is diluted 1:5,000.

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3.3 Preparation of the sample

Dilution of test serum: The serum is heat-inactivated at 56°C for 30 minutes. Serial twofold dilutions are made in sterile tubes containing diluent. Transfers are made with a 1-mL pipette and mixing is done by using a mixer (Vortex or similar type).

Twofold dilutions are made as follows:

3.3.1 One mL diluent is added to tubes 2, 3, 4, and 5.

3.3.2 One mL serum is added to tubes 1 and 2. Pipette is discarded and tube 2 is mixed. Tube 1 contains 1 mL of undiluted serum. Tube 2 contains a 1:2 dilution of the serum.

3.3.3 One mL is transferred from tube 2 to tube 3. Pipette is discarded and tube 3 is mixed. Tube 3 contains a 1:4 dilution of the serum.

3.3.4 This process is continued until the desired number of serum dilutions are made. One mL from the last serum dilution tube is discarded.

4. Performance of the Test

4.1 Serum neutralization of virus

An equal volume of stock virus (1 mL) is added to each serum dilution tube (1 mL), mixed, and allowed to incubate at room temperature for 45 minutes. Each of 5 cell culture tubes is inoculated with 0.2 mL of the serum-virus mixture. The mixing of equal volumes of serum and virus results in a further twofold dilution of serum. Thus, the undiluted serum (tube 1) becomes a 1:2 final dilution, the initial 1:2 becomes 1:4, etc.

4.2 Controls

4.2.1 The stock virus is titrated by preparing serial tenfold dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4}) and allowing them to remain at room temperature along with the serum-virus mixtures. Five cell culture tubes are inoculated with 0.1 mL of each virus dilution.

4.2.2 A known negative serum control is tested at a 1:2 dilution along with the test serums.

4.2.3 Five uninoculated cell culture tubes are incubated and processed along with the other cultures as a check on the test system.

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5. Interpretation of the Test Results

The 50% endpoints of the serum and virus are calculated by the method of Reed and Muench or Spearman-Kärber. The neutralization dose₅₀ of serum is based on the dilution showing complete neutralization of the test dose of virus.

The stock virus 50% endpoint must be between 100 and 500 TCID₅₀/0.1 mL for a test to be valid. The negative serum control must not neutralize virus. The cells in the uninoculated control tubes must remain normal.

6. Report of Test Results

6.1 Bovine Rhinotracheitis

The inoculated BEK roller tubes are incubated at 35°- 37°C for 4 to 6 days. The tubes are examined for cytopathic effect (CPE) typical of IBR virus. The number of tubes found positive and negative for CPE is recorded and the 50% endpoints calculated.

6.2 Bovine Virus Diarrhea

The inoculated Leighton tubes are incubated at 35°- 37°C for 4 to 6 days. The coverslips are removed from the tubes and processed for reading by the fluorescent antibody (FA) technique. The cells on the coverslips are stained as follows:

- 6.2.1** Coverslips are removed from the tubes and placed in racks.
- 6.2.2** They are rinsed in PBS, then in distilled water (DW), and dried.
- 6.2.3** They are fixed in cold acetone for 15 minutes, then dried thoroughly.
- 6.2.4** The cells are covered with conjugated BVD-specific immune serum and held in a high-humidity incubator at 37°C for 30 minutes.
- 6.2.5** Conjugate is drained and the coverslips are washed in a gently circulating PBS bath for 10 minutes, rinsed in DW, and dried.
- 6.2.6** Coverslips are mounted on glass slides with the cells down using FA mounting fluid.

Monolayer cells are examined by fluorescence microscopy with dry darkfield condenser. The number of slides positive and negative for fluorescence is recorded and the 50% endpoints calculated.

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6.3 Parainfluenza-3

The inoculated BEK roller tubes are incubated at 35°- 37°C for 4 to 6 days. The cell layers are examined by one or both of the following methods:

6.3.1 Cytopathic effect

The tubes are examined for CPE typical of PI₃ virus. The number of tubes found positive and negative for CPE is recorded and the 50% endpoints calculated.

6.3.2 Hemadsorption test

1. Fluids are poured from the tubes.
2. The cells are washed once with PBS.
3. To each tube is added 1 mL of a 0.5% suspension of RBCs.
4. The tubes are placed so that the cell monolayer is covered with the RBC suspension and allowed to stand 15 to 20 minutes at room temperature.
5. The suspension of RBCs is poured off and the monolayers are washed 3 times with PBS.
6. The PBS is drained from the tubes and the monolayers are examined microscopically from hemadsorption.

The number of tubes positive and negative for HAd is recorded and the 50% endpoints calculated.

7. References

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

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8. Summary of Revisions

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

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 Contact information has been updated.

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Appendices

Appendix I

Growth Medium

Lactalbumin hydrolysate	0.5%
Hanks BSS q.s. ad	100.0%
Antibiotics	
Penicillin	100 units/mL
Streptomycin	100 mcg/mL
Kanamycin	160 mcg/mL
Amphotericin B	2 mcg/mL

Ten percent fetal calf serum is added.

Appendix II

Maintenance Medium

Lactalbumin hydrolysate	0.5%
MEM (Eagle) q.s. ad	100.0%
Antibiotics	
Penicillin	100 units/mL
Streptomycin	100 mcg/mL
Kanamycin	160 mcg/mL
Amphotericin B	2 mcg/mL

Two percent fetal calf serum is added.

Appendix III

Alsever's Solution

Dextrose	2.05%
Sodium citrate	0.8%
Sodium chloride	0.42%
Citric acid	0.55%
Distilled H ₂ O q.s. ad	100%

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Appendix IV

Phosphate Buffered Saline (PBS-Dulbecco)

NaCl	0.8%
KCl	0.02%
Na ₂ HPO ₄	0.115%
KH ₂ PO ₄	0.02%
CaCl ₂ (anhy.)	0.01%
MgCl ₂ •6H ₂ O	0.01%
Distilled H ₂ O q.s. ad	100%

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