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Testing Protocol

SAM 122

Supplemental Assay Method for the Titration of Porcine Rotavirus Antibody (Constant Virus-Varying Serum Method)

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Standard Requirement:

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* serum neutralization (SN) method which utilizes cytopathic effects (CPE) or an indirect fluorescent antibody (IFA) technique in a cell culture system for determining the SN antibody titer against Group A porcine rotavirus (PROTA). The SN assay uses a constant amount of virus to test varying dilutions of serum.

Note: For this SAM, the dilution terminology of 1:10, 1:20, etc., specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.

2. Materials

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

2.1 Equipment/instrumentation

2.1.1 Incubator, 36°± 2°C, 5% ± 1% CO_2, high humidity (Model 3158, Forma Scientific Inc.)

2.1.2 Water bath

2.1.3 Microscope, inverted light (Model CK, Olympus America Inc.)

2.1.4 Microscope, ultraviolet (UV) light (Model BH2, Olympus America Inc.)

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

2.1.6 Micropipetters: 200-µL and 1000-µL single channel; 300-µL x 12-channel

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 PROTA Positive Control Viruses

1. Serotype 4 (Gottfried strain)

2. Serotype 5 (OSU strain)

2.2.2 Rhesus monkey kidney cells (MA-104) free of extraneous agents as tested by title 9, *Code of Federal Regulations* (9 CFR), (available from the Center for Veterinary Biologics (CVB))

2.2.3 Minimum essential medium (MEM) (National Centers for Animal Health (NCAH) Media #20030)

1. 9.61 g MEM with Earles salts without bicarbonate

2. 1.1 g sodium bicarbonate ($NaHCO_3$)

3. Q.S. to 1000 mL with deionized water (DI); adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

4. Sterilize through a 0.22-µm filter.

5. Aseptically add:

a. 10 mL L-glutamine (200 mM)

b. 50 µg/mL gentamicin sulfate

6. Store at 2°- 7°C.

2.2.4 Growth Medium

1. 900 mL of MEM

2. Aseptically add 100 mL of gamma-irradiated fetal bovine serum (FBS).

3. Store at 2°- 7°C.

2.2.5 Diluent Medium

1. 100 mL MEM

2. 83.3 µL pancreatin (4XNF 10X)

3. Store at 2°- 7°C.

2.2.6 Anti-PROTA monoclonal antibody (MAb)

1. MAb against Serotype 4 (Gottfried strain)

2. MAb against Serotype 5 (OSU strain)

2.2.7 Rabbit anti-mouse fluorescein isothiocyanate labeled conjugate (Rabbit Anti-mouse Conjugate)

2.2.8 0.01 M Phosphate buffered saline (PBS) (NCAH Media #30054)

1. 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2. 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

3. 8.5 g sodium chloride (NaCl)

4. Q.S. to 1000 mL with DI.

5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 2.0 N HCl.

6. Sterilize by autoclaving at 15 psi, 121°± 2°C for 35 ± 5 minutes.

7. Store at 2°- 7°C.

2.2.9 80% Acetone

1. 80 mL acetone

2. 20 mL DI

3. Store at room temperature.

2.2.10 Cell culture plates, 96-well

2.2.11 Polystyrene tubes, 12 x 75-mm

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have training in the immunological basis of SN assays, cell culture techniques, the principles of IFA, and aseptic technique.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of test initiation, set a water bath at 56°± 2°C.

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

3.3 Preparation of reagents/control procedures

3.3.1 MA-104 Plates. Two days prior to test initiation, seed 96-well cell culture plates with MA-104 cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of incubation at 36°± 2°C in a CO_2 incubator. These become the MA-104 Plates. Two sera can be tested on each MA-104 Plate. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent after incubation.

3.3.2 Stock Virus Preparation. On the day of test initiation, rapidly thaw vials of each PROTA Positive Control Virus in a 36°± 2°C water bath. Dilute each virus in Diluent Medium to contain 50-300 50% tissue culture infective dose ($TCID_{50}$)/100 µL.

3.3.3 Virus Back Titration. On the day of test initiation, make 4 serial tenfold dilutions of each Stock Virus.

1. Place 900 µL of MEM into 2 sets of 4, 12 x 75-mm polystyrene tubes, labeled 10^{-1} to 10^{-4}. Label each set with the appropriate stock virus.

2. Transfer 100 µL of each Stock Virus into the appropriate 10^{-1} tubes; mix by vortexing. Discard pipette tip.

3. Transfer 100 µL from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing. Discard pipette tip.

4. Repeat **Step 3** for the remaining tubes, transferring 100 µL sequentially from the previous dilution to the next dilution until the dilution sequence is completed.

3.3.4 Working Anti-PROTA MAb. On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the appropriate Anti-PROTA MAb in PBS, as determined for that specific MAb.

3.3.5 Working Rabbit Anti-mouse Conjugate. On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the Rabbit Anti-mouse Conjugate in PBS, according to the manufacturer's recommendations.

3.4 Preparation of the sample

3.4.1 On the day of test initiation, heat inactivate all Test Sera in a 56°± 2°C water bath for 30 ± 5 minutes.

3.4.2 Prepare serial twofold dilutions of Test Sera in 96-well cell culture plates, which become the Dilution Plates (see **Appendix**). Place each Test Serum onto 2, 96-well cell culture plates, 1 for each PROTA serotype to be tested. Make twofold dilutions as follows:

1. Add 150 µL Diluent Medium to all wells in Rows B-H.

2. Add 150 µL of a Test Serum to Rows A and B. Change pipette tips. Mix Row B with a multichannel micropipettor (6-8 fills).

3. Transfer 150 µL from Row B to Row C. Change pipette tips. Mix Row C with a multichannel micropipettor (6-8 fills).

4. Continue as in **Step 3** for the remaining rows. Discard 150 µL from all wells in Row H.

5. Add 150 µL of Stock Virus to all wells of the Dilution Plate. Tap plates gently to mix.

6. Incubate for 60 ± 10 minutes at 36°± 2°C to allow for neutralization of virus. This is an additional twofold dilution of the Test Sera. This becomes the Virus-Test Sera Mixture.

4. Performance of the Test

4.1 On the day of test initiation, decant Growth Medium from the MA-104 Plates.

4.2 Add 200 µL/well Diluent Medium to the MA-104 Plates. Decant the Diluent Medium.

4.3 Again, add 200 µL/well Diluent Medium to the MA-104 Plates. Incubate for 60 ± 10 minutes at 36°± 2°C. Decant the Diluent Medium.

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4.4 Inoculate 200 µL/well of each Virus-Test Sera Mixture into 5 wells/dilution of an MA-104 Plate, using a multichannel pipettor.

4.5 Inoculate 100 µL/well of each dilution (10^0 to 10^{-4}) of Virus Back Titration-Diluent Medium mixture into 5 wells of an MA-104 Plate, using a multichannel pipettor. Add 100 µL Diluent Medium to all wells of back titration.

4.6 Add 200 µL/well of Diluent Medium to 2 columns on each MA-104 Plate, to serve as uninoculated cell controls.

4.7 Incubate the MA-104 Plates for 5 days postinoculation at 36°± 2°C.

4.8 CPE counting is the primary method of determining the $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$).

4.8.1 Five days ± 1 day postinoculation, examine the wells with an inverted light microscope. The CPE of PROTA is visible as cell death in the cell monolayer.

4.8.2 Record the number of wells/dilution showing any characteristic CPE of PROTA for each Test Serum and Virus Back Titration.

4.8.3 Calculate the $TCID_{50}$ of each Virus Back Titration using the Spearman-Kärber method as commonly modified by Finney.

4.8.4 Calculate each endpoint of the Test Sera using the Spearman-Kärber method as commonly modified. The endpoints of the Test Sera are reported as SN titer which corresponds to the reciprocal of the highest serum dilution that neutralizes PROTA.

Example:

1:2 dilution of Test Sera = 5 of 5 wells CPE
1:4 dilution of Test Sera = 5 of 5 wells CPE
1:8 dilution of Test Sera = 3 of 5 wells CPE
1:16 dilution of Test Sera = 0 of 5 wells CPE

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Titer = (**X - d/2 + [d • S]**) where:

X = Log_{10} of lowest dilution (=0.3)
d = Log_{10} of dilution factor (=0.3)
S = Sum of proportion of CPE

$$\frac{5}{5} + \frac{5}{5} + \frac{3}{5} + \frac{0}{5} = \frac{13}{5} = 2.6$$

Titer = (0.3 - 0.3/2 + [0.3 • 2.6]) = 0.93
antilog of 0.93 = 8.5

Titer of the Test Serum is 9

4.9 Certain strains of PROTA may not exhibit pronounced CPE, thus an IFA may be conducted to determine the titer:

4.9.1 Decant the Growth Media from the MA-104 Plates.

4.9.2 Rinse the MA-104 Plates with PBS; incubate at room temperature for 5 ± 2 minutes. Decant the PBS.

4.9.3 Fill wells with 80% acetone.

4.9.4 Incubate at room temperature for 15 ± 5 minutes.

4.9.5 Decant the 80% acetone from the MA-104 Plates and air dry at room temperature.

4.9.6 Pipette 35 µL of the Working Anti-PROTA MAb into all wells. Incubate for 45 ± 15 minutes at room temperature.

4.9.7 Fill the wells completely with PBS; incubate at room temperature for 5 ± 2 minutes. Decant the PBS.

4.9.8 Repeat for a total of 2 washes.

4.9.9 Gently tap the MA-104 Plates onto paper towels to remove excess moisture.

4.9.10 Pipette 35 µL of the Working Rabbit Anti-mouse Conjugate into all wells. Incubate for 40 ± 10 minutes at room temperature.

4.9.11 Repeat **Sections 4.9.7 through 4.9.9.**

4.9.12 Fill wells completely with DI and decant. Allow to air dry or dry at 36°± 2°C.

4.9.13 Examine the MA-104 Plates with a UV-light microscope at 100 to 200X magnification.

4.9.14 A well is considered positive if typical cytoplasmic, apple-green fluorescence is observed.

4.9.15 Record and calculate as in **Sections 4.8.2 through 4.8.4**

5. Interpretation of the Test Results

5.1 The test is invalid if CPE, fluorescence, or bacterial/fungal contamination is observed in any of the control wells.

5.2 For a valid assay, the Virus Back Titration must be between 50 and 300 $TCID_{50}$/100 µL.

6. Report of Test Results

Record all test results on the test record.

7. References

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

7.2 Conrath TB. Handbook of Microtiter Procedures. In: *Clinical and Research Applications Laboratory*. Alexandria, Virginia: Cooke Engineering Co, 1972.

7.3 Finney DJ. *Statistical Method in Biological Assay*. 3rd ed. London: Charles Griffin and Co., 1978, pp. 394-401.

7.4 Rose NR, Friedman H, and Fahey JL, eds. Neutralization Assays. In: *Manual of Clinical Laboratory Immunology*. Washington, DC, ASM, 1986.

7.5 Parker RA, Pallansch MA. Using the virus challenge dose in the analysis of virus neutralization assays. Statistics in Medicine 11:1253-1262, 1992.

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.
- References to NVSL have been changed to NCAH throughout the document.

Version .03

- The Contact has been changed from Kenneth Eernisse to Joseph Hermann.
- The term "Reference" has been changed to "Positive Control" throughout the document.

Version .02

This document was revised to clarify 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:

- **2.2.3.2** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.
- **2.2.3.5** Penicillin, streptomycin, and amphotericin B have been removed.
- **2.2.4.2** "Heat–inactivated serum" has been changed to "gamma-irradiated serum".
- **3.3.2** 100-700 $TCID/_{50}$ has been changed to 50-300 $TCID/_{50}$.
- **3.3.3.1** Two sets of 8 tubes has been changed to 2 sets of 4 tubes.
- **3.3.3.5** This has been deleted.

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- **4.5** The volume of back titration dilutions added to the plate has been changed to be compatible with test conditions.

- **4.8.1** 120 hours ± 12 hours has been changed to 5 days ± 1 day.
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- **4.9.12** "Dip the plate" has been changed to "fill wells completely with DI".

- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.

- 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.

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Appendix
Transfer Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 1:2	TS1	TS1	TS1	TS1	TS1	CC	CC	TS2	TS2	TS2	TS2	TS2
B 1:4												
C 1:8												
D 1:16												
E 1:32												
F 1:64												
G 1:128												
H 1:256												

TS= Test Serum
CC= Cell Control