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Testing Protocol

SAM 114

Supplemental Assay Method for Titration of Porcine
Transmissible Gastroenteritis Virus

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**Supplemental Assay Method for Titration of Porcine
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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method which utilizes cytopathic effect (CPE) in a cell culture system to determine viral titers of modified live porcine transmissible gastroenteritis (TGE) virus vaccines.

2. Materials

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

2.1 Equipment/instrumentation

2.1.1 36°± 2°C, 5% ± 1% CO₂, high-humidity incubator (Model 3158, Forma Scientific Inc.)

2.1.2 Water bath

2.1.3 Inverted microscope (Model CK, Olympus America Inc.)

2.1.4 96-well microtiter plates

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 TGE Reference Virus, Purdue Strain [available from the Center for Veterinary Biologics (CVB)]

2.2.2 Swine testicular (ST) cells free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR) 113.52 [available from CVB]

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2.2.3 Minimum Essential Medium (MEM) (Media 20030)

2.2.3.1 9.61 g MEM with Earles salts without bicarbonate

2.2.3.2 1.1 g sodium bicarbonate

2.2.3.3 Q.S. to 1000 ml with deionized water, adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

2.2.3.4 Sterilize through 0.22- μ m filter.

2.2.3.5 Aseptically add:

1. 10 ml L-glutamine (200 mM)
2. 5 ml lactalbumin hydrolysate or edamine
3. 50 μ g/ml gentamicin sulfate

2.2.3.6 Store at 2°- 7°C.

2.2.4 Growth Medium

2.2.4.1 900 ml MEM

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS).

2.2.4.3 Store at 2°- 7°C.

2.2.5 Dilution Medium

2.2.5.1 98 ml of MEM

2.2.5.2 2 ml of FBS

2.2.5.3 2% sodium pyruvate

2.2.5.4 Store at 2°- 7°C

2.2.6 12 x 75-mm polystyrene tubes

2.2.7 Self-refilling, 2-ml repetitive syringe

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2.2.8 Graduated cylinders 25-, 50-, 100-, and
250-ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in the basis of cell-culture techniques, virus titration assays, and in the principles of aseptic technique.

3.2 Preparation of equipment/instrumentation

3.2.1 Set the water bath at $36^{\circ} \pm 2^{\circ}\text{C}$

3.3 Preparation of reagents/control procedures

3.3.1 Two days prior to test performance

3.3.1.1 Seed 96-well microtiter plates with ST cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of incubation at $36^{\circ} \pm 2^{\circ}\text{C}$. These become the ST plates. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent 2 days after incubation.

3.3.2 On day of test performance

3.3.2.1 Rapidly thaw a vial of TGE Reference Virus in $36^{\circ} \pm 2^{\circ}\text{C}$ water bath.

3.3.2.2 Reference Virus titration
Make 5 serial tenfold dilutions of Reference Virus as follows:

1. Dispense 1.8 ml of Dilution Medium into 5, 12 x 75-mm polystyrene tubes labeled 10^{-1} to 10^{-5} respectively using a repetitive syringe.

2. Transfer 200 μl of Reference Virus to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

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3. Transfer 200 μ l from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing. Discard pipette tip.
4. Repeat **3.3.2(3)** to the remaining tubes, transferring 200 μ l from previous dilution to the next dilution.

3.4 Preparation of the test vaccine (on day of test performance)

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 test initiation, remove the seal and stopper from both the Test Vaccine and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the Test Vaccine's total volume indicated on the manufacturer's label (e.g., for a 100-dose container of 2-ml-per-dose, reconstitute with 200 ml of diluent). Aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing. Porcine rotavirus, if present in the test vaccine, is not usually blocked with specific antisera due to its poor growth on ST cells in the absence of proteolytic enzymes.

NOTE: If the vaccine being evaluated is a bivalent TGE/Porcine rotavirus product, it is not necessary to neutralize the Porcine rotavirus fraction due to its poor growth on ST cells in the absence of proteolytic enzymes.

3.4.2 Prepare serial tenfold dilutions of Test Vaccine. Serial tenfold dilutions may be made as follows:

1. Dispense 1.8 ml of Diluent Medium into labeled tubes using repetitive syringe.
2. Pipette 200 μ l of Test Vaccine to the 10^{-1} tube, mix by vortexing. Discard pipette tip.
3. Repeat **step 3.4.2(2)** to the remaining tubes transferring 200 μ l from previous dilution to the next dilution. Continue as needed (10^{-2} to 10^{-5}).

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4. Performance of the test

- 4.1** Decant the Growth Media from ST plates.
- 4.2** Pipette 200 μ l/well from each Test Vaccine tube to 5 wells on an ST plate.
- 4.3** Pipette 200 μ l/well of each Reference Virus titration tube to 5 wells on an ST plate.
- 4.4** Maintain 5 or more wells as uninoculated cell-culture controls.
- 4.5** Incubate the ST plates undisturbed at $36^{\circ} \pm 2^{\circ}\text{C}$ in the CO_2 incubator for 4 days \pm 12 hours.
- 4.6** After 4 days \pm 12 hours inoculation, examine the wells with an inverted microscope. The CPE of TGE is visible as cellular death in the cell monolayer where the cells have been destroyed by the virus.
- 4.6.1** Record the number of wells/dilution, showing any characteristic CPE of TGE for each Test Vaccine and Reference Virus Titration.
- 4.6.2** The 50% tissue culture infective dose (TCID_{50}) of the Test Vaccine and Reference Virus Titration are calculated using the Spearman-Kärber method as modified by Finney. The titers are expressed as \log_{10} TCID_{50} per dose.

Example:

10^{-3} dilution of vaccine = 5/5 wells CPE positive
 10^{-4} dilution of vaccine = 4/5 wells CPE positive
 10^{-5} dilution of vaccine = 1/5 wells CPE positive
 10^{-6} dilution of vaccine = 0/5 wells CPE positive

Spearman-Kärber formula:

Test Vaccine titer = $(X - d/2 + [d \cdot S])$, where:

X = \log_{10} of dilution with all wells infected (3)
d = \log_{10} of dilution factor (1)
S = sum of proportions of wells CPE positive for all dilutions tested:

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$$\frac{5}{5} = 1.0 + \frac{4}{5} = 0.8 + \frac{1}{5} = 0.2 + \frac{0}{5} = 0 = 2.0$$

$$\text{Test Vaccine titer} = (3 - 1/2) + (1 \cdot 2.0) = 4.5$$

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

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

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this test vaccine, the recommended dose is 2 ml)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this test vaccine, the inoculation dose is 0.2 ml)

$$\frac{2 \text{ ml dose}}{0.2 \text{ ml inoculum}} = 10$$

B. calculate \log_{10} of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

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

$$\text{Test Vaccine titer} = 4.5 + 1.0 = 5.5$$

Therefore the titer of the **Test Vaccine** is $10^{5.5}$ TCID₅₀/2 ml

5. Interpretation of the test results

5.1 The test is invalid if visible contamination is observed.

5.2 The test is invalid if CPE is observed in any of the control wells.

5.3 For a valid assay, the Reference Virus Titration 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.

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

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5.5 If the validity requirements are met and the titer of the vaccine is greater than or equal to the titer contained in the approved Outline of Production for the product under test, the product is considered **SATISFACTORY**.

5.6 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum, it must be retested according to 9 CFR 113.8.

6. Report of test results

Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Cottral, G.E., (Ed.), 1978. *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, a division of Cornell University Press/Ithaca and London, pg 731.

7.2 Finney, D.J. 1978. *Statistical methods in biological assay*. Griffin, London. 3rd edition, 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** The amount of sodium bicarbonate (NaHCO₃) has been changed from 2.2 g to 1.1 g. Penicillin, streptomycin and amphotericin B have been removed from the media formulation.
- **3.4.1** This has been rewritten from "reconstituting with syringe and needle" to "graduated cylinder". Also that a test is from a single vial.

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- **4.6.2** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- 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.