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Supplemental Assay Method for the Detection of Extraneous Bovine Viral Diarrhea Virus in Modified-Live Vaccines

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Supplemental Assay Method for the Detection of Extraneous Bovine Viral Diarrhea Virus in Modified-Live Vaccines

1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method using a cell culture procedure and a direct or an indirect fluorescent antibody technique (DFAT, IFAT) for detecting extraneous bovine viral diarrhea virus (BVDV) in modified-live vaccines (MLV) produced in cell cultures which support the growth of BVDV.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Incubator, 36°± 2°C, high humidity, 5 ± 1% CO_2

2.1.2 Microscope, inverted light

2.1.3 Microscope, ultraviolet light (UV-light microscope)

2.1.4 Microscope slide, glass staining dish with rack (glass staining dish)

2.1.5 Laboratory stirrer/hot plate and magnetic stir bar

2.1.6 Centrifuge with rotor

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Monospecific antiserum that is neutralizing, nontoxic to cell cultures, free of BVDV1 and BVDV2 antibodies, and specific to the agent(s) in the test serial.

2.2.2 Bovine turbinate (BT) secondary cell culture or other permissive cells free of extraneous agents as tested by title 9, *Code of Federal Regulations*, (9 CFR).

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. Dissolve **Steps 1 and 2** with 900 mL deionized water (DI).

4. Add 5 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to 60°± 2°C until dissolved. Add to **Step 3** with constant stirring.

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

6. Sterilize through a 0.22-µm filter.

7. Aseptically add:

a. 10 mL L-glutamine

b. 50 ug/mL gentamicin sulfate

8. Store at 2°- 7°C.

2.2.4 Growth Medium

1. 900 mL of MEM

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

3. Store at 2°- 7°C.

2.2.5 Maintenance Medium

1. 980 mL of MEM

2. Aseptically add 20 mL gamma-irradiated fetal bovine serum (FBS).

3. Store at 2°- 7°C.

2.2.6 BVDV Reference Viruses

1. Noncytopathic BVDV type 1, e.g., New York-1 strain

2. Noncytopathic BVDV type 2, e.g., 890 strain

3. Other BVDV strains (optional), e.g., Singer strain (cytopathic type 1), 125 strain (cytopathic type 2)

2.2.7 For DFAT, anti-BVDV fluorescein isothiocyanate labeled polyclonal antiserum (anti-BVDV FITC conjugate), reactive to all type 1 and type 2 strains of BVDV.

2.2.8 For IFAT, a polyclonal antiserum reactive to all type 1 and type 2 strains of BVDV. For additional testing, BVD types 1 and 2 monoclonal antibodies (MAb) may also be used.

2.2.9 For IFAT, appropriate anti-species IgG (H+L) fluorescein isothiocyanate labeled antiserum (anti-species FITC conjugate)

2.2.10 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 to 7.6 with 0.1 N sodium hydroxide (NaOH) or 2N HCl.

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

7. Store at 2°- 7°C.

2.2.11 Acetone

2.2.12 Cell culture flask, 25-cm^2

2.2.13 Cell culture slides, 8-chamber (Lab-Tek® slide)

2.2.14 Polystyrene tubes, 12 x 75-mm

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in aseptic techniques, ultraviolet light microscopy, and cell culture techniques.

3.2 Preparation of equipment/instrumentation

On the day of the DFAT or IFAT, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DI.

3.3 Preparation of reagents/control procedures

3.3.1 One day prior to test initiation (first passage) and one day prior to second passage, seed one 25-cm^2 cell culture flask per test serial, one 25-cm^2 cell culture flask for a media control, and one 25-cm^2 cell culture flask for an uninoculated cell control, using 10 mL of approximately $10^{5.2}$ to $10^{5.5}$ BT cells/mL in Growth Medium. Incubate at 36°± 2°C in a CO_2 incubator, to 50 to 75% confluency. These become the BT flasks.

3.3.2 One day prior to inoculation of the Lab-Tek® slides, seed one Lab-Tek® slide per test serial with 0.3 to 0.4 mL of approximately $10^{5.0}$ to $10^{6.5}$ BT cells/mL in Growth Medium. Incubate at 36°± 2°C in a CO_2 incubator, to 60 to 90% confluency in 24 hours.

3.3.3 Preparation of BVDV Positive Controls

On the day of inoculation of the Lab-Tek® slides, dilute the BVDV type 1 and type 2 reference viruses in MEM, according to the Center for Veterinary Biologics (CVB) Reagent Data Sheet or the manufacturer's recommendations.

3.3.4 Preparation of working anti-BVDV FITC conjugate for DFAT

On the day of the DFAT, dilute anti-BVDV FITC conjugate in PBS to the working dilution according to the CVB Reagent Data Sheet or the manufacturer's recommendations.

3.3.5 Preparation of working polyclonal antiserum and BVDV type 1 and 2 MAb for IFAT

On the day of the IFAT, dilute the polyclonal antiserum or BVDV types 1 and 2 MAb in PBS to the IFA working dilution as determined for that specific antiserum or MAb according to the CVB Reagent Data Sheet or the manufacturer's recommendations. The polyclonal antiserum should be used as a screening antibody, and the MAb may be used if further identification of genotype of virus is necessary.

3.3.6 Preparation of working anti-species FITC conjugate for IFAT

On the day of the IFAT, dilute the anti-species FITC conjugate in PBS to the working dilution according to the manufacturer's recommendations.

3.4 Preparation of the sample

3.4.1 The initial test of a test serial will be with a single vial (a single sample from 1 vial).

3.4.2 On the day of test initiation, rehydrate the test serial according to the manufacturer's instructions. If the test serial contains a bacterin for the diluent, sterile DI is used to replace the diluent. Mix by vortexing.

3.4.3 Test serials may require neutralization. (See **Appendix II** for a listing of viruses able to replicate in BT.)

1. Monovalent test serial: 0.5 mL of rehydrated test serial and 0.7 mL of monospecific antiserum against virus able to replicate in BT that is a fraction in the test serial are mixed in a labeled 12 x 75-mm polystyrene tube. Depending on the titer of the virus to be neutralized and the antiserum neutralization titer, it may be necessary to increase the amount of antiserum used for neutralization of a given virus, as determined empirically. Incubate for 60 ± 10 minutes at room temperature.

2. Bivalent test serial: 0.5 mL of rehydrated test serial and 0.5 mL of each monospecific antisera against viruses able to replicate in BT that are a fraction in the test serial are mixed in a labeled 12 x 75-mm polystyrene tube. Depending on titer of the virus to be neutralized and the antiserum neutralization titer, it may be necessary to increase the amount of antiserum used for neutralization of a given virus, as determined empirically. Incubate for 60 ± 10 minutes at room temperature.

3. Test serial containing 3 or more fractions: 0.5 mL of the rehydrated test serial and 0.3 mL of each monospecific antisera against viruses able to replicate in BT that are a fraction in the test serial are mixed in a labeled 12 x 75-mm polystyrene tube. Depending on the titer of the virus to be neutralized and the antiserum neutralization titer, it may be necessary to increase the amount of antiserum used for neutralization of a given virus. Incubate for 60 ± 10 minutes at room temperature.

4. Performance of the Test

4.1 Just prior to test initiation, decant the Growth Medium from all but one of the BT flasks.

4.2 Inoculation

4.2.1 Inoculate 0.5 mL of test serial not requiring neutralization into a BT flask; q.s. to 2.5 mL with Maintenance Medium. This becomes the test flask.

4.2.2 When neutralization is required, inoculate the entire volume of the neutralized test serial (**Section 3.4.3**) after the incubation period into a BT flask, q.s. to 2.5 mL with Maintenance Medium. This becomes the test flask.

4.3 Maintain 1 BT flask per test as an unopened, uninoculated cell control.

4.4 Maintain 1 BT flask per test as a media control. Inoculate the media control with 2.5 mL Maintenance Medium.

4.5 Allow each test flask and media control to absorb at 36°± 2°C for 60 ± 10 minutes.

4.6 Add 7.5 mL Maintenance Medium to each test flask and the media control (i.e., q.s. to 10 mL).

4.7 Incubate the flasks at 36°± 2°C in a CO_2 incubator. Observe each test flask, media control, and cell control not less often than every 3 days with an inverted light microscope for any signs of cytopathic effect (CPE) or contamination.

4.8 Four days ± 1 day postinoculation observe each test flask, media control, and cell control for any signs of CPE. Discard the cell control if no CPE is evident. Freeze the remaining flasks at -70°± 5°C for a minimum of 30 minutes. Flasks may be maintained at -70°± 5°C until the next passage. Thaw each flask at room temperature and shake

contents. When the contents of the flask have thawed completely, pour into a sterile 12 x 75-mm plastic tube, and centrifuge at 1000 X g for 10 minutes.

4.9 Decant Growth Medium from all but one of the new BT flasks seeded one day previously, as described in **Section 3.3.1**.

4.10 Pipette 1.0 mL of the supernatant fluid from the first passage (see **Step 8**) of a test serial into a new labeled BT flask from which the Growth Medium has been removed; q.s. to 2.5 mL with Maintenance Medium. Repeat for the media control. These are the new test flask and media control. A new BT flask is maintained as an unopened, uninoculated cell control. Pour off the remaining portion of the supernatant fluid into another sterile 12 x 75-mm tube and freeze at -70°C.

4.10.1 Additional neutralizing antisera may be necessary to suppress replication of specific viral fractions in the vaccine. If so, the cell suspension neutralization may be performed according to **Sections 3.4.3(1), 3.4.3(2),** or **3.4.3(3)**.

4.10.2 Inoculate the entire volume of the neutralized cell suspension after the incubation period into a BT flask; q.s. to 2.5 mL with Maintenance Medium. This becomes the test flask.

4.11 Allow each test flask and media control to absorb at 36°± 2°C for 60 ± 10 minutes, then q.s. to 10 mL with 7.5 mL Maintenance Medium, as in **Steps 5 and 6**.

4.12 Incubate flasks at 36°± 2°C in a CO_2 incubator. Observe all flasks not less than every 3 days for signs of CPE or contamination.

4.13 Four days ± 1 day after the second inoculations repeat **Step 8**. This completes the second passage of the test serial.

4.14 Inoculate 0.1 mL/well of the cell suspension from each test flask into at least 4 wells of a Lab-Tek® slide, seeded one day previously, as described in **Section 3.3.2**. For every assay, at least 4 wells serve as uninoculated cell control wells. Incubate at 36°± 2°C in a CO_2 incubator for 4 days ± 1 day postinoculation.

4.14.1 Additional neutralizing antisera may be necessary to suppress replication of specific viral fractions in the vaccine. If so, add antiserum directly to the Lab-Tek® slide.

4.14.2 Alternately, the cell suspension neutralization may be performed similar to **Sections 3.4.3(1), 3.4.3(2),** or **3.4.3(3)**.

4.15 Add 0.1 mL/well of each BVDV positive control to at least 4 wells of a Lab-Tek® slide, seeded 1 day previously, as described in **Section 3.3.2**. Incubate at 36°± 2°C in a CO_2 incubator for 4 days ± 1 day postinoculation.

4.16 Add 0.1 mL/well of the cell suspension from the media control flask to at least 4 wells of a Lab-Tek® slide, seeded one day previously, as described in **Section 3.3.2**. Incubate at 36°± 2°C in a CO_2 incubator for 4 days ± 1 day postinoculation.

4.17 Following incubation, decant the media from all Lab-Tek® slides. Remove the plastic walls by twisting them away from the Lab-Tek® slides, leaving the gasket attached.

4.18 Place the Lab-Tek® slides in a slide rack; place the rack in a glass staining dish filled with PBS. (Optional: A magnetic stir bar can be placed to rotate slowly in the dish, which is set on a laboratory stir plate.) Let stand 15 ± 5 minutes at room temperature. This step may be omitted in the event cells are in danger of sloughing from the Lab-Tek® slides. In that case, cells are immediately fixed in acetone without a PBS rinse (see **Step 19**).

4.19 Discard the PBS, replace it with acetone, and fix the Lab-Tek® slides for 15 ± 5 minutes at room temperature. Remove and allow to air dry.

4.20 Either DFAT or IFAT may be used to detect possible extraneous BVDV. A suitable anti-BVDV FITC conjugate used as a DFAT offers the advantage of less steps in processing the Lab-Tek® slides. For IFAT use polyclonal BVDV antiserum. If further characterization of the BVDV is required, it can be accomplished by using type 1- and type 2-specific antibodies provides information on the genotype of BVDV strains.

4.20.1 For DFAT, pipette 75 ± 25 µL of a working anti-BVDV FITC conjugate into each well of the Lab-Tek® slides. Incubate in a CO_2 incubator at 36°± 2°C for 30 ± 5 minutes.

4.20.2 For IFAT, pipette 75 ± 25 µL of a working polyclonal BVDV antiserum into at least 4 wells of a Lab-Tek® slide inoculated with a given test serial. If desired, pipette 75 ± 25 µL of a working BVDV type 1 MAb or type 2 MAb into at least 4 wells each of slide inoculated with the same test serial. Repeat this for every test serial. Pipette 75 ± 25 µL of each working BVDV polyclonal antiserum and MAb into at least 4 wells of cell control, and at least 4 wells of media control. Incubate in a CO_2 incubator at 36°± 2°C for 30 ± 5 minutes.

1. Wash per **Step 18**. Discard the PBS.

2. Pipette 75 ± 25 µL of the working anti-species FITC conjugate into each well of the Lab-Tek® slides; incubate in a CO_2 incubator at 36°± 2°C for 30 ± 5 minutes.

4.21 Wash per **Step 18**. Discard the PBS. Briefly dip each Lab-Tek® slide in DI.

4.22 Allow the Lab-Tek® slides to air dry.

4.23 Read within 1 hour at 100-200X with a UV-light microscope. Alternatively, the Lab-Tek® slides may be stored in the dark at 2°- 7°C for no longer than 48 hours before reading. Examine the cell monolayer for typical apple-green cytoplasmic fluorescence.

4.24 Wells containing 1 or more cells displaying specific fluorescence for BVDV are positive.

5. Interpretation of the Test Results

5.1 Validity requirements

5.1.1 For a valid test, the media control and the cell control wells on Lab-Tek® slides shall not exhibit signs of contamination, CPE, or positive DFAT or IFAT.

5.1.2 The BVDV positive controls must show specific fluorescence for BVDV.

5.1.3 If either validity requirement in **Sections 5.1.1 or 5.1.2** is not met, the test is considered a **NO TEST** and may be repeated without prejudice.

5.2 Results

5.2.1 If the initial test is valid and all wells of the test serial on the Lab-Tek® slides are free of BVDV contamination, the serial is **SATISFACTORY**.

5.2.2 Retests

1. If the initial test is valid and 1 or more wells of the test serial on the Lab-Tek® slides exhibits signs of BVDV contamination, the test is repeated (1st retest), using a new vial of the test serial. Retests are conducted using an additional BT flask for each passage and at least 4 wells of a Lab-Tek® slide to serve as serum control flask and wells, respectively. These will have contents identical to the media control flask and wells, plus addition of equivalent amounts of antiserum as used in the test flask and wells to neutralize viral fractions of the test serial.

2. If the second valid test (1st retest) confirms the initial result and the serum control is negative for BVDV, the test serial is **UNSATISFACTORY**.

3. If the second valid test (1st retest) fails to confirm the initial result, the test serial is tested a third time (2nd retest). The test is repeated using a new vial of test serial and a serum control as described in **Step 1**.

a. If the second and third valid tests (1st and 2nd retests) result in no signs of BVDV contamination, the test serial is **SATISFACTORY**.

b. If the third valid test (2nd retest) confirms the initial result and the serum control is negative for BVDV, the test serial is **UNSATISFACTORY**.

4. If at any time in a valid retest the serum control in the flasks or on the Lab-Tek® slides is positive for BVDV, the blocking antiserum used in the testing is suspect and that test and all preceding testing is a **NO TEST**. A test serial may be retested without prejudice after adequate evaluation and assurance of BVDV free reagents.

6. Report of Test Results

Record all test results on the test record.

7. References

7.1 Title 9, *Code of Federal Regulations,* part 113.300, U.S. Government Printing Office, Washington, DC.

7.2 G. E. Cottral ed. *Manual of Standardized Methods for Veterinary Microbiology*. Comstock Publishing Assoc. Ithaca, NY.

8. Summary of Revisions

Version .06

- **Updated coversheet**

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.

- **3.3.2:** Revised section to include a larger range of cell density and confluency for seeded :LabTek slides.

Version .03

- The Contact has been changed from Kenneth Eernisse and Marsha Hegland to Joseph Hermann and Peg Patterson.

- **2.1:** The 36° aerobic incubator has been deleted and a centrifuge and rotor have been added.

- **4.20:** The use of an aerobic incubator has been changed to a CO_2 incubator.

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** The forumlation of MEM has been changed to include 1.1 grams sodium bicarbonate per liter instead of 2.2 grames, and the removal of penicillin, streptomycin, and amphotericin B.

- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C throughout the document.

Appendix I
Cell lines that support BVDV replication

Based on testing conducted at the CVB and other scientific institutions, the cell lines that are permissive for BVDV include:

1) all bovine, ovine, and swine cell lines,

2) cat cell lines, especially Crandell feline kidney (CRFK),

3) rabbit cell lines,

4) MA104 (African green monkey) cell lines,

5) chick embryo fibroblast cells and cell lines,

6) equine dermal cell lines (ED), though the BVDV titer is much reduced.

Products grown on cells that are not readily available to the CVB, such as ferret embryo cells, are also considered candidates for testing until such time as BVDV replication on these cells is determined.

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Appendix II
Agents that require neutralization during testing

Live vaccine viruses or live agents that replicate when grown in BT and that may potentially interfere with the detection of extraneous BVDV are neutralized with homologous antisera.

Agents requiring neutralization	Agents not requiring neutralization
bovine coronavirus	canine coronavirus
bovine parvovirus	canine parvovirus
bovine rotavirus	equine viral arteritis virus
bovine respiratory syncytial virus	feline calici virus
bluetongue virus	feline infectious peritonitis virus
*Chlamydia psittaci**	feline panleukopenia virus
canary pox virus	feline rhinotracheitis virus
canine distemper virus	porcine parvovirus
canine parainfluenza virus	West Nile virus
equine herpesvirus 1 and 4	
equine influenza virus	
infectious bovine rhinotracheitis virus	
infectious canine hepatitis virus	
measles virus	
parainfluenza virus 3	
pseudorabies virus	

* When present in viral vaccines, *Chlamydia psittaci* may cause unwanted effects on BT. These effects can be neutralized by the use of tetracycline.