United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 320

Supplemental Assay Method for Titration of *Chlamydophila felis* (formerly Feline *Chlamydia psittaci*) in Embryonated Chicken Eggs

Date: **November 20, 2014**

Number: SAM 320.04

Supersedes: SAM 320.03, June 30, 2011

Standard Requirement: 9 CFR 113.71

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1. Introduction

This Supplemental Assay Method (SAM) is a titration method for assaying live chicken-embryo-adapted *Chlamydophila felis* (formerly feline *Chlamydia psittaci*) vaccines for potency. The method uses embryonated chicken eggs as the indicator host system. This assay is used to demonstrate the presence of live chlamydia and to determine the titer of a vaccine serial by the chicken embryo death pattern 10 days after inoculation.

2. Materials

2.1 Equipment/instrumentation

2.1.1 Egg incubator, 36°± 2°C, humidified

2.1.2 Cabinet, laboratory biosafety level-2

2.1.3 Water bath, 36°± 2°C

2.1.4 Vortex mixer

2.1.5 Micropipettor, 1000-µL, and tips

2.2 Reagents/supplies

2.2.1 *C. felis* Positive Control, Cello strain

2.2.2 Embryonated chicken eggs, 6- to 7-day, specific pathogen free, in accordance with Title 9, *Code of Federal Regulations* (9 CFR)

2.2.3 7.5% Sodium Bicarbonate

1. 7.5 g sodium bicarbonate

2. Q.S. to 100 mL with deionized water (DI**).**

3. Sterilize by autoclaving at 121°± 2°C**,** 15 psi for 30 ± 10 minutes.

4. Store at 2°- 7°C.

2.2.4 1% Phenol Red

1. 1.0 g phenol red

2. Q.S. to 100 mL with DI.

3. Store at 2°- 7°C**.**

2.2.5 Sucrose phosphate buffer of Bovarnick (Chlamydophila Diluent)

1. 74.6 g sucrose ($C_{12}H_{22}O_{11}$)

2. 0.42 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)

3. 1.25 g potassium phosphate, dibasic, anhydrous (K_2HPO_4)

4. 0.92 g monosodium glutamate ($C_5H_8NO_4Na$)

5. 1.0 mL gentamicin

6. 1 mL 1% Phenol Red

7. Dissolve ingredients in DI to make 1 L.

8. Filter through a 0.22-µm filter.

9. Adjust pH to 7.0-7.2 with 7.5% Sodium Bicarbonate.

10. Store at 2°- 7°C**.**

2.2.6 Ethanol Solution, 70%

1. 718 mL ethanol, 95%

2. 282 mL DI

3. Store at room temperature.

2.2.7 Tincture of Iodine, 2%

1. 2 g iodine

2. 100 mL 70% Ethanol Solution

3. Store at room temperature.

2.2.8 Egg-candling light

2.2.9 Etcher/engraver, electric, or egg drill

2.2.10 Needles, 22-gauge x 1 1/2-inch and 18-gauge x 1 1/2-inch

2.2.11 Syringe, 1-mL tuberculin

2.2.12 Duco cement

2.2.13 Polystyrene tubes, 17 x 100-mm

2.2.14 Pipette-aid

2.2.15 Pipettes

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel shall have experience in the propagation and maintenance of chlamydophila agents and inoculation techniques of embryonated chicken eggs. Personnel shall be proficient with chlamydophila titration techniques using embryonated chicken eggs.

3.2 Preparation of equipment/instrumentation

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

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of eggs

1. On the day of test initiation, using an egg-candling light, check each egg for viability, proper embryo growth, and integrity of the egg shell. Appropriately dispose of eggs which do not meet these criteria.

2. Label each egg by writing a number with a pencil just below the base line of the air sac. Numbered eggs are placed in double layer cardboard flats.

3. Disinfect the air sac end by swabbing with 2% Tincture of Iodine. Allow to air dry.

4. Drill a small hole through the disinfected egg shell using an electric etcher/engraver or egg drill.

5. Retain 5 uninoculated viable embryonated chicken eggs to serve as controls and to monitor nonspecific embryo death.

3.3.2 Preparation of *C. felis* Positive Control

1. On the day of test initiation, rapidly thaw a vial of Chlamydophila Positive Control in a 36°± 2°C water bath.

2. With a 10-mL pipette, dispense 4.5 mL Chlamydophila Diluent into each of an appropriate number of 17 x 100-mm polystyrene tubes. Label each tube, bracketing the expected Chlamydophila Positive Control endpoint titer specified in the Center for Veterinary Biologics (CVB) Reagent Data Sheet (e.g., label 7 tubes from 10^{-1} through 10^{-7}, respectively).

3. With a micropipettor, transfer 500 µL of the Chlamydophila Positive Control to the tube labeled 10^{-1}; mix by vortexing.

4. Using a new pipette tip, transfer 500 µL from the 10^{-1}-labeled tube (**Section 3.3.2[3]**) to the 10^{-2} tube; mix by vortexing.

5. Repeat **Section 3.3.2(4)** for each of the subsequent dilutions until the tenfold dilution series is completed.

3.4 Preparation of the sample

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 inoculation, using a sterile 1.0-mL syringe and an 18-gauge x 1 1/2-inch needle, rehydrate a vial of the Test Vaccine with the provided diluent by transferring 1.0 mL for a 1-mL-dose vaccine, 0.5 mL for a 1/2-mL-dose vaccine, etc., into the vial containing the lyophilized Test Vaccine; mix by vortexing. Incubate for 15 ± 5 minutes at room temperature.

3.4.2 Multifraction Test Vaccines containing feline rhinotracheitis virus (FRV), feline calicivirus (FCV), or feline panleukopenia virus (FPV) are prepared the same as chlamydophila single-fraction Test Vaccines. (FRV, FCV, and FPV do not replicate in embryonated chicken eggs.)

3.4.3 Using the same method described for diluting the Chlamydophila Positive Control in **Section 3.3.2(2)** through **Section 3.3.2(5)**, prepare an appropriate number of tenfold serial dilutions of the reconstituted Test Vaccine to bracket its expected endpoint titer specified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production.

4. Performance of the Test

4.1 Mix the dilution tubes by vortexing, and aspirate 0.5 mL of the highest (most dilute) tenfold dilution of the Test Vaccine with a 1-mL syringe and 22-gauge x 1 1/2-inch needle. Holding the syringe vertically, insert the needle to a depth of 1.25 ± 0.25 inch. Inoculate 100 µL of the Test Vaccine into the yolk sac of each of 5 eggs/dilution.

4.2 With the same syringe, aspirate an equal volume of the vortexed next lower Test Vaccine dilution and inoculate the yolk sacs of 5 more eggs.

4.3 Inoculate the remaining dilutions. Separate syringes are not necessary between dilutions in a dilution series when dispensing from the most dilute to the most concentrated within that series but are required between series.

4.4 In a similar manner, inoculate 5 eggs/dilution of the Chlamydophila Positive Control (dilutions 10^{-7} through 10^{-4}, **Section 3.3.2(2)**).

4.5 Seal the inoculation hole of all eggs with Duco cement and allow to air dry 5 ± 2 minutes.

4.6 Return all inoculated embryonated chicken eggs to the egg incubator. (At this time, inoculated eggs can be incubated in egg flats for the required 11 ± 1 days time interval. Use double egg flats to prevent accidental egg rupture during candling/handling.)

4.7 On the second day postinoculation, candle the inoculated and uninoculated control eggs. Identify, record day of death, and appropriately discard dead embryonated chicken eggs. Deaths occurring prior to 3 days postinoculation are regarded as nonspecific deaths.

4.8 Candle the embryonated chicken eggs daily through 11 ± 1 days postinoculation. Identify, record day of death, and appropriately discard dead embryonated chicken eggs. Deaths occurring on 3 days postinoculation through 11 ± 1 days postinoculation are regarded as specific and are used to calculate the titer of the Test Vaccine.

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4.9 Calculate the *C. felis* endpoints of the Test Vaccine and the Chlamydophila Positive Control using the Spearman-Kärber method as commonly modified. The titers are expressed as $\log_{10}$ 50% chicken embryo lethal dose ($CELD_{50}$) per mL.

Example:

10^{-4} dilution of Test Vaccine = 5/5 embryos dead
10^{-5} dilution of Test Vaccine = 5/5 embryos dead
10^{-6} dilution of Test Vaccine = 2/5 embryos dead
10^{-7} dilution of Test Vaccine = 0/5 embryos dead

Test Vaccine titer = (X - d/2 + [d • s] where:

X = reciprocal $\log_{10}$ of highest dilution with all chicken embryos dead due to chlamydophila infection (5)
d = $\log_{10}$ of tenfold dilution factor (1)
s = sum of proportions of infected dead chick embryos for all dilutions tested starting at X:

$$\frac{(5+2+0)}{5} = \frac{7}{5} = 1.4$$

Test Vaccine titer = (5 - 1/2 + [1 • 1.4]) = 5.9

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

A. Divide the Test Vaccine Dose by the Inoculation Dose where:

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this Chlamydophila Test Vaccine, the recommended dose is 1 mL).
Inoculation Dose = amount of diluted Test Vaccine with which each chicken embryo is inoculated (for this Chlamydophila Test Vaccine, the inoculation dose is 0.1 mL)

$$\frac{\text{1 mL dose}}{\text{0.1 mL inoculum}} = 10$$

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

Log of 10 = 1

Test Vaccine titer 5.9 + 1 = 6.9

Therefore, the Test Vaccine Titer = $10^{6.9}$ $CELD_{50}$/mL.

5. Interpretation of the Test Results

5.1 For a valid assay

5.1.1 All 5 of the uninoculated embryonated chicken eggs must be viable at the end of the test.

5.1.2 At least 4 eggs/dilution must be viable 3 days postinoculation for each Test Vaccine and the Chlamydophila Positive Control.

5.1.3 The calculated $CELD_{50}$ titer of the Chlamydophila Positive Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established by a minimum of 10 previous titrations.

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

5.2 In a valid test, if the titer of the Test Vaccine is equal to or greater than the titer specified in an APHIS filed Outline of Production, the Test Vaccine is considered **SATISFACTORY**.

5.3 In a valid test, if the titer of the Test Vaccine is lower than the titer specified in an APHIS filed Outline of Production, the Test Vaccine shall be retested in accordance with 9 CFR 113.8(b).

6. Report of Test Results

Report results as $CELD_{50}$ per dose.

7. References

7.1 Title 9, Code of Federal Regulations, part 113.71, U.S. Government Printing Office, Washington, DC.

7.2 Cottral, GE (Ed.). *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, New York, 1978, pg. 731.

7.3 Finney, DJ. *Statistical method in biological assay*, 3rd edition. Griffin, London, 1978, pg. 508.

7.4 Richmond JY, McKinney RW (Eds.). *Biosafety in microbiological and biomedical laboratories*. U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington, DC, 1993, pg. 177.

7.5 Storz, J. *Chlamydia and chlamydia-induced diseases*. Charles C. Thomas Publishing Company, Springfield, Illinois, 1971, pg. 358.

8. Summary of Revisions

Version .04

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

Version .03

- The Contact information has been updated.

Version .02

This document was rewritten 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:

- The document number has been changed from MVSAM0320 to SAM 320.
- The Contact has been changed from Ione Stoll to Victor Becerra and Sandra Conrad.
- **1.2:** "Key Words" has been deleted.
- **4.9:** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- The refrigeration temperatures have been changed from 4°± 2°C to 2°- 7°C.
- The term "chicken embryo infective dose (CEID)" has been changed to "chicken embryo lethal dose (CELD)" throughout the document.
- Based on the present nomenclature "Feline *Chlamydia psittaci*" has been changed to *Chlamydophila felis*"; therefore, the Genus "Chlamydia" has been changed to "Chlamydophila" and the species "felis" has been introduced.

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- The term "Reference" has been changed to "Positive Control" throughout the document.

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

- "Test Serial" has been changed to "Test Vaccine" through the document.

- The footnotes have been deleted with any pertinent references now noted next to the individual items.