United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Detection of Pathogens by the Inoculation Test in Embryonated Chicken Eggs

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1. Introduction

This testing protocol (PRO) describes a procedure for detection of extraneous pathogens, per title 9, *Code of Federal Regulations* (9 CFR), section 113.37. The master seed or vaccine is reconstituted and inoculated into embryonated chicken eggs. Deaths and/or abnormalities attributed to the inoculum occurring will constitute disposition of the product as unsatisfactory.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Laminar flow biological safety cabinet (BSC) (NuAire Inc., Labgard)

2.1.2 Humidified, rotating egg incubator set at 98°± 2°F (Midwest Incubators, Model 252)

2.1.3 Vortex mixer

2.1.4 Digital timer

2.1.5 Pipetting apparatus, (Whisper 600 model Pipet-Aid or Matrix Cell Mate II)

2.1.6 Speed King candling light

2.1.7 Curved forceps

2.1.8 Scissors, sharp pointed

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 Specific-Pathogen-Free (SPF) embryonated chicken eggs, 9- to 11-days-old

2.2.2 Cotton balls/cotton swabs

2.2.3 Syringe, 1-cc tuberculin single use

2.2.4 Syringe, 10-cc single use

2.2.5 Surgical gloves, latex or nitrile

2.2.6 Needle, 25-gauge x 5/8-inch

2.2.7 Needle, 26-gauge x 3/8-inch

2.2.8 Glass test tubes, 13 x 100-mm, with Morton closures

2.2.9 Duco cement

2.2.10 Serological pipettes, 10-mL and 25-mL

2.2.11 Permanent marker

2.2.12 Water, distilled or deionized, or water of equivalent purity

2.2.13 Firm's diluent if supplied

2.2.14 Specific antiserum for test

2.2.15 Clean glassware of appropriate size and type

2.2.16 Tryptose Phosphate Broth (TPB), National Centers for Animal (NCAH) Media #10426

2.2.17 Penicillin/Streptomycin (Pen/Strep), NCAH Media #30044

2.2.18 Iodine 2% in alcohol, NCAH Media #30013

iodine	2 g
ethyl alcohol (70%)	100 mL

2.2.19 TPB with Pen/Strep

TPB (NCAH Media #10426) 100 mL
Pen/Strep (NCAH Media #30044) 2 mL

This results in a final concentration of 500 IU penicillin and 1500 IU streptomycin per 1.0 mL TPB.

2.2.20 70% alcohol

200 Proof	70 mL
Deionized water	30 mL

3. Preparation for the Test

3.1 Personnel qualifications/training

The personnel performing this procedure must have experience or training in this protocol. Personnel must also have a knowledge base of the lesions for the various avian viruses that will be tested. This includes knowledge of aseptic biological laboratory techniques, preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures, policies, and guidelines; with training in the operation of the necessary laboratory equipment to conduct this test.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer's instructions and monitor in compliance with current corresponding standard operating procedures (SOPs). Maintain aseptic conditions in a laminar flow biological safely cabinet.

3.3 Preparation of eggs

3.3.1 For this test, a minimum of 20, 9- to 11-day-old SPF embryonated eggs are recommended for the test. Also needed are 10 eggs of the same age and lot for the control. The eggs are prepared in accordance with the current version of **VIRSOP0023**, *Chicken Egg Preparation and Inoculation: Dropped Chorioallantoic Membrane (CAM) Method*.

3.3.2 Label the eggs with a permanent marker, putting the identification mark and dilution clearly on the side of each egg. These markings must correspond with the ones on the current version of **VIRTWS0004**, *Virus Titration in Chick Embryos, Test Record*. Label 20 eggs per antiserum and master seed/vaccine dilution. Label 10 eggs as controls.

3.4 Preparation of master seed/vaccine

3.4.1 For liquid master seed/vaccine, thaw vial at room temperature. Thaw appropriate antiserum at the same time and in the same way. For 20 eggs at 0.2 mL per egg, you will need total of 4.0 mL recoverable inoculum. This is a combination of master seed/vaccine and antiserum.

3.4.2 For lyophilized master seed/vaccine, rehydrate with sterile distilled water at the rate of 30 mL per 1000 doses if a vaccine. For master seed, see firm's recommended rehydration rate. See **Section 3.4.1** for antiserum directions.

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Vaccine Dilution Table

Number of doses per vial	*Reconstituted Volume of Vaccine*	*Additional Dilution to reach 33.3 doses/mL*
500	15mL	None
1000	30 mL	None
2,000	60 mL	None
2,500	10 mL	1 mL vaccine plus 6.5 mL sterile distilled water
5,000	20 mL	1 mL vaccine plus 6.5 mL sterile distilled water
10,000	20 mL	1 mL vaccine plus 14 mL sterile distilled water
15,000	30 mL	1 mL vaccine plus 14 mL sterile distilled water
20,000	40 mL	1 mL vaccine plus 14 mL sterile distilled water
25,000	50 mL	1 mL vaccine plus 14 mL sterile distilled water

3.5 Preparation of the sample

3.5.1 Obtain and store samples for testing as described in the current version of **VIRWI2040**, *Sample Tracking in the CVB Virology Section*.

3.5.2 For master seed, one volume of prepared product is mixed with one equal volume of appropriate antiserum. Mix well on the vortex mixer for approximately 15 seconds. Place in a 37°C incubator for 60 minutes.

3.5.3 With the vaccine, one volume of prepared product is mixed with up to nine volumes of appropriate antiserum. Mix well on the vortex mixer for approximately 15 seconds. Place in a 37°C incubator for 60 minutes.

3.5.4 Prepare inoculum for control eggs with 100 mL TPB and 2.0 mL pen/strep.

4. Performance of the Test

4.1 Egg inoculation

4.1.1 Retrieve the labeled eggs from the incubator. Using the Modified Gorham Technique listed here, inoculate first the normal control eggs using the TPB with antibiotics.

Modified Gorham Technique:

All inoculations will be done in the BSC. Place the Speed King mounted egg candling light into the BSC. Darken room and turn off BSC light. Only light that will be needed is the egg candling light. Using a 25-gauge 5/8 inch needle on a 1 cc syringe, draw up the appropriate amount of inoculum mixture. Inoculate into the egg at approximately a 45° angle and dispense 0.1 mL on the CAM. Continue into the AAF to dispense 0.1 mL in the fluid by rolling the egg slightly after you

have put the 0.1 mL on CAM to catch the AAF. Seal inoculation site with Duco cement.

Another way to inoculate would be to drop the CAM and inoculate the 0.1 mL as previously stated but to then go to the hole at the top of the egg for the AAF injection of the 0.1 mL and reseal the hole when you seal the CAM hole.

4.1.2 Vortex the inoculum and then inoculate the master seed/vaccine as noted in **Section 4.1.1** and then seal the eggs with Duco cement. Be careful not let the glue run over the egg and stick the shell to the flat.

4.2 Egg incubation

4.2.1 Place the eggs, which are positioned horizontally on flats in the humidified egg incubator baskets, in a 98°± 2°F humidified egg incubator. At 24 hours post-inoculation (PI), candle the eggs and record and discard all dead eggs. The 24 hour deaths are considered to be due to trauma and are not used in the results. Eighteen (18) viable embryos shall survive 24 hours PI for a valid test.

4.2.2 Candle eggs daily, excluding weekends. Record all deaths occurring after 24 hours and place them into refrigeration of 4°C for later opening and inspection.

4.3 Egg CAM and Embryo examination

4.3.1 On the 7th day PI, candle final time and place all surviving embryos in to refrigeration for a minimum of two (2) hours. Then open all surviving eggs. Open and examine all normal controls first.

4.3.2 For CAM examination: Remove and briefly examine the yolk and fluids, then discard. Look into the egg for lesions on the inside and underside of the CAM. Slowly and gently peel the CAM from the inside of the shell with a forceps, while at the same time looking for firm discolored or whitish plaques. These can be on the top, sides, and between the major blood vessels of the CAM. These plaques may be: 1) large or small masses; 2) discolored protruding masses; or 3) small measles-like dots on the CAM. Record CAMs exhibiting any of these lesions as positive and comment as to the appearance of the lesions. Be aware of the possible needle trauma on the top of the CAM by the inoculation site, and do not record these as positive lesions.

For Embryo examination: Remove and briefly examine the yolk and fluids, then discard. Examine the surviving embryos for typical lesions indicating the presence of virus. These lesions will depend upon the strain of virus being tested. The following is a listing of the most common lesions that could be found for the following:

Newcastle and some Bronchitis strains:
Check for stunting, curling, and clubbed down.

Bronchitis-additional strains: Open the embryos and check for kidney urates and bile stasis (dark green liver).
Record all embryos exhibiting these lesions as positive.

4.3.3 When necessary, embryo subcultures shall be made to determine the cause of death. Subcultures of CAM may be passaged again to further determine nature of lesion.

5. Interpretation of the Test Results

5.1 Controls

All controls will show no lesions for a valid test.

5.2 Inoculated embryos results

If death and/or abnormality attributable to the inoculum occur, the master seed/vaccine is unsatisfactory provided that if there is a master seed/vaccine virus override, the test may be repeated, using a higher tittered antiserum if available.

5.3 Evaluation of the test results

5.3.1 Title 9, *Code of Federal Regulations*, section 113.37(d) defines the criteria for a satisfactory (SAT) or unsatisfactory (UNSAT) serial.

6. Report of Test Results

Results are reported to agent in charge. Report the results as described in the current version of **VIRSOP0027**, *Testing Roles, Responsibilities, and Procedures for Reporting Test Results in the Virology Section*.

7. References

7.1 Title 9, *Code of Federal Regulations*, subchapter E. section 113.37, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .03

- **3.4.2:** Dilution table updated.

Version .02

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