Center for Veterinary Biologics and
National Veterinary Services Laboratories
Reagent Production Protocol

Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

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Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test

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1. Introduction

1.1 Background

Avian influenza (AI) agar gel immunodiffusion (AGID) antiserum is used as a positive control serum with AI AGID antigen to enhance the detection of group specific antibodies to type A influenza virus in serum. The method for antiserum preparation is based on procedures outlined by J. E. Pearson and D. A. Senne in Proceedings of the Third International Symposium on Avian Influenza. (Reference 7.1).

1.2 Key words

Avian influenza, AI, antiserum, agar-gel immunodiffusion, AGID

2. Materials

2.1 Equipment/instrumentation

2.1.1 Refrigerated (4 C) low speed centrifuge (Beckman model J-6B or equivalent)

2.1.2 Refrigerated (4 C) ultracentrifuge (Beckman model L8-80 or equivalent)

2.1.3 Fixed angle ultracentrifuge rotor (Beckman model Ti-60 or equivalent)

2.1.4 Refrigerator (4 C)

2.1.5 Incubator (37 C)

2.1.6 Automatic dispensing pump or Cornwall syringe capable of dispensing 10-ml quantities

2.1.7 Ultra-low freezer (-70 C)

2.1.8 Freezer (-20 C)

2.1.9 Lyophilizer

2.1.10 Autoclave

2.1.11 Biological safety cabinet (class II)
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2.1.12 Common laboratory apparatus, supplies and glassware--top loading balance (capable of measuring 0.01 g), magnetic stir plate, magnetic stir bar, vortex mixer, beakers, Erlenmeyer flasks, graduated cylinders, pipettes, 50-ml conical centrifuge tubes, 15 × 75-mm snap cap tubes, 30- and 60-cc disposable syringes, 3- and 5-cc syringes, 25-gauge × 5/8-inch and 18-gauge × 1½-inch needles, 12-gauge × 2½-inch stainless steel needles, disposable latex gloves, 8-10-mm thick glass rod, 25 × 89-mm thick-walled polycarbonate ultracentrifuge tubes, 10-ml glass serum bottles, and 20-mm slotted stoppers and aluminum seals.

Note: All glassware and disposable labware should be sterile unless otherwise stated.

2.2 Reagents/supplies

Note: All chemicals should be reagent grade unless specified.

2.2.1 Phosphate buffered saline (PBS), 0.01 M, pH 7.2 (National Veterinary Services Laboratories [NVSL] media number 30054, see appendix 8.1)

2.2.2 10T antibiotic medium (NVSL media number 10411, see appendix 8.2)

2.2.3 Sodium N-lauroyl sarcosine -- C\textsubscript{15}H\textsubscript{28}NO\textsubscript{3}Na

2.2.4 Glycine -- C\textsubscript{2}H\textsubscript{5}NO\textsubscript{2}

2.2.5 Sodium Azide -- NaN\textsubscript{3}

2.2.6 Ketamine hydrochloride (100 mg/ml)

2.2.7 Iodine-based disinfectant

2.2.8 Water -- distilled or deionized water or water of equivalent purity. Heat sterilized.

2.3 Biological materials

2.3.1 Specific pathogen free (SPF) embryonating chicken eggs, 10- to 11-day-old.

2.3.2 SPF mature white Leghorn chickens
3. Preparation for production

3.1 Personnel qualifications/training

Personnel must be familiar with:

3.1.1 Preparation and proper handling of test reagents and biological materials.

3.1.2 Calibration, maintenance, and use of instruments listed in section 2.1.

3.1.3 Proper care and handling of laboratory animals as outlined in the NVSL Animal Users Manual.

3.2 Preparation of equipment/instrumentation

Equipment is calibrated and certified according to respective NVSL Standard Operating Procedures (SOPs).

3.3 Preparation of reagents/control procedures

Note: All reagents can be stored at 4 C for up to 3 mo unless otherwise indicated.

3.3.1 10% Sodium N-lauroylsarcosine (Sarkosyl Reagent)

Dispense 70 ml water into a 250-ml beaker and place on a magnetic stir plate. Add, slowly, a little at a time, 10 g of Sodium N-lauroylsarcosine and mix with a magnetic stir bar until dissolved. Adjust pH to 7.50–8.00 with 10% H₂SO₄ or 10% NaOH. Add water QS to 100 ml.

3.3.2 Glycine-Sodium N-lauroylsarcosine (Glycine Sarkosyl Buffer)

Add 9.25 g glycine to 200 ml water in an Erlenmeyer flask. Mix until dissolved and adjust pH to 9.00 ± 0.05. If pH is below 8.95, adjust by adding a few drops of 10 N NaOH. Add 25 ml 10% Sarkosyl Reagent to the pH-adjusted glycine solution. Add water QS to 250 ml and mix thoroughly.
3.4 Propagation/Preparation of virus

Critical control point: Parts 3.4.1 and 3.4.2 should be performed in a biological safety cabinet.

3.4.1 Preparation of the type A influenza ribonucleoprotein (RNP) for chicken inoculation.

3.4.1.1 Propagate virus in SPF embryonating chicken eggs as outlined in current version of AVRPP0100, “Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test”, section 3.4.

3.4.1.2 Remove amnionic allantoic fluid (AAF) from freezer and thaw at room temperature (25 C).

3.4.1.3 Centrifuge AAF at 1500 × g (2500 rpm) for 20 min at 4 C to clarify. Pour supernatant through a funnel containing 2 layers of sterile gauze into an Erlenmeyer flask(s).

3.4.1.4 Evaluate the AAF (supernatant) for viral hemagglutinating (HA) activity (see current version of AVPRO0805). If no HA activity is present, then the AAF is not suitable for RNP production.

3.4.1.5 If HA activity is satisfactory, pipette supernatant into 8 thick-walled ultracentrifuge tubes (20 ml per tube). Place tubes in a fixed angle rotor (Ti-60) and ultracentrifuge at 251,720 × g (50,000 rpm) for 90 min to pellet virus.

3.4.1.6 Decant supernatant into a container with an iodine-based disinfectant. Gently rinse pellet with approximately 5 ml PBS then decant PBS into the container with the disinfectant.

3.4.1.7 Add 0.1 ml glycine-sarkosyl buffer to each tube and break up pellet with a glass rod. Incubate at room temperature (25 C) for 60 min.
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3.4.1.8 Add 1.0 ml PBS to each tube, pool RNP and freeze at -70 C in a closed container until needed.

3.4.1.9 Disinfect thick-walled ultracentrifuge tubes with an iodine-based or other suitable disinfectant. Scrub out the tubes with a soft-bristled test tube brush and rinse tubes thoroughly with distilled water and dry. Autoclave sterilize. Tubes can be reused several times but should be discarded if any cracks or signs of wear are noted.

3.4.2 Preparation of live virus chicken inoculum.

3.4.2.1 Select a type A influenza virus that grows to a high titer in embryonating chicken eggs and has a hemagglutinin (H) and neuraminidase (N) surface antigen different from the virus used to produce the AAF in section 3.4.1, e.g., A/Eq1-Bel (H7N1) subtype.

3.4.2.2 Follow the same procedure for virus propagation and collection of AAF as in step 3.4.1.1. Store AAF at -70 C until ready to use.

4. Production of the product

4.1 Remove RNP inoculum from the freezer, thawing only what is needed for the number of birds being inoculated.

4.2 Inoculate mature white Leghorn chickens via the brachial vein (main wing vein) with 0.5 ml of RNP solution.

Note: All intravenous injections are made using a 3- or 5-cc syringe with a 25-gauge × 5/8-inch needle. Latex gloves should be worn for protection.

4.3 Twenty-seven to 29 days after the first injection, thaw at room temperature (25 C) the AI virus prepared in part 3.4.2 and centrifuge at 1500 × g (2500 rpm) for 20 min to clarify. Save the supernatant and refrigerate at 4 C until ready to use. Inoculate each chicken with 5.0 ml of live virus inoculum via the brachial vein.
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Note: Virus inoculum may be loaded into syringes and stored at 4°C one day prior to bird inoculation.

4.4 Thirteen to 15 days after the second inoculation, thaw RNP and prepare a water-in-oil emulsion of equal parts of the antigen in Freund's complete adjuvant (FCA). Inject 0.8 ml RNP-adjuvant mixture per chicken into 3 or 4 sites subcutaneously at the base of the neck. Inoculations are performed with a 5-cc syringe and 18-gauge × 1½-inch needle.

Note: Follow NVSL guidelines for proper use of FCA in animals.

4.5 Exsanguinate birds 13 to 15 days after the last inoculation, as follows:

4.5.1 Inject each chicken intramuscularly with 7 to 10 mg/lb Ketamine hydrochloride.

4.5.2 When birds appear sedated, exsanguinate by cardiac puncture through the thoracic inlet using a 12-gauge × 2½-inch stainless steel needle attached to a 60-cc syringe.

4.5.3 Fill syringe no more than 2/3 full as sufficient air space is needed in the syringe along the length of the clot for good separation of the serum from the clot.

4.5.4 It may be necessary to use more than 1 syringe to fully exsanguinate each bird. After filling the first syringe 2/3 full, quickly detach the syringe from the needle without removing needle from the thoracic inlet and attach a new 60- or 30-cc syringe. Use care not to move the needle during the exchange of syringes. Continue to exchange syringes until the bird is fully exsanguinated.

4.5.5 Label all syringes from each bird with the same identification (I.D.) number. Make sure the cap is placed back on the tip of each syringe.
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4.6 Position the syringes horizontally with the capped end slightly raised to allow an air space to form along the entire length of the syringe barrel and incubate at room temperature (25 C) for 18 to 24 hr. Serum separation may be enhanced by incubating syringes at 37 C for ≤ 2 hr before incubation at room temperature.

Critical control point: Serum from each bird should be kept separate and tested individually before pooling.

4.7 Remove plunger from syringe barrel and decant serum into a 50-ml centrifuge tube. Serum from each bird should be pooled and the centrifuge tube labeled with the corresponding I.D. number. Syringes and 50-ml tubes can be refrigerated overnight at 4 C so that additional serum can be allowed to separate from the clots. Any additional serum can be decanted into the same 50-ml centrifuge tube as before. Centrifuge tubes at 1200 × g (2000 rpm) for 15 min.

4.8 Carefully pipette or decant serum off red blood cell (RBC) pellet into another labeled 50-ml centrifuge tube. Refrigerate tubes of serum at 4 C. Discard the tube with the RBC pellet. An average yield of serum from an adult leghorn chicken is 15 to 20 ml.

5. Quality control testing

5.1 Evaluation of sera

5.1.1 Test an aliquot of serum from each bird by the AI AGID test (current version of protocol AVPRO0100). Test undiluted serum as well as dilutions of 1/2, 1/4, 1/6, 1/8, and 1/10. Prepare serum dilutions with PBS. Sera exhibiting strong precipitin lines at the 1/10 dilution are further diluted and tested by AI AGID to determine the optimum dilution for use. Discard sera which exhibit lines of nonidentity (i.e., non-specific or double lines) at all dilutions. Save all sera exhibiting clear, sharp precipitin lines, including those which may have non-specific lines at lower dilutions but not at the higher dilutions.
5.1.2 Pool undiluted sera according to optimum dilution range, e.g., pool sera with sharpest precipitin lines at lower dilutions and make a separate pool of the higher titered sera. Centrifuge each serum pool at 1500 x g (2500 rpm) for 20 min and save supernatant. Each pool can be used as a separate lot of AI AGID antiserum or pooled with other lots of serum of similar titers.

Note: At this point, undiluted serum pools can be frozen at -20 C and used later or proceed to the next step.

5.1.3 Thaw antiserum pool(s), if frozen, and evaluate small aliquots of each antiserum pool against several lots of AI AGID antigen (current version of protocol AVRPP0100) by the following method:

5.1.3.1 Make dilutions of antiserum in a range which is appropriate for each serum pool made in step 5.1.2.

5.1.3.2 Make dilutions of antigen to test against the antiserum dilutions (see current version of protocol AVRPP0100 step 5.2).

5.1.3.3 Place AI negative control serum in alternate wells of the test patterns. A control pattern consisting of a known matched set of antigen and antiserum with AI negative control serum in alternate wells should also be included on each plate as a reference.

5.1.3.4 The optimal dilution(s) of antigen and antiserum should produce a single, sharp precipitin line that forms midway between the antigen and antiserum wells. Complete precipitin lines should extend to but do not bend into the negative serum wells.

5.1.3.5 Usually, more than 1 pattern, i.e. antigen and antiserum dilution appears to be optimal. A final evaluation of the optimal dilutions by AI AGID should include a weak positive control serum in at least 1 well of each pattern. This will aid in determining the best dilution of antigen and antiserum to use for a matched set.
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5.2 Sensitivity testing

5.2.1 Testing by Viral Reagents Unit

Evaluate optimally diluted antiserum and matching antigen against a panel of a minimum of 4 each of weak positive, negative, and strong positive reference serums. Compare results to one or more previous lots of antiserum and antigen. Results obtained with panel serums must concur with previous test results. Reevaluate the antiserum and matching antigen with the reference sera panel after final bottling and lyophilization.

5.2.2 Testing by Avian Viruses Section

Send the matching antiserum and antigen (final lyophilized product) to the Avian Viruses Section of the Diagnostic Virology Laboratory for evaluation by the AI AGID test with a panel of reference sera.

6. Post preparatory steps

6.1 Bottling

6.1.1 Centrifuge the bulk antiserum pool (thaw first if frozen) at 1500 × g (2500 rpm) for 20 min to clarify.

6.1.2 Pipette or pour the antiserum off the pellet into a measuring cylinder then into a large Erlenmeyer screw-cap flask. Discard the pellet.

6.1.3 Dilute the antiserum with sterile PBS (if necessary) to obtain the optimal dilution for use as determined in section 5.1.3.

6.1.4 Add sodium azide to the diluted antiserum to achieve a final concentration of 0.1% (w/v). Thoroughly mix on a stir plate with a magnetic stir bar at 4 C for a minimum of 30 min.

6.1.5 Dispense 6 ml aliquots of antiserum into sterile 10-ml glass serum vials with a sterile dispensing pump or Cornwall syringe. Cap each vial loosely with a sterile 13 × 20-mm slotted stopper.
6.1.6 Freeze the antiserum at -70 C for at least 24 hr, then lyophilize for ≥36 hr.

6.1.7 Vacuum seal vials of lyophilized antiserum before removing from lyophilizer and cap with 20-mm aluminum closures.

6.2 Assign expiration date

Not available.

6.3 Labeling instructions

Label bottles with a green label containing the following information: product name, serial number, amount of sterile water required to reconstitute the antiserum to the original volume, and laboratory name and location.

6.4 Place product on inventory

6.4.1 Reevaluate as in step 5.2.1 before adding to the inventory.

Note: This step is not necessary if the product has been evaluated within 6 mo prior to placing on inventory.

6.4.2 A Reagent Data Sheet (see current version of NVSLSOP0008) should be created for each new lot of product.

6.4.3 A minimum of 3 vials of each lot or serial should be placed into the repository before placing product on inventory.

6.5 Product storage

Store lyophilized antigen at 4 C.
6.6 Directions for use

Use antiserum as specified in current version of protocol AVPRO0100.

7. References


7.2 Version .02, was a revision superseding the June 3, 1998, February 24, 1997, version. Section 6.4.2 was added and there were some minor changes in wording.

7.3 Version .03, September 14, 1999, was a revision superseding the June 3, 1998 version. Section 1 was updated to include format changes (key words). Changes and/or more details were added to steps 3.4.1.3, 3.4.1.5, 3.4.1.6, and 3.4.1.9. An additional step 3.4.1.4 was added to section 3.4.1 and the amount of PBS added in step 3.4.1.8 was changed from 2 ml to 1 ml. A range of days for injections or bleeding was added to steps 4.3 through 4.5. Procedural changes were added to steps 4.3, 4.6, 4.7 and 5.1.1. Section 5.1.3 was changed and divided into steps. An additional step 5.1.3.5 was added to section 5.1.3. Additions/changes were made to steps 6.1.4, 6.1.6, 6.3, and 6.4.1. Step 6.4.3 was added. There were some minor wording changes made to add clarity.

7.4 Version .04, May 10, 2001, was a revision superseding the September 14, 1999, version. Changes or additions were made to steps 4.4 and 5.1.2. There were some minor wording changes.

8. Appendices

8.1 Phosphate buffered saline:

Sodium phosphate dibasic, 11.9 gm
Sodium phosphate monobasic, 2.2 gm
Sodium chloride, 85.0 gm
Distilled water, QS to 10 L.

Adjust final pH to 7.2. Autoclave on slow exhaust.
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8.2 10T antibiotic medium

8.2.1 Basal (make in advance and store in desired quantities in refrigerator)

Trizma base, 1.21 gm
Tryptose broth, 26 gm
Distilled water, 1,000 ml

Autoclave 20 min on slow exhaust.

8.2.2 Antibiotics for 10T

Penicillin (1,586 U/mg), 6.3 gm
Streptomycin (747 U/mg), 2.68 gm

Place 15 ml of sterile Dulbecco’s PBS into a sterile 250-ml cylinder. Add penicillin and streptomycin and stir briefly to dissolve. Aseptically add:

13.0 ml kanamycin sulfate (50 mg/ml)
20.0 ml gentocin (50 mg/ml)
0.04 ml mycostatin (5,000,000) --to make new stock, rehydrate with 10 ml sterile PBS

Adjust the pH of the antibiotic solution to 6.6 with 1 N NaOH. QS to 50 ml in a measuring cylinder with sterile PBS and add to 950 ml basal. Aseptically dispense in desired quantities in sterile 12 x 75-mm snap-cap tubes. Freeze at -20 C.
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9. Quick reference

_____ Prepare buffers and reagents
_____ Prepare RNP inoculum
_____ Prepare live virus inoculum
_____ Inoculate chickens with RNP
_____ Inoculate chickens with live virus
_____ Inoculate chickens with RNP-adjuvant
_____ Bleed out chickens
_____ Evaluate antiserum from individual birds by AI AGID
_____ Sensitivity tests
_____ Make optimal dilution of antiserum
_____ Add preservative
_____ Bottle antiserum and freeze
_____ Lyophilize antiserum (optional)
_____ Label antiserum
_____ Store antiserum
_____ File worksheet and testing results
_____ Place antiserum in inventory