Avian Influenza Agar Gel Immunodiffusion Test to Detect Serum Antibodies to Type A Influenza Viruses

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1. Introduction

1.1 Background

The avian influenza (AI) agar gel immunodiffusion (AGID) test is used to detect circulating antibodies to Type A influenza group-specific antigens, namely the ribonucleoprotein (RNP) and matrix (M) proteins. Therefore, this test will detect antibodies to all influenza A virus subtypes. The AGID test can also be used as a group-specific test to identify isolates as Type A influenza viruses. The method used is similar to that described by Beard1.

The basis for the AGID test is the concurrent migration of antigen and antibodies toward each other through an agar gel matrix. When the antigen and specific antibodies come in contact, they combine to form a precipitate which is trapped in the gel matrix and produces a visible line. The precipitin line forms where the concentration of antigen and antibodies is optimum. An extreme variation in the concentration of antigen or antibodies will alter the location of the line or cause it to be dissolved. Electrolyte concentration, buffer, pH and temperature also affect precipitate formation.

1.2 Key Words

avian influenza, AI, agar gel, AGID

2. Materials

2.1 Equipment/instrumentation

2.1.1 Refrigerator (4 C)
2.1.2 Freezer (-20 C)
2.1.3 Incubator or closed plastic container for room temperature (25 C) incubations.
2.1.4 Autoclave
2.1.5 Hot plate (optional)
2.1.6 Vacuum pump
2.1.7 Microscope illuminator
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2.1.8 Template cutter, 7-well pattern—a center well surrounded by 6 evenly spaced wells. Wells are 5.3 mm in diameter and 2.4 mm apart.

2.1.9 Top loading balance (capable of measuring 0.1 gm)

2.1.10 Micropipettor

2.2 Reagents/supplies

Note: All chemicals should be reagent grade unless specified.

2.2.1 Sodium phosphate monobasic (NaH$_2$PO$_4$) and dibasic (Na$_2$HPO$_4$)

2.2.2 Agarose (Type II Medium grade) agar (Sigma Chemical Co. Cat. number A-6877)

2.2.3 Sodium Chloride (NaCl)

2.2.4 Avian influenza AGID antigen (current version of AVRPP0100) and antiserum (current version of AVRPP0101)

2.2.5 Strong positive, weak positive and negative reference sera (optional)

2.2.6 Water—distilled or deionized water or water of equivalent purity. Heat sterilized.

2.2.7 Common laboratory supplies and glassware—Erlenmeyer flasks, graduated cylinders, pipettes, 100 x 15-mm and/or 60 x 15-mm disposable plastic petri plates, flexible silicone or rubber tubing, side-arm flask (500 ml or larger), and a 12- to 14-gauge blunt-ended cannula.

Note: All glassware and disposable labware should be sterile unless otherwise stated.
3. **Preparation for the test**

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.

3.2 **Preparation of equipment/instrumentation**

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

3.3 **Preparation of reagents/control procedures**

3.3.1 Phosphate buffered saline (PBS), 0.01 M, pH 7.2 (NVSL media number 30054, see appendix 8.1)

3.3.2 Preparation of AI AGID agar:

3.3.2.1 Weigh 9.0 gm of Agarose (see 2.2.2) and 80 gm of NaCl and add to 1 liter of PBS (0.01 M, pH 7.2) in a 2 liter Erlenmeyer flask.

Note: Larger or smaller volumes of agar can be prepared by multiplying or dividing each ingredient by the same factor. The size of flask used should be at least twice the volume of the contents so that when heated, the contents will not boil over.

3.3.2.2 Dissolve the mixture by bringing to a boil on a hot plate using a magnetic stir bar to mix the contents in the flask while heating.
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Autoclave the mixture for 10 min and mix the contents by swirling after removing from the autoclave to ensure a homogeneous mixture of ingredients.

3.3.2.3 After boiling, allow the agar to cool at room temperature (25 C) for 10 to 15 min before dispensing into petri plate(s).

3.3.2.4 Agar can be stored in the flask at 4 C for several months and melted and dispensed into plate(s) as needed.

OR

The liquid agar solution can be kept in a 45 C waterbath for several weeks and used as needed.

Note: Do not use agar if mold or precipitate is observed.

3.3.3 AI AGID antigen is prepared according to the current version of protocol AVRPP0100.

3.3.4 AI AGID control antiserum is prepared according to the current version of protocol AVRPP0101.

4. Performance of the test

4.1 Detection of serum antibodies

4.1.1 Dispense 15 to 17 ml of melted agar into a 100 x 15-mm petri plate or 5 to 6 ml agar into a 60 x 15-mm petri plate using a 25-ml pipette. The agar thickness should be approximately 2.8 mm.

4.1.2 Allow plates to cool in a relatively dust-free environment with the lids off to permit escape of water vapor. The lids should be left off for at least 15 min, but not longer than 30 min, as electrolyte concentration of the agar may change due to evaporation and adversely affect formation of precipitin lines.
Note: Plates should be used the same day they are poured.

4.1.3 Fill out an AGID test worksheet in ink with sample identification, reagent lot numbers, test date, initials of the person(s) performing and reading the test, as well as any other pertinent information.

4.1.4 Using a template, cut the agar after it has hardened. Up to 7 template patterns can be cut in a 100 x 15-mm plate and 2 patterns can be cut in a 60 x 15-mm plate.

4.1.5 The agar plugs are removed by aspiration with a 12- to 14-gauge cannula connected to a side arm flask with a piece of silicone or rubber tubing which is connected to a vacuum pump with tubing. Adjust the vacuum so that the agar surrounding the wells is not disturbed when removing the plugs.

4.1.6 Place approximately 50 µl of each unknown sera in the appropriate well (the well should be filled as near level as possible without overflowing), using a micropipette with an attached pipette tip. A clean tip must be used for each new serum sample tested. Place approximately 50 µl AI AGID positive control antiserum (current version of protocol AVRPP0101) in each of three alternate peripheral wells. Place approximately 50 µl of AI AGID antigen (current version of protocol AVRPP0100) in the center well (see Figure 1). This arrangement provides a positive control line on each side of the test serum, thus facilitating accurate determination of lines of identity.

Note: A pattern should be included with positive, weak positive and negative reference serum in the test sera wells to aid in the interpretation of results.

4.1.7 Cover each plate after filling all wells and allow plate(s) to set for a few minutes before moving. This will reduce the possibility of spillage.

4.1.8 Incubate the plate(s) for 24 hr at room temperature (25 C) in a closed chamber to prevent evaporation. Humidity can be provided, if necessary,
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by placing a damp paper towel at the bottom of the incubation chamber.

Note: Temperature changes during migration may lead to artifacts.

4.2 Alternate procedure for identifying isolates as type A influenza viruses

4.2.1 Dispense agar and cut plates as in steps 4.1.1 to 4.1.5.

4.2.2 Place approximately 50 µl of AI AGID positive control antiserum (current version of protocol AVRPP0100) in the center well using a micropipette.

4.2.3 Dispense AI AGID antigen (current version of protocol AVRPP0100) in alternate wells around the center well. Test antigen consisting of amnionic-allantoic fluid or a crude chorio-allantoic membrane suspension is placed in the remaining alternate peripheral wells.

4.2.4 Plates are covered and incubated as in steps 4.1.7 and 4.1.8.

5. Interpretation of test results

5.1 Serum antibody detection

5.1.1 Remove the lid and read plate(s) over an intense narrow beam of light against a dark background. A microscope illuminator works well and allows for varying intensities of light and positions.

5.1.2 The type of reaction will vary with the concentration of antibody in the sample being tested. The positive control serum line is the basis for reading the test, and if the line is not distinct, the test is not valid and must be repeated. The following types of reactions are observed:

5.1.2.1 Negative reaction--the control lines continue into the test sample well without bending or with a slight bend away
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from the antigen well and toward the positive control serum well (see Figure 1).

5.1.2.2 Positive reaction--control lines join with and form a continuous line (line of identity) with the line between the test serum and antigen. The location of the line will depend on the concentration of antibodies in the unknown sample. Weakly positive samples may not produce a complete line between the antigen and test serum but may only cause the tip or end of the control line to bend inward toward the test well (see Figure 1).

![Figure 1. Immunodiffusion test pattern with AI antigen in the center well; AI positive control serum in wells A, C, and E; negative test serum in well B; positive test serum in well D; and weak positive test serum in well F.](image)

5.1.2.3 Non-specific lines--these lines occasionally are observed between the antigen and test serum well. The control lines will pass through the non-specific line and continue on into the test serum well. The non-specific line does not form a continuous line (line of identity) with the positive control lines.
Figure 2. Immunodiffusion test pattern with examples of nonspecific line formation (wells B and F). These reactions are not specific for AI and should be disregarded.

5.2 Antigen detection (alternate procedure)

5.2.1 Plates are read as in step 5.1. The unknown virus is identified as type A influenza virus if a line of identity forms with the positive control antigen.

6. Report of test results

6.1 Record test results, in ink, on the AGID worksheet using the following notations: “+” = positive reaction, “−” = negative reaction, “I” = inconclusive results, and “NSL” designates a nonspecific line. Positive results may also be recorded in degrees to denote strength of the reaction, ie., “+4” = strong positive, “+3” = positive, “+2” = weak positive, and “+1” = very weak positive.

6.2 Record test results from AGID worksheet to summary worksheet.

OR

If AI AGID results of all samples are negative, and no additional test is performed, results are recorded on original submission sheet.
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Note: If new reagents are being evaluated, then record results on the appropriate reagent production worksheets, then go to step 6.6.

6.3 Enter the number of serums tested in the specimens block of program 1 (log new cases) in the Avian Isolation Menu of Reflections (HP3000) so serology summaries can be electronically generated.

6.4 Enter status of the case and summary of results in program 7 (update case status) on the Avian Isolation Menu.

6.5 Give case report (APHIS form 10-4 or equivalent) and summary worksheet to Head of Avian Viruses Section for reporting.

6.6 File worksheet(s) and copies of summary sheet(s).

7. References


7.2 Version .02, April 13, 1998, was a revision superseding the February 28, 1997 version. Some wording changes were made.

7.3 Version .03, June 15, 1999, was a revision superseding the April 13, 1998 version. Additions were made in part 5.1.2 to include figures 1 and 2.

7.4 Version .04, May 10, 2001, was a revision superseding the June 15, 1999 version. Some minor wording changes were made.

7.5 Version .05, April 1, 2003, was a revision superseding the May 10, 2001 version. Additions were made in part 4.1.3, part 6.2, and part 6.3. Changes were made in part 4.1.6 and 4.2.2.
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 10L

Adjust final pH to 7.2. Autoclave on slow exhaust.

9. Quick reference

_____Prepare buffers and reagents
_____Prepare AI AGID agar
_____Pour AGID plates
_____Fill out worksheet
_____Cut and remove agar plugs from agar gel plates
_____Fill plates with reagents and samples
_____Read plates after 24 hr incubation
_____Record test results
_____Enter test results into the computer (Avian Isolation Menu number 1 and number 7)
_____Give results to Section Head for reporting
_____File worksheet(s) and testing results