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Agar Gel Immunodiffusion Test to Detect Antibodies to Type A Influenza Virus

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Notes:

Agar Gel Immunodiffusion Test to Detect Antibodies to Type A Influenza Virus

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1 Purpose/Scope

The agar gel immunodiffusion (AGID) test detects circulating antibodies to type A influenza group-specific antigens, namely the ribonucleoprotein (RNP) and matrix (M) proteins, therefore this assay will detect antibodies to any influenza A subtype (6.4, 6.6). The AGID can detect IgM antibodies to RNP antigens within 5-7 days after infection in *gallinaceous* poultry. Sera from these species may test AGID positive for up to 3 months after infection. However, AGID is not recommended for the detection of influenza A virus (IAV) antibodies in ducks and other wild birds due to the variation in immune response and inconsistent antibody production to RNP (6.6, 6.7, 6.8, 6.9) The method described in this protocol is similar to that described by Beard (6.4).

The AGID is set up using a pattern of six wells around a center well in agar. The basis for the AGID test is the simultaneous 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. The visual precipitin lines can be detected within 24 hours after setting up the tests; however, a weak positive reaction may take up to 48 hours to develop. Several factors such as electrolyte concentration, buffer, pH, temperature, and also variation in the concentration of antigen or antibodies could affect precipitate formation, its location, or even cause it to dissolve (6.4, 6.10).

AGID is compatible with samples from serum, plasma and egg yolk (6.3), which have been shown to be adequate for the detection of IAV antibodies (7). Concentration of antibodies in plasma may be lower than those found in serum and positive reactions may not be able to be confirmed by subtyping. Ideally specimens should be tested within a week of collection and may be stored in the refrigerator. Samples stored long term should be frozen to at least -20°C, and repeated freeze-thaw cycles should be avoided (6.10).

While AGID is typically used for the detection of antibodies to IAV, it can also be used as a type-specific test for viral isolates; for example, confirmation of IAV infection in allantoic fluid from embryonated chicken eggs. In this case, the agar pattern would be set up in the reverse order with the reference antiserum in the center well and the reference RNP antigen in alternating peripheral wells (6.5, 6.6).

2 Definitions

Not Applicable (N/A)

3 Special Precautions

N/A

4 Equipment and Materials Required

- **Equipment**
  - Refrigerator 4°C (± 5°C recommended)
  - Freezer -20°C (± 5°C recommended)
  - Incubator or closed plastic container for room temperature 25°C (± 5°C recommended) incubations.
  - Water bath (optional) 56°C (± 5°C recommended)
  - Autoclave
  - Hot plate (optional)
  - Vacuum pump
  - Microscope illuminator or other light source
  - 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. Before use inspect template for damage that would affect the shape of the well (record on worksheet). The template cutter (5.3 mm diameter) can be purchased from Veterinary Diagnostic Technology INC, (Wheat Ridge, CO) Catalog # T-02.
  - Top loading balance (capable of measuring 0.1 gm)
  - Micropipettor that is calibrated to dispense 50-100µl

Note: Temperature ranges listed for equipment are assumed when listed as a single temperature in the document.

- **Reagents/Supplies**
  - Sodium phosphate monobasic (NaH2PO4) and dibasic (Na2HPO4)
  - Agarose (Type II Medium Electroendosmosis (EEO 0.16-0.19) agar (Sigma Chemical Co., St. Louis, MO, Cat. number A6877).
  - Sodium Chloride (NaCl)
  - Avian influenza AGID antigen 300 ADV (current version of SOP-AV-0048) and antiserum 305 ADV (current version of SOP-AV-0046). The 300 ADV and 305 ADV reagents are quality control (QC) tested as matched reagents, for this reason the same lot of 300 ADV and 305 ADV must be used to assure quality precipitin lines.
  - Strong positive (902 ADV), weak positive (903 ADV), and negative (905 ADV) reference sera.

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

- 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. All chemicals should be reagent grade unless specified.

5 Procedure

Per 2018 NPIP ruling, a current version of the SOP will be maintained at Avian_AGID_SOP.pdf (usda.gov).

5.1 Personnel Qualifications/Training

Personnel must be familiar with:

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

5.1.2 Calibration, maintenance, and use of instruments listed in section 4.1.

5.1.3 Must complete and pass a proficiency test each calendar year. Proficiency tests are produced by the National Veterinary Services Laboratories (NVSL), Diagnostic Virology Laboratory (DVL) Proficiency Test & Reagents Section (PTR).

5.2 Preparation of Equipment/Instrumentation

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

5.3 Preparation of Reagents/Control Procedures

5.3.1 Phosphate buffered saline (PBS), 0.01 M, pH 7.2 (±0.1) (NVSL media number 30054, see appendix 8.1)

5.3.2 Preparation of AGID agar:

5.3.2.1 Weigh 9.0 gm (± 0.2 g) of Agarose (see 4.2.2.) and 80 gm (± 0.2 g) 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.

5.3.2.2 Weigh out reagents, add the salt to the PBS, and be sure the salt is completely dissolved in the PBS before the addition of the agarose.

5.3.2.3 Dissolve the agarose in the PBS by bringing it to a boil on a hot plate using a magnetic stir bar to ensure a homogeneous mixture of ingredients.

OR

Dissolve the agarose in the PBS by autoclave for 20 minutes and mix the contents by swirling after removing from the autoclave to ensure a homogeneous mixture of ingredients.

OR

Dissolve the agarose in the PBS by heating in microwave; mix the contents by swirling after removing from the microwave to ensure a homogeneous mixture of ingredients. The time varies between volumes of agar and microwaves. The agar should be microwaved long enough to generate a rolling boil.

Note: Be Cautious – Agar has the potential to flash boil.

5.3.2.4 After boiling, allow the agar to cool at room temperature for 5-15 minutes, depending upon volume of agar, before dispensing into petri plate(s) or into a flask for longer storage. It is important to monitor the temperature of the agar before dispensing into plate. Agar that is too hot will warp the petri plate and agar that is too cool will not seal tightly to the petri plate.

5.3.2.5 The liquid agar solution can be kept in a 56°C water bath for 5 days and used as needed. If the agar sits for more than 5 days the salt concentration may changes and results in unreliable readings.

OR

Agar can be stored in the flask at 4°C for several months and melted and dispensed into plate(s) as needed. If re-liquidified after storage, it should be autoclaved for 7-10 min.

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

Note: The AGID antigen and antiserum are prepared by the DVL Proficiency Test & Reagent Section according to the current version of protocols SOP-AV-0048, Preparation of Antigen for the Avian Influenza Agar Gel Immunodiffusion Test, and SOP-AV-0046, Preparation of Antiserum for the Avian Influenza Agar Gel Immunodiffusion Test. Lyophilized reagents can be stored at 4°C. Following reconstitution, reagents can be stored at 4°C or frozen at -20°C for long-term storage.

5.4 Performance of the Test

5.4.1 Detection of antibodies in serum, plasma or extracted egg yolk

5.4.1.1 Dispense 16 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. The agar thickness should be approximately 2.8 mm.

5.4.1.2 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 minutes, but not longer than 30 minutes, 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 prepared.

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

5.4.1.4 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.

5.4.1.5 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. Alternative methods may be used.

5.4.1.6 Place approximately 55-65 µl of each unknown sample in alternating peripheral wells, (3 samples per pattern) using a micropipette. The volume added to each well should be adjusted so wells are filled as near level as possible without under filling or

overflowing. A clean tip must be used for each new sample tested.

5.4.1.7 Vortex all reagents before placing approximately 55-65 µl AGID positive control antiserum (NVSL 305-XX) in each of three alternating peripheral wells. Place approximately 55-65 µl of AGID antigen (NVSL 300- XX) in the center well (see Figure 1). The same tip may be used to load antigen or antiserum into all wells. This arrangement provides a positive control line on each side of the test serum, thus facilitating accurate determination of lines of identity.

Note: For each group of specimens tested, a positive, weak positive and negative reference serum should be included to aid in interpreting results and to ensure that test conditions are satisfactory.

5.4.1.8 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.

5.4.1.9 Incubate the plate(s) for approximately 24 hours (refer to 5.5.1.4.4) at room temperature (25°C) in a closed chamber to prevent evaporation. Humidity should be provided, by placing a damp paper towel, or a dish of water at the bottom of the incubation chamber.

Note: Temperature changes during migration may lead to artifacts.

5.5 Interpretation of test results

5.5.1 Serum antibody detection

5.5.1.1 After 24 hours (from set-up) 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.

5.5.1.2 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. Refer to section 5.5 for troubleshooting suggestions if atypical reactions are observed. The following types of reactions are observed:

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

- 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 (wells D and F in Figure 1).

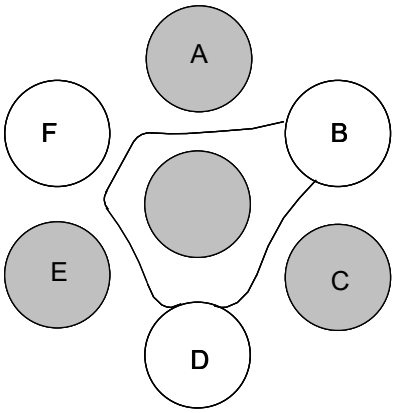

Figure 1. Immunodiffusion test pattern with reference antigen in the center well; positive reference control serum in wells A, C, and E; negative test serum in well B; positive test serum in well F; and weak positive test serum in well D.

- Non-specific lines--these lines are occasionally observed between the antigen and test serum well. The control lines will pass through the non-specific line and continue 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).

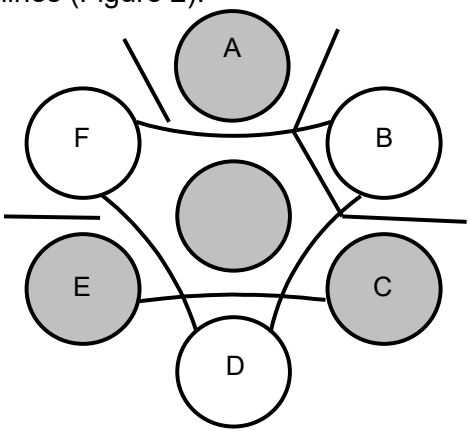

Figure 2. Immunodiffusion test pattern with examples of nonspecific line formation (wells B, D and F). These reactions are not specific for IAV and should be disregarded as long as the specific precipitin lines can be visualized. If the specific precipitin lines are too weak for interpretation, the specimen should be reported as a **"QCF"** (quality control failure).

5.6 Report of test results

5.6.1 Record test results, in ink, on the AGID worksheet using the following recommended notations: "+" = positive reaction, "-" = negative reaction, "QCF" = quality control failure, and "NSL" designates a nonspecific line. Positive results may also be recorded in degrees to denote strength of the reaction, i.e., "+4" = strong positive, "+3" = positive, "+2" = weak positive, and "+1" = very weak positive.

5.6.2 Transfer test results from AGID worksheet to summary worksheet. If AGID results of all samples are negative, and no additional test is performed, results may be recorded on original submission sheet.

5.6.3 Enter specimen(s) and test result in NVSL Laboratory Information Management System (LIMS) for approval by Head of Avian Viruses Section or senior microbiologist, or Veterinary Medical Officer (VMO).

5.6.4 Give case report (APHIS form 10-4 or equivalent) and summary worksheet to approving officer for reporting.

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

5.7 Troubleshooting

5.7.1 Problems that may occur are given below with recommended solutions where available. In any of these situations, if an interpretation of the test lines cannot be made the samples should be reported as QCF (quality control failure) and forwarded to NVSL for additional testing.

5.7.1.1 Wells have no or reduced volume of reagents after 24 hours:

5.7.1.1.1 Temperature of the agar was too cool for the agar to seal to the plate causing the liquid in the wells to leak out.

5.7.1.1.2 The humidity was too low in the incubation chamber and the liquid evaporated from the wells.

5.7.1.1.3 Uneven levels of agar in the plate changes the volume of reagent/sample dispensed into the wells.

5.7.1.2 Fuzzy control lines

5.7.1.2.1 Incorrect formulation of PBS, salt or agar.

5.7.1.2.2 Improper storage of agar prior to use changed the salt concentration. The change in the salt concentration results in unreliable readings.

5.7.1.2.3 Using mismatched antigen and antiserum.

5.7.1.2.4 Contamination of reagents – reagents should be checked before every use. If mold or precipitate is observed, do not use and discard the reagents.

5.7.1.2.5 Improper storage of reagents. If the reagents were not stored at 4°C either lyophilized or re-constituted, quality may be affected.

5.7.1.2.6 Reagents not mixed appropriately. Reagents, especially the antigen, should be vortexed thoroughly before adding to the plate. This ensures the reagents are a homogenate solution when pipetting.

5.7.1.3 Non-specific lines interfering with the lines of identity (line of precipitation)

5.7.1.3.1 Plates not incubated for adequate period of time. Incubate >24 hours, may help intensify the lines of identity or complete the precipitation of a weak positive.

5.7.1.3.2 Unable to determine if lines are specific or non-specific. Have multiple trained laboratory personnel read the plate.

5.7.1.3.3 Hemolysed or poor quality sample. Centrifuge the sample to remove any lysed red blood cells for 500xg for 10 minutes and then repeat the AGID. May also follow up with submitter to obtain a new sample.

5.7.1.3.4 Follow up with submitter to obtain a new sample.

5.7.1.3.5 Report as a No Test.

5.7.1.4 Lines not entering wells

5.7.1.4.1 Not allowing the plate to incubate for 24 hours before attempting to read.

5.7.1.4.2 Poor sample quality.

5.7.1.4.3 Under filling the wells. Wells should be filled as near level as possible without overflowing them.

5.7.1.4.4 Slow precipitating specimen. Some specimens will not have strong lines of precipitation at 24 hr. These specimens should be incubated for 24-48 hours or until the lines have migrated into the well.

5.7.1.5 Precipitin lines do not form a positive reference line or are distorted

5.7.1.5.1 Template wells may be distorted. Distortion of wells may be caused by gouge the agar around the wells during aspiration.

5.7.1.5.2 Improper distance between the side of the plate and between other templates.

5.7.1.6 Inconsistent reference sera controls

5.7.1.6.1 Overfilling wells. If wells are over filled positive controls can spill into samples and negative controls, giving false positives.

5.7.1.6.2 Disturbing the plates may cause controls and sample to become mixed causing inconsistent readings.

5.7.2 If problems still occur with any of the following reagents AGID antigen (300-), antiserum (305-), and reference sera, (strong positive 902-ADV, weak positive 903-ADV or negative 905-ADV) please contact the Diagnostic Virology Laboratory-Proficiency Test & Reagent Section, 515-337-7877.

6 Associated NVSL Quality Documents/References

- Beard, C. W. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bull. Wld. Hlth. Org. 42:779-785. 1970.
- Spackman, E., Suarez, D. L. and Senne, D. A. (2009) Avian Influenza Diagnostics and Surveillance Methods, in Avian Influenza (ed D. E. Swayne), Blackwell Publishing Ltd., Oxford, UK.
- Swayne, D. E. Suarez, D. L. and Senne, D. A. (2008) Avian Influenza, in A Laboratory Manual For The Isolation and Identification of Avian Pathogens (ed L. Durour-Zavala), OminiPress, Inc., Madison, WI
- Swayne, D. E., and Halvorson, D. A. (2008) Influenza, in Diseases of Poultry (ed Y. M. Saif), Blackwell Publishing Ltd., Oxford, UK.
- Avian Influenza in OIE *Terrestrial Manual* (2012), Version adopted by the World Assembly of Delegates of OIE in May 2012.

- Stallknecht, D. E., Nagy, E., Hunter, D. B. and Slemons, R. D. (2008) Avian Influenza, in Infectious Diseases of Wild Birds (eds N. J. Thomas, D. B. Hunter and C. T. Atkinson), Blackwell Publishing Professional, Ames, Iowa, USA.
- Thayer, S. G., and Beard C. W. (2008) Serologic Procedures, in A Laboratory Manual for the Isolation and Identification of Avian Pathogens (ed L. Durour-Zavala), OminiPress, Inc., Madison, WI.

7 Revision History

- NVSL-SOP-0045.03 supersedes SOP-AV-0045.02 due to document number process updates.
    - Statement was added in section 5: (Per 2018 NPIP ruling, a current version of the SOP will be maintained at Avian_AGID_SOP.pdf (usda.gov)).

- SOP-AV-0045.02 April 2017; Changes as followed
    - Expanded Purpose/Scope to give more detail on AGID as a diagnostic method in poultry. Also included information on compatible samples and storage conditions of samples.
    - 4.1 updated temperature ranges on equipment and throughout
    - 5.1.3 Updated Section name change
    - 5.4.1.1 Changed agar volume from 15 ml-17 ml to 16 ml-17 ml
    - 5.5.1.3.3 Added troubleshooting comment for under filling wells
    - 5.5.1.2.6 Added information about vortexing reagents
    - 5.5.1.3 Added to troubleshooting Non-specific lines interference with the lines of identity, (line of precipitation)
    - 6.0 Associated NVSL Quality Documents/Reference: Added more external reference and updated associated NVSL QA documents.

- SOP-AV-0045.01 supersedes AVPRO0100.07 due to uploading into new electronic document management system.

8 Appendices

8.1 Formulas

- **Phosphate Buffered Saline (**NVSL media number 30054)

    - 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.

8.2 Quick Reference

_____ Prepare buffers and reagents
_____Prepare 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
_____Give results to Section Head/Micro/VMO for reporting
_____File worksheet(s) and testing results

Signature Manifest

Document Number: NVSL-SOP-0045 **Revision:** 03

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NVSL-SOP-0045 03 Agar Gel Immunodiffusion Test to Detect Antibodies to Type A Influenza...

Step 3: Review for Quality Content

Name/Signature	Title	Date	Meaning/Reason
AMBER GUSTAFSON (AMGUSTAFSON)	Quality Mgr/Micro, DVL	20 Oct 2021, 08:48:37 **AM**	Approved

Step 3: Review for Doc Format

Name/Signature	Title	Date	Meaning/Reason
LINDA HOFER (LHOFER)	Quality Mgmt Assistant, DO QA	20 Oct 2021, 10:48:35 **AM**	Approved

Step 4: Supervisor Final Approval

Name/Signature	Title	Date	Meaning/Reason
KRISTINA LANTZ (KLANTZ)	VMO, DVL	08 Dec 2021, 11:26:58 **AM**	Approved

Step 5: Final Quality Check and Assign Training

Name/Signature	Title	Date	Meaning/Reason
AMBER GUSTAFSON (AMGUSTAFSON)	Quality Mgr/Micro, DVL	08 Dec 2021, 11:53:40 **AM**	Approved