

(5) Sera controls (well A of each test sera) must not have non-specific agglutination or hemolysis. If negative, report as "negative with non-specific agglutination or non-specific hemolysis" or "unable to evaluate due to non-specific agglutination or hemolysis" or treat the serum to remove the non-specific agglutination and repeat the test. (See paragraph (e)(2)(v) of this section.)

(v) *Treatment to remove non-specific agglutination.*

(A) *Purpose.* Treatment of serum to remove non-specific agglutination that is interfering with HI assays.

(B) *Specimen.* Serum.

(C) *Materials.* Homologous RBC's (chicken or turkey), 50 percent solution PBS, centrifuge, incubator, 4C (refrigerator).

(D) *Procedure.* (1) Prepare a 1:5 dilution of test serum by adding 50 µL of serum to 200 µL of PBS.

(2) Prepare a 50 percent solution of RBC's by adding equal volumes of packed RBC's to PBS. Mix well.

(3) Add 25 µL of 50 percent RBC solution to the serum dilutions.

(4) Vortex gently to mix.

(5) Incubate at 4 °C for 1 hour.

(6) Centrifuge to pellet the RBC's.

(7) Use the supernatant to perform the HI assay. Modify the dilution scheme in the assay to consider the initial 1:5 dilution prepared in the treatment. For the 1:5 dilution scheme, do not add PBS to row A. Add 50 µL of the 1:5 treated supernatant to row A. Serially dilute 25 µL from rows A through H. This prepares a serum dilution of 1:10 through 1:640 in rows B through H.

[49 FR 19803, May 10, 1984, as amended at 57 FR 57342, Dec. 4, 1992; 59 FR 12799, Mar. 18, 1994; 63 FR 3, Jan. 2, 1998]

§ 147.8 Procedures for preparing egg yolk samples for diagnostic tests.

The following testing provisions may be used for retaining the classification U.S. M. Gallisepticum Clean under § 145.23(c)(1)(ii)(C) and § 145.33(c)(1)(ii)(C), and for retaining the classification U.S. M. Synoviae Clean under § 145.23(e)(1)(ii)(b) and § 145.33(e)(1)(ii)(b) of this chapter.

(a) Under the supervision of an Authorized Agent or State Inspector, the eggs which are used in egg yolk testing must be selected from the premises

where the breeding flock is located, must include a representative sample of 30 eggs collected from a single day's production from the flock, must be identified as to flock of origin and pen, and must be delivered to an authorized laboratory for preparation for diagnostic testing.

(b) The authorized laboratory must identify each egg as to the breeding flock and pen from which it originated, and maintain this identity through each of the following:

(1) Crack the egg on the round end with a blunt instrument.

(2) Place the contents of the egg in an open dish (or a receptacle to expose the yolk) and prick the yolk with a needle.

(3) Using a 1 ml syringe without a needle, aspirate 0.5 ml of egg yolk from the opening in the yolk.

(4) Dispense the yolk material in a tube. Aspirate and dispense 0.5 ml of PBS (phosphate-buffered saline) into the same tube, and place in a rack.

(5) After all the eggs are sampled, place the rack of tubes on a vortex shaker for 30 seconds.

(6) Centrifuge the samples at 2500 RPM (1000 x g) for 30 minutes.

(7) Test the resultant supernatant for *M. gallisepticum* and *M. synoviae* by using test procedures specified for detecting IgG antibodies set forth for testing serum in § 147.7 (for these tests the resultant supernatant would be substituted for serum); except that a single 1:20 dilution hemagglutination inhibition (HI) test may be used as a screening test in accordance with the procedures set forth in § 147.7.

NOTE.— For evaluating the test results of any egg yolk test, it should be remembered that a 1:2 dilution of the yolk in saline was made of the original specimen.

[50 FR 19900, May 13, 1985; 63 FR 3, Jan. 2, 1998]

§ 147.9 Standard test procedures for avian influenza.

(a) The agar gel immunodiffusion (AGID) test should be considered the basic screening test for antibodies to Type A influenza viruses. The 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 viruses, regardless of subtype. 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 Beard.⁶ 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 that is trapped in the gel matrix and produces a visible line. The precipitin line forms where the concentration of antigen and antibodies is optimum. Differences in the relative concentration of the antigen or antibodies will shift the location of the line towards the well with the lowest concentration or result in the absence of a precipitin line. Electrolyte concentration, pH, temperature, and other variables also affect precipitate formation.

(1) *Materials needed.*

- (i) Refrigerator (4 °C).
- (ii) Freezer (-20 °C).
- (iii) Incubator or airtight container for room temperature (approximately 25 °C) incubations.
- (iv) Autoclave.
- (v) Hot plate/stirrer and magnetic stir bar (optional).
- (vi) Vacuum pump.
- (vii) Microscope illuminator or other appropriate light source for viewing results.
- (viii) Immunodiffusion template cutter, seven-well pattern (a center well surrounded by six evenly spaced wells). Wells are 5.3 mm in diameter and 2.4 mm apart.
- (ix) Top loading balance (capable of measuring 0.1 gm differences).
- (x) Pipetting device capable of delivering 50µl portions.
- (xi) Common laboratory supplies and glassware—Erlenmeyer flasks, graduated cylinders, pipettes, 100 × 15 mm or 60 × 15 mm petri dishes, flexible vacuum tubing, side-arm flask (500 mL or larger), and a 12- or 14-gauge blunt-ended cannula.

(2) *Reagents needed.*

(i) Phosphate buffered saline (PBS), 0.01M, pH 7.2 (NVSL media #30054 or equivalent).

(ii) Agarose (Type II Medium grade, Sigma Chemical Co. Cat.# A-6877 or equivalent).

(iii) Avian influenza AGID antigen and positive control antiserum approved by the Department and the Official State Agency.

(iv) Strong positive, weak positive, and negative control antisera approved by the Department and the Official State Agency (negative control antisera optional).

(3) *Preparing the avian influenza AGID agar.*

(i) Weigh 9 gm of agarose and 80 gm of NaCl and add to 1 liter of PBS (0.01 M, pH 7.2) in a 2 liter Erlenmeyer flask.

(ii) To mix the agar, either:

(A) Autoclave the mixture for 10 minutes and mix the contents by swirling after removing from the autoclave to ensure a homogeneous mixture of ingredients; or

(B) 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. After boiling, allow the agar to cool at room temperature (approximately 25 °C) for 10 to 15 minutes before dispensing into petri plates.

(iii) Agar can be dispensed into small quantities (daily working volumes) and stored in airtight containers at 4 °C for several weeks, and melted and dispensed into plates as needed.

NOTE: Do not use agar if microbial contamination or precipitate is observed.

(4) *Performing the AGID.* (i) *Detection of serum antibodies.*

(A) Dispense 15 to 17 mL of melted agar into a 100 × 15 mm petri plate or 5 to 6 mL agar into a 60 × 15 mm petri plate using a 25 mL pipette. The agar thickness should be approximately 2.8 mm.

(B) Allow plates to cool in a relatively dust-free environment with the lids off to permit the 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.

⁶Beard, C.W. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bull. Wld. Hlth. Org. 42:779-785. 1970.

NOTE: Plates should be used within 24 hours after they are poured.

(C) Record the sample identification, reagent lot numbers, test date, and identification of personnel performing and reading the test.

(D) Using the template, cut the agar after it has hardened. Up to seven template patterns can be cut in a 100×15 mm plate and two patterns can be cut in a 60×15 mm plate.

(E) Remove the agar plugs by aspiration with a 12- to 14-gauge cannula connected to a side arm flask with a piece of silicone or rubber tubing that 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.

(F) To prepare the wells, either:

(1) Place 50 µl of avian influenza AGID antigen in the center well using a micropipette with an attached pipette tip. Place 50 µl AI AGID positive control antiserum in each of two opposite wells, and add 50 µl per well of test sera in the four remaining wells. This arrangement provides a positive control line on one side of the test serum, thus providing for the development of lines of identity (see figure 1); or

(2) Place 50 µl AI AGID positive control antiserum in each of three alternate peripheral wells, and add 50 µl per well of test sera in the three remaining wells. This arrangement provides a positive control line on each side of the test serum, thus providing for the development of lines of identity on both sides of each test serum (see figure 2).

NOTE: A pattern can be included with positive, weak positive, and negative reference serum in the test sera wells to aid in the interpretation of results (see figure 3).

(G) Cover each plate after filling all wells and allow the plates to incubate for 24 hours at room temperature (ap-

proximately 25 °C) in a closed chamber to prevent evaporation. Humidity should be provided by placing a damp paper towel in the incubation chamber. Note: Temperature changes during migration may lead to artifacts.

(ii) *Interpretation of test results.*

(A) Remove the lid and examine reactions from above by placing the plate(s) over a black background, and illuminate the plate with a light source directed at an angle from below. A microscope illuminator works well and allows for varying intensities of light and positions.

(B) 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. If the line is not distinct, the test is not valid and must be repeated. The following types of reactions are observed (see figure 3):

(1) *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.

(2) *Positive reaction.* The 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 test serum. 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.

(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 with positive control lines.

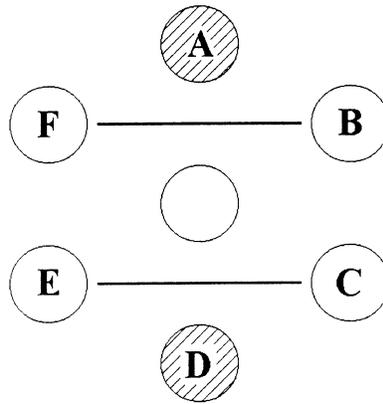


FIGURE 1.—*Immunodiffusion test that uses AI AGID antigen in the center well; AI-positive control serum in wells A and D; and AI-negative test serum in wells B, C, E, and F.*

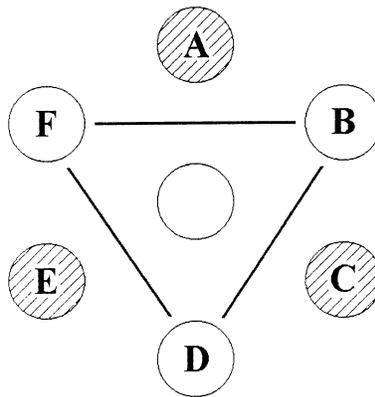


FIGURE 2.—*Immunodiffusion test that has AI AGID antigen in the center well; AI-positive control serum in wells A, C, and E; and AI-negative test serum in wells B, D, and F.*

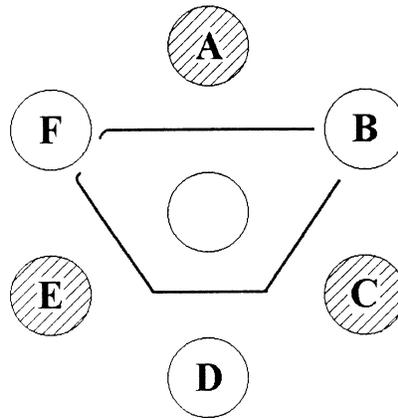


FIGURE 3.—Immunodiffusion test that has AI AGID antigen in the center well; AI-positive control serum in wells A, C, and E; AI-negative test serum in well B; AI-positive test serum in well D; and weak positive test serum in well F.

(b) The enzyme-linked immunosorbent assay (ELISA) may be used as a screening test for avian influenza. Use only federally licensed ELISA kits and follow the manufacturer's instructions. All ELISA-positive serum samples must be confirmed with the AGID test conducted in accordance with paragraph (a) of this section.

[65 FR 8019, Feb. 17, 2000]

Subpart B—Bacteriological Examination Procedure

§ 147.10 Laboratory procedure recommended for the bacteriological examination of egg-type breeding flocks with salmonella enteritidis positive environments.

Birds selected for bacteriological examination from egg-type breeding flocks positive for *Salmonella enteritidis* after environmental monitoring should be examined as described in §147.11(a) of this subpart, with the following exceptions and modifications allowed due

to the high number of birds required for examination:

(a) Except when visibly pathological tissues are present, direct culture, §147.11(a)(1) of this subpart, may be omitted; and

(b) Enrichment culture of organ (non-intestinal) tissues using a non-selective broth, §147.11(a)(2) of this subpart, may be omitted.

[59 FR 12801, Mar. 18, 1994]

§ 147.11 Laboratory procedure recommended for the bacteriological examination of salmonella.

(a) For egg- and meat-type chickens, waterfowl, exhibition poultry, and game birds. All reactors to the Pullorum-Typhoid tests, up to at least four birds, should be cultured in accordance with both *direct* (paragraph (a)(1)) and *selective enrichment* (paragraph (a)(2)) procedures described in this section. Careful aseptic technique should be used when collecting all tissue samples.

(1) Direct culture (refer to illustration 1). Grossly normal or diseased