

where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of *S. pullorum*.)

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**§ 147.4 The tube agglutination test for *S. typhimurium*.**

(a) The procedure for the collection and delivery of blood samples in the tube agglutination test for *S. typhimurium* is the same as that described in § 147.1(a).

(b) The "O" antigen should be prepared as follows:

(1) The antigen shall consist of a representative nonmotile strain of *S. typhimurium* which is of known antigenic composition and high agglutinability but is not sensitive to negative and nonspecific sera. Strain P 10 meets these requirements.

(2) The stock culture is maintained on 1 percent nutrient agar deeps, which have been incubated for 18-24 hours at 37° C. They are stored at room temperature.

(3) A satisfactory medium used for growing the organism is veal infusion agar (Difco). It is dispensed in 50 ml. amounts into 500 ml. medicine bottles, with screw caps, and sterilized at 15 pounds pressure for 20 minutes. The bottles are then laid flat upon an even surface until the medium has solidified.

(4) The inoculum used for preparation of "O" antigen is a nonmotile strain of *S. typhimurium*. The organism is grown in veal infusion broth (Difco) for 18-24 hours at 37° C.; then plated, for single colony isolation, on veal infusion agar plates. These plates are incubated for 18-24 hours at 37° C. After incubation, single colonies are picked and transferred to veal infusion agar slants, which are incubated for 18-24 hours at 37° C. After this, the cultures are tested for smoothness by using a 1:500 dilution of acriflavine.

(5) Smooth cultures are inoculated into flasks containing veal or beef infusion broth which is incubated for 18-24 hours at 37° C. The incubated broth sus-

pension of organisms is dispensed into the antigen bottles containing veal infusion agar. The suspension is distributed evenly over the agar surface by gently tilting the bottles from side to side. The inoculated bottles are then laid flat, agar side down, for 10-20 minutes. They are subsequently incubated, agar side upward, for 24-48 hours at 37° C. before harvesting.

(6) The harvesting of the organism consists of washing the growth from each antigen bottle with 0.5 percent phenolized physiological saline. The bacterial suspension from each bottle is filtered through sterile milk pad filters into a large sterile container or through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. To each 100 ml. of the bacterial suspension is added additional phenol to make the final concentration 0.5 percent. The concentrated antigen is tested for sterility at intervals after 24 hours. After sterility is proved, the stock antigen is standardized to determine the density according to the McFarland nephelometer scale.

(7) The diluted antigen to be used in routine testing is prepared from stock antigen, by diluting with 0.25 percent phenolized saline, and is standardized to a turbidity corresponding to 0.75-1.00 of the McFarland nephelometer scale.

(c) The maximum serum dilution employed for the "O" antigen tube test must not exceed 1:25. In all official reports on the blood test, the serum dilutions should be indicated. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37° C.

(d) The results shall be recorded as described in § 147.1(h).

**§ 147.5 The microagglutination test for *pullorum-typhoid*.**

Routinely, the microagglutination test is applied as a single-dilution test and only a single 18-24 hour reading is made.

(a) The procedure for the collection and delivery of blood samples in the

microagglutination test is the same as that described in §147.1(a). A method that has proven advantageous is to transfer the serum samples from the blood clot to a microplate as described in "Applied Microbiology," volume 24, No. 4, October 1972, pages 671-672. The dilutions are then performed according to paragraphs (d) or (e) of this section.

(b) Stained microtest antigen for pullorum-typhoid is supplied as concentrated stock suspension and must be approved by the Department.<sup>4</sup> Directions for diluting will be provided with the antigen. The stock as well as the diluted antigen prepared each day should be kept sealed in the dark at 5° to 10° C. when not in use.

(c) Available data indicate that a 1:20 dilution for the microagglutination test is most efficient for the detection of pullorum-typhoid agglutinins in both chickens and turkeys. In all official reports on the blood test, the serum dilutions shall be indicated.

(d) The recommended procedure for the 1:20 dilution in the microagglutination test is as follows:

(1) Add 100 microliters (0.10 cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) Using a microdiluter or a multimicrodiluter handle fitted with twelve 10 microliter microdiluters, transfer 10 microliters (0.01 cc.) of the serum sample from the collected specimen to the corresponding well of the microplate. This is accomplished by touching the surface of the serum sample with the microdiluter and then transferring and mixing with the diluent in the microplate well. The microdiluter is removed, blotted, touched to the surface of the distilled water wash, and again blotted. Other acceptable methods of serum delivery are described in "Applied Microbiology," volume 21, No. 3, March 1971, pages 394-399.

(3) Dilute the microtest antigens with 0.50 percent phenolized saline and add 100 microliters (0.1 cc.) to each microplate well.

<sup>4</sup>Information as to criteria and procedures for approval of concentrated stock suspension of stained microtest antigens may be obtained from the National Poultry Improvement Plan, Veterinary Services, APHIS, USDA, 1500 Klondike Road, Suite A-102, Conyer, GA 30207.

(4) Seal each plate with a plastic sealer or place unsealed in a tight incubation box as described in "Applied Microbiology," volume 23, No. 5, May 1972, pages 931-937. Incubate at 37°C. for 18-24 hours.

(5) Read the test results as described in paragraph (f) of this section.

(e) The recommended procedure for a microagglutination test titration is as follows:

(1) Add 50 microliters (0.05cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) To the wells representative of the lowest dilution in the titration, add an additional 50 microliters (0.05 cc.) of 0.85 percent physiological saline making a total of 100 microliters in these wells.

(3) Transfer each serum sample as described in §147.5(d)(2) of this section to the first well containing 100 microliters (0.10cc.) in the titration, which represents the lowest dilution.

(4) Make twofold serial dilutions of each serum by transferring 50 microliters (0.05cc.) of diluted serum from one well to the next using twelve 50 microliter microdiluters fitted in a multimicrodiluter handle. When transfers have been made to all of the wells of the desired series, the 50 microliters remaining in the microdiluters are removed by blotting, touching the microdiluters to the surface of the distilled water wash, and blotting again.

(5) Dilute the desired microtest antigen with 0.50 percent phenolized saline and add 50 microliters (0.05 cc.) to each microplate well.

(6) Seal each plate with a plastic sealer or place the unsealed microplates in a tight incubation box and incubate at 37° C. for 18-24 hours.

(7) Read the test results as described in paragraph (f) of this section.

(f) Read the test results with the aid of a reading mirror. Results are interpreted as follows:

(1) N, or - (negative) when the microplate well has a large, distinct button of stained cells; or

(2) P, or + (positive) when the microplate well reveals no antigen button; or

(3) S, or ? (suspicious) when the microplate well has a small button. Suspicious reactions may tend to be

more positive than negative [ $\pm$ ] or vice versa [ $\mp$ ] and can be so noted if desired.

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**§ 147.6 Procedure for determining the status of flocks reacting to tests for *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Mycoplasma meleagridis*.**

The macroagglutination tests for *Mycoplasma* antibodies, as described in "Standard Methods for Testing Avian Sera for the Presence of *Mycoplasma Gallisepticum* Antibodies" published by the Agricultural Research Service, USDA, March 1966, and the microagglutination tests, as reported in the Proceedings, Sixteenth Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, 1973, shall be the official tests. Procedures for isolation and identification of *Mycoplasma* may be found in Isolation and Identification of Avian Pathogens, published by the American Association of Avian Pathologists and §§ 147.15 and 147.16 of this part.

(a) When reactors are submitted to a laboratory as prescribed by the Official State Agency, the following criteria shall be used to determine if the flock is positive for *M. gallisepticum*, *M. synoviae*, or *M. meleagridis*:

(1) Active air sac lesions, sinusitis, synovitis, or other clinical signs of a respiratory disease;

(2) Recovery by culture of the *Mycoplasma* for which the flock was tested;

(3) Supplemental serological test.

(b) If all of these tests are negative, the flock shall be deemed to have had no reactors for the *Mycoplasma* for which the flock was tested. If the *Mycoplasma* for which the flock was tested is isolated bacteriologically or identified as infected by a polymerase chain reaction (PCR)-based procedure approved by the Department, the flock shall be considered infected. If any of the other tests described in paragraphs (a)(1) or (3) of this section is positive, the flock shall be considered suspicious, and additional culturing proce-

dures, and agglutination and hemagglutination inhibition (HI) tests shall be conducted according to the following sequence:

(1) If the tube agglutination or the serum plate test is negative, the flock qualifies.

(2) If the tube agglutination or the serum plate test is positive, the hemagglutination inhibition (HI) test and/or the Serum Plate Dilution (SPD) test shall be conducted. *Provided*, that for egg-type and meat-type chicken and waterfowl, exhibition poultry, and game bird flocks, if more than 50 percent of the samples are positive for either *Mycoplasma gallisepticum*, *M. synoviae*, or both, the HI and/or the SPD test shall be conducted on 10 percent of the positive samples or 25 positive samples, whichever is greater. The results of the HI and/or SPD tests must be followed by the action prescribed in paragraphs (b)(3), (b)(4), and (b)(5) of this section.

(3) If the tube agglutination or serum plate tests are positive and HI and/or the SPD tests are negative, the flock shall be retested in accordance with paragraph (b)(6) of this section.

(4) If HI titers of 1:40 or SPD titers of 1:5 are found, the flock shall be considered suspicious and shall be retested in accordance with paragraph (b)(6) of this section.

(5) If HI titers of 1:80, positive enzyme-labeled immunosorbent assay (ELISA) titers, or SPD titers of 1:10 or higher are found, in conjunction with any of the criteria described in paragraph (a)(1) of this section, the Official State Agency shall presume the flock to be infected. If the indicated titers are found, but none of the criteria described in paragraph (a)(1) of this section are evident, tracheal swabs from 30 randomly selected birds shall be taken promptly and cultured individually or a PCR-based procedure conducted on these specimens for *Mycoplasma*, and additional tests conducted in accordance with paragraph (b)(6) of this section before final determination of the flock status is made.

(6) Fourteen days after the previous bleeding date, all birds or a random sample comprised of 75 birds shall be