Serological Diagnosis of Pullorum Disease with the Microagglutination System

J. E. WILLIAMS AND A. D. WHITTEMORE
Southeast Poultry Research Laboratory, Veterinary Sciences Research Division, Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia 30601

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The application of a tetrazolium-stained Salmonella pullorum antigen in the microagglutination test is described and compared with the macroscopic tube agglutination test for detecting carriers of pullorum disease and fowl typhoid in chickens. Titers revealed by both testing procedures are similar; however, the microagglutination test is preferred because of the savings in time, space, and cost.

Since the introduction by Jones (4) in 1913 of the macroscopic tube agglutination test (macrotest) for pullorum disease, this procedure with some modifications has been routinely used by animal diagnostic laboratories all over the world as the standard serological test for the detection of carriers of Salmonella pullorum-S. gallinarum (P-T) infections in both chickens and turkeys. Progress made in the eradication of pullorum disease (P) and fowl typhoid (T) in all avian species attests to the efficacy of this procedure in detecting the infection in adult breeding flocks (6).

The unstained antigen used in the United States since 1931 for the macrotest for pullorum disease has been prepared from S. pullorum strains 17, 19, and 20 by standard methods (1, 7).

Under the official National Poultry and Turkey Improvement Plans (1), a maximum serum dilution of 1:50 is permitted in testing chickens and a maximum dilution of 1:25 in testing turkeys. The 1:25 is the most frequently applied diagnostic dilution in chickens.

The microagglutination system (microtest) has been adapted for a wide variety of serological procedures (2, 8) and has been cited for detecting agglutinins for several salmonella serotypes other than S. pullorum (8). We have reported on the use of the microagglutination procedure for the detection of experimentally stimulated agglutinins to S. pullorum (5). It was found in these studies, however, that an unstained antigen did not afford a readable microtest.

This report describes the preparation of a standard tetrazolium-stained S. pullorum antigen for application in the microtest for the detection of carriers of P-T. Procedures and advantages of applying the microtest by using stained pullorum antigen are described and evaluated, along with the standard macrotest, as a routine diagnostic test for the detection of chronic carriers of S. pullorum and S. gallinarum.

MATERIALS AND METHODS
Preparation of tetrazolium-stained S. pullorum antigen. S. pullorum strains 17, 19, and 20 (7) were plated on Veal Infusion Agar (Difco), and a smooth single colony selection was made for each strain and transferred into Veal Infusion Broth (Difco) which was incubated at 37 °C for 24 hr. The broth culture of each of the three strains was used to seed separate Erlenmeyer flasks, each containing Veal Infusion Broth. These were incubated for 24 hr at 37 °C, at which time each flask revealed dense growth.

A 1:100 sterile aqueous stock solution of neotetrazolium chloride (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared by dissolving 10 g in a liter of distilled water by using a hot-plate magnetic stirrer. This solution was sterilized by filtration through a Hormann filter with a D-7 Sterilfilo pad into sterile screw-capped Erlenmeyer flasks and stored in the dark at room temperature.

The stock neotetrazolium solution was added aseptically in the amount of 0.5% to the 24-hr broth cultures. The flasks were agitated and returned to the incubator for an additional 24 hr.

After the second 24-hr incubation period, liquefied phenol was added to each flask in an amount to provide a final concentration of 0.5%, and the flasks were returned for 2 hr to the incubator for inactivation.

The tetrazolium-stained broth suspension of each of the S. pullorum strains was filtered through a separate sterile milk filter pad and combined into a single large sterile dispensing bottle equipped with a bottom spout and sterile rubber tubing. The combined broth suspension was slowly fed through a continuous-flow Sorvall centrifuge operating at 37,000 × g for cell collection. The supernatant fluid was decanted, and the packed cells were suspended in 0.5% phenolized saline by vibrating on a Genie mixer with a few sterile glass beads. A dense suspension of deeply stained cells resulted.
Cells from all of the Sorvall tubes were collected into a thick-walled 250-ml Pyrex centrifuge bottle containing glass beads. The mouth of each bottle was sealed, and the bottles were placed on a shaking machine for 1 hr to resuspend the cells which were then stored at 5°C overnight. The following day the bottles were shaken for 5 min and the cell suspension was filtered through a sterile milk filter pad into a sterile flask. The milk filter pad was rinsed into the collection flask by using 0.5% phenolized saline.

The cell density of the stock antigen was adjusted to a value equivalent to a reading of 50X tube no. 1 of the McFarland scale by the following procedure. By using a 10-ml Hopkins vaccine tube with 0.01-ml gradations in its tip, 1 ml of an unstained S. pullorum tube agglutination antigen, having a known density of 50X McFarland tube no. 1, was added to 9 ml of distilled water. Duplicate tubes were prepared and centrifuged at 3,000 rev/min for 80 min, and a reading of 0.02 ml was obtained. By following the same procedure with the unknown tetrazolium-stained suspension, a reading reflecting its density was made. The total volume of the unknown tetrazolium-stained antigen suspension was determined, and its density was adjusted to 50X McFarland tube no. 1 by using the following formula: Hopkins tube reading × volume of unknown/0.02 = final total volume (to be arrived at by dilution with 0.5% phenolized saline or increased cell density through centrifugation).

The cell density of the antigen in earlier stages of preparation should be kept above a reading of 50X McFarland tube no. 1 since the antigen will only need to be diluted with 0.5% phenolized saline rather than subjected to further concentration.

After making the necessary adjustments by dilution or concentration and repeating the procedure outlined above to determine the cell density, the final antigen will have a density reading of 0.02 ml.

Preparation of unstained S. pullorum antigen. The unstained pullorum antigen for use in the macroscopic tube agglutination test was prepared by recommended standard procedures (1).

Sera. All negative sera were collected from adult White Rock hens maintained in strict isolation on the laboratory premises. Source flocks have been blood-tested yearly for P-T by the macrostest for the past 7 years with negative results. Also birds hatched and reared in Horsfall units were used as a source of negative sera.

Positive sera from chickens from which S. pullorum was subsequently isolated on culture were supplied by Charles F. Smyser from the P-T serum repository at the Department of Veterinary and Animal Sciences, Univ. of Massachusetts, Amherst.

Agglutinins were experimentally stimulated in adult chickens by subcutaneous inoculations of killed cell suspensions of S. pullorum emulsified in mineral oil. The first group of seven chickens received 0.5 ml; the second, 1.0 ml; and the third, 2.0 ml. Sera were collected from these birds weekly for 3 weeks starting after 1 week.

Macroscopic tube agglutination test. For the conventional 1:25 dilution, 0.04 ml of undiluted serum was deposited in each 13- by 100-mm disposable tube. To the serum was added 1 ml of unstained S. pullorum antigen diluted with 0.25% phenolized saline to a density equivalent to tube no. 1 of the McFarland scale. The pH of the diluted antigen was adjusted to 8.4 by the addition of dilute NaOH. The tests were incubated at 37°C and read after 18 to 24 hr.

Sera were titrated by preparing twofold serum-saline dilutions ranging from 1:12.5 to 1:1,600. To 0.5 ml of each serum dilution was added 0.5 ml of antigen suspension diluted with 0.5% phenolized saline to have a density of 2X tube no. 1 of the McFarland scale. This resulted in final serum-antigen dilutions ranging from 1:25 to 1:3,200. Titration tests were incubated at 37°C for 18 to 24 hr. Various degrees of agglutination were recorded, from + down to a trace of agglutination (±). Any reaction below a 3+ was interpreted as suspicious, whereas 3+ and 4+ were considered as positive. Negative sera revealed no agglutination.

Microagglutination test: microplates. Tests were conducted in rigid styrene microplates (Cook Engineering Co., Alexandria, Va.), with 96 rounded (U-shaped) wells per plate. Plates with V-shaped wells and those constructed of flexible vinyl plastic can be used but were not preferred in this work. Agglutination titers observed in testing positive sera were, in general, found to be slightly higher when tests were conducted in V-shaped plates. Unless otherwise indicated, all microtest equipment used in these studies was obtained from Cooke Engineering Co.

Titrations. In titrating sera with the microagglutination system, a 1:12.5 dilution was prepared by adding 40 μlilts of serum to 500 μlilts of saline in a 10- by 75-mm tube. With a 50-μl pipet disposable pipette, a two-drop amount (100 μlilts) of the 1:12.5 serum dilution was delivered into the first plate of the plate. To each of the remaining wells included in the titration series, 50 μlilts of saline was added by a single-channel reagent dispenser. By employing the multi-micro diluting handle and the 50-μl channel microdilutors, serial dilutions for up to 12 sera were accomplished at once by mixing and moving from well to well. The resulting dilutions of serum to saline were 1:12.5 to 1:1,600. By using the single-channel reagent dispenser, 50 μlilts of stained pullorum antigen was added to each well. The antigen was diluted to a density of tube no. 4 of the McFarland scale with 0.5% phenolized saline. The resulting final dilutions of serum to antigen were 1:25 to 1:3,200. Each plate was tightly sealed with an adhesive plate sealer.

For addition of a uniform quantity of either saline, antigen, or other reagent to the microwells, experimental use has also been made of a 12-channel and a 96-channel hand dispenser (Fig. 1) supplied by Cooke. By varying the total volume of liquid introduced into the dispensers, any volume desired can be rapidly delivered to the microplate wells. Both dispensers have been operated with a Brewer automatic pipetting
machine with foot control and with a Cornwall Luer-Lok syringe. Their use in routine serology is under further study.

Undiluted sera tested at a routine diagnostic dilution of 1:25 or 1:50. Sera were added to the microwells by one of the following methods. (i) For a dilution of 1:25, a manually operated Oxford sampler with disposable tip (Oxford Laboratories, Inc., San Mateo, Calif.; Fig. 2) was used to deliver 10 μliters of undiluted serum into each microwell in accordance with manufacturer's recommendations. For the diagnostic 1:50 dilution, 5 μliters of serum was delivered into each well with a sampler obtained on special order. The tip was rapidly rinsed in fresh saline by depressing the plunger to the first stop and slowly releasing it to the starting position several times to remove any trace of serum. The tip was then touched to an absorbent lint-free material with the plunger at the lowest position before moving on to the next serum to be tested. A new tip was inserted on the sampler after every 100 sera; however, it was felt that unlimited deliveries could be made with only one tip if it was properly rinsed between samples. Hand fatigue can occur if the sampler is used over a long period of time. This method of serum delivery is most applicable when a limited number of sera are to be tested. (ii) In a second method, a Cooke microdiluter (Fig. 3) was used to add 10 μliters of serum to each well containing 250 μliters of stained pullorum antigen diluted to a density of tube no. 2 of the McFarland scale with 0.25% phenolized saline for the 1:25 dilution. A 5-μliter diluter was used for the 1:50 dilution. Both of these microdiluters were obtained on special order. The antigen was added to each well by using a Cornwall Syringe with a 15-gauge cannula or with the 12-channel dispenser discussed above. It is essential that the serum be added within 1 hr after the wells are loaded with antigen so that the cells do not settle excessively in the bottom of the wells. To eliminate any problem with antigen settling, saline can first be added to the wells, the serum dilution made, and an equal volume of antigen having a density of McFarland tube no. 4 subsequently added. The microdiluter was filled by touching its tip to the surface of the serum sample and the serum then mixed with the antigen or saline by rotating the diluter in the microplate well a few times. The microdiluter was quickly rinsed in clean saline and the excess liquid was removed by touching its tip to thick blotter paper before moving on to the next sample. This method of serum dispensing has the advantages of being easily applied and very rapid. (iii) Serum was also rapidly dispensed into the microwells by graduated micropipettes or conventional serological pipettes with capacities of 5 and 10 μliters. These pipettes can be conveniently used with a rubber suction bulb and rapidly rinsed between samples. In these studies, such pipettes were easily employed by trained technicians working with the macrotest in field labora-

![Fig. 1. 12- and 96-channel hand dispensers for adding reagents in the microtest.](image1)

![Fig. 2. Manually operated sampler with disposable tip for serum delivery and titration.](image2)

![Fig. 3. Microdiluter (10 μliter) for serum delivery and titration, lateral view.](image3)
tories; however, the experience of other workers may make it advisable to use one of the other methods of serum delivery described above. The microdiluter was the method favored by personnel engaged directly in work on this project.

Tetrazolium-stained antigen was diluted without pH adjustment to a density of tube no. 2 of the McFarland scale with 0.25% phenolized saline as a diluent. Portions (250 µl) were dispensed into each well after depositing the sera by method i or iii above. The quantity of antigen and serum added by method i, ii, or iii above can be varied to arrive at any dilution between 1:25 and 1:50 that may be desired. For the 1:25 dilution it is also possible to utilize 5 µl of serum and 125 µl of antigen in smaller volume U- or V-shaped plates. The top of each plate was sealed with an individual plate sealer.

Incubation. Tests for titrations and routine diagnostic procedures were incubated for 18 to 24 hr at 37°C in a standard bacteriological incubator.

Reading and interpretation of microagglutination tests. After incubation, the plates were tilted at an angle of approximately 70° for at least 3 min (3). With U-shaped plates, readings must be made promptly after plates are tilted since the cells tend to run back readily to the center of these plates when they are returned to the horizontal position.

The test results were read with the aid of a reading mirror, and reactions were interpreted as follows: (i) positive, a well revealing no antigen button or one with a button that did not run when the plate was tilted. In a positive well, a tint of purple was also noted since the agglutinated cells were often deposited in a thin sheet across the bottom of the well. These cell sheets tended to pile up as colored strips when the plates were tilted (Fig. 4 and 5). (ii) Suspicious, a well showing a small button approximately one-half that of the negative control which ran only slightly when the plate was tilted. A faint purple color was also observed with such samples (Fig. 4 and 5). (iii) Negative, a well with a large distinct button corresponding to the negative control. The well was clear, showing no color tint as was observed in positive and suspicious tests. The cells ran freely when the plate was tilted (Fig. 4 and 5).

With each series of sera tested, a positive and negative serum control can be included. A well containing only antigen should be set up with each series to provide a negative reference button.

RESULTS

The results of comparative serial titrations by using the macrotest with unstained S. pullorum antigen and the microtest with stained antigen did not reveal a significant titer difference. Serum to antigen dilutions from 1:25 through 1:3,200 were employed.

Representative tests with the sera of five chickens confirmed to be naturally infected with pullorum disease by isolation of the causative organism are shown in Table 1. Forty sera of this type were titrated during these studies. End titers with the unstained antigen in the macrotest were essentially identical to those obtained in the microtest with the stained antigen.

Comparative titrations of five sera from chickens inoculated with oil suspensions of heat-killed S. pullorum cells are presented in Table 2. These sera were titrated in the same manner as those presented in Table 1, and the analysis of variance also demonstrated no significant differences. A total of 21 sera of this type were titrated.

Approximately 30 doubtful or nonspecific pullorum sera from field sources were tested. These sera were collected from chickens which revealed generally low-titered reactions to the pullorum tube agglutination test but from which S. pullorum was not isolated. Some revealed infections with other salmonellae. End titers to both the macro- and the microtest were quite comparable. The latter testing method was no more sensi-

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**Fig. 4.** Typical appearance of microagglutination tests prior to tilting plate.

**Fig. 5.** Same microagglutination tests shown in Fig. 4 after tilting plate for 3 min. Wells A-1 through A-8 are positive tests with no button; B-6 and C-6 are positives with buttons that do not run; D-3 and E-2 are suspicious; and A-9 through A-12 are negative.
Table 1. Comparative titrations of sera from chickens naturally infected with pullorum disease by employing the macroscopic tube agglutination and microagglutination testing techniques

| Serum | Antigen and procedure | Dilution (serum:antigen) |
|-------|-----------------------|--------------------------|
|       | 1:25                  | 1:50                     |
|       | 1:100                 | 1:200                    |
|       | 1:400                 | 1:800                    |
|       | 1:1,600               | 1:3,200                  |
| 1     | A                     | 4                       |
|       | +                     | 4                       |
|       | +                     | 2                       |
|       | +                     | ±                       |
| 2     | A                     | 4                       |
|       | +                     | 4                       |
|       | +                     | 2                       |
| 3     | A                     | 4                       |
|       | -                     | 2                       |
| 4     | A                     | 4                       |
|       | +                     | 4                       |
|       | S                     | 3                       |
|       | 2                     | ±                       |
| 5     | A                     | 4                       |
|       | 4                     | 4                       |
|       | ±                     | +                       |
|       | S                     | ±                       |

a A = macroscopic tube agglutination test with unstained reference antigen, pH 8.4. B = microagglutination test with tetrazolium-stained S. pullorum antigen, pH unadjusted.
b 1 to 4, Increasing degrees of agglutination; ±, trace of agglutination; +, no agglutination.
c +, Positive microagglutination reaction; S, suspicious reaction; —, no reaction.

tive to such nonspecific type agglutinins than were conventional macrotest procedures. Twenty negative sera were tested by the macro- and microtest with stained antigen. All gave negative results, indicating that the stained antigen was not hypersensitive with negative sera. More than 5,000 sera were tested under routine laboratory conditions at the Georgia Poultry Diagnostic Laboratory with the macro- and microtest. Very satisfactory agreement was obtained between the two methods with these sera which were all negative.

To determine if there was any agglutinin carry-over from positive into negative sera by using the serum delivery methods with saline rinses between, as outlined above, positive and negative sera were used in succession employing all the suggested methods. Test results showed no evidence of transfer of agglutinins from positive to negative sera. When transfers were made from twofold dilutions of positive sera with each of the three serum delivery methods outlined above in the microagglutination test, identical results were obtained.

Discussion

The microagglutination technique has distinct advantages in routine serological detection of pullorum disease and fowl typhoid in poultry. Serum titers and test results in the microtest are essentially identical to those in the conventional macrotest.

Preparation of a satisfactory tetrazolium-stained antigen was essential to the successful use of the microtest. Other developments in microtiter equipment and technology occurring while this work was in progress have greatly aided the successful application of the test on a time and money-saving basis. It is anticipated that yet other developments may occur in the future that will require the updating of some phases of procedures as outlined here.

The dilution ratio of serum to antigen in the microtest is the same as that presently employed in the macrotest, but the volume of antigen and serum required has been reduced three-fourths. Use of an antigen with a higher cell count in the microtest was found to result in closer agreement of test results with the two methods. The mechanics of adding the reagents for the test, especially the antigen, have been greatly simplified, resulting in speed and ease of application.

The microtest offers several distinct advantages over the standard macrotest which will be discussed in the major categories of savings in time, space, and cost. The average number of tests set up per hour should be essentially the same or slightly more by the micromethod. The time re-

Table 2. Comparative titrations of sera from chickens inoculated with oil suspensions of killed S. pullorum cells employing the macroscopic tube agglutination and microagglutination testing techniques

| Serum | Antigen and procedure | Dilution (serum:antigen) |
|-------|-----------------------|--------------------------|
|       | 1:25                  | 1:50                     |
|       | 1:100                 | 1:200                    |
|       | 1:400                 | 1:800                    |
|       | 1:1,600               | 1:3,200                  |
| 1     | A                     | 4                       |
|       | +                     | 4                       |
|       | +                     | 4                       |
| 2     | A                     | 4                       |
|       | +                     | 4                       |
|       | 1                     | ±                       |
| 3     | A                     | 4                       |
|       | +                     | 4                       |
|       | 1                     | —                       |
| 4     | A                     | 4                       |
|       | ±                     | S                       |
|       | 4                     | —                       |
| 5     | A                     | 4                       |
|       | 2                     | ±                       |
|       | S                     | ±                       |

a A = macroscopic tube agglutination test with unstained reference antigen, pH 8.4. B = microagglutination test with tetrazolium-stained S. pullorum antigen, pH unadjusted.
b 1 to 4, Increasing degrees of agglutination; ±, trace of agglutination; —, no agglutination.
c +, Positive microagglutination reaction; S, suspicious reaction; —, no reaction.
required to read the test will be decreased about 90% since results are interpreted for all 96 tests per microplate at once rather than requiring the handling and reading of individual test tubes. The time presently used to fill racks with tubes and to clean dirty tubes when disposable tubes are not used will all be saved through the use of completely disposable microplates. Only a minimal amount of training is required for the inexperienced worker to become proficient in the use of the microtest.

The microtest offers a great saving of space. An area 13 by 11 by 8 inches is adequate to store 100 microplates. This number of plates will accommodate 9,600 tests. Equipment for the microtest is compact, and the working space needed for operations in setting up the test is greatly reduced. Incubation space is saved to about the same degree as storage space, allowing smaller incubators to be used.

As a result of a national survey of pullorum testing laboratories, it is conservatively estimated that the cost of conducting the conventional macrotest is about $0.07 per test. We have estimated that it costs approximately $0.033 to test each serum sample by the micromethod. Thus, the microtest costs slightly less than one-half that of the macrotest per sample. In six Northeast states where the macroscopic tube agglutination test is used exclusively, use of the microtest would have resulted in a savings of approximately $83,000 during the fiscal year ending June 30, 1970.

The microagglutination test has been found to be of equal accuracy to the macrotest in detecting carriers of pullorum-typhoid in chicken flocks and is a great saver of time, space, and cost. Microagglutination techniques are being studied further for application in the serological detection of carriers of other salmonella serotypes in poultry flocks.

LITERATURE CITED
1. Anonymous. 1969. The national poultry and turkey improvement plans and auxiliary provisions. Animal Science Research Division, Agricultural Research Service, USDA, Beltsville, Md.
2. Anonymous. 1970. A selected bibliography of micro methods in microbiology with special emphasis on microrititration techniques. Cooke Engineering Co., Alexandria, Va.
3. Chaffin, A. J., O. Smithies, and R. K. Meyer. 1966. Antibody responses in bursa-deficient chickens. J. Immunol. 97:693-699.
4. Jones, F. S. 1913. The value of the macroscopic agglutination test in detecting fowls that are harboring Bacterium pullorum. J. Med. Res. 27:481-495.
5. Thaxton, P., J. E. Williams, and H. S. Siegel. 1970. Microtitration of Salmonella pullorum agglutinins. Avian Dis. 14:813-816.
6. Van Roekel, H. 1964. Is eradication of pullorum disease realistic? J. Amer. Vet. Med. Ass. 144:19-23.
7. Williams, J. E., and A. D. MacDonald. 1955. The past, present, and future of salmonella antigens for poultry. Proc. Book Amer. Vet. Med. Ass. 92nd Annu. Meet., p. 333-339. Minneapolis.
8. Witlin, B. 1967. Detection of antibodies by microtitration techniques. Mycopathol. Mycol. Appl. 33:241-257.