Avian *Salmonella*-Stained Microtest Antigens Produced on Solid Media

J. E. WILLIAMS and A. D. WHITTEMORE

Southeast Poultry Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia 30601

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Procedures are described for the preparation of *Salmonella pullorum* and *Salmonella typhimurium* tetrazolium-stained microagglutination and microantitglobulin antigens with solid media for culture propagation. Cell suspensions were found to be more easily harvested from solid medium than from previously used liquid medium, and greater yields of antigen were obtained. Liquid medium microagglutination and microantitglobulin test antigens were found to be more sensitive than those prepared on solid medium. The microtest antigens produced on solid medium were easier to prepare and in serological tests gave titers more comparable to those demonstrated with the standard macroscopic tube agglutination test.

In prior publications (3, 4) procedures were described for the preparation of *Salmonella pullorum* and *Salmonella typhimurium* microagglutination antigens with broth medium. Use of liquid medium for antigen production has several disadvantages. Rather large volumes of medium must be handled and special equipment must be available for harvesting and processing cell suspensions. Compared to solid medium, the yield of final antigen from a given volume of liquid medium is low. Finally, in compiling comparative data on macro- and microtests for salmonellosis, it is preferred that all antigenic materials be prepared on media of as nearly identical composition as possible. Presently, *S. pullorum* and *S. typhimurium* macroagglutination antigens, which are unstained suspensions, are prepared on solid media incubated for 48 h at 37°C (1). This report describes procedures for the preparation of tetrazolium-stained *S. pullorum* and *S. typhimurium* microtest antigens with bacterial growth from solid media harvested after only 24 h at 37°C. Solid and liquid media antigens were compared, along with conventional tube agglutination antigens, for the serological detection of *Salmonella* agglutinins.

**MATERIALS AND METHODS**

**Cultures.** Three strains of *S. pullorum* (strains 17, 19, and 20) and one strain of *S. typhimurium* (strain P-10) were used as antigen preparation cultures. These were the same strains presently used for production of *S. pullorum* and *S. typhimurium* macroscopic tube agglutination antigens and liquid medium microagglutination antigens.

**Media.** The *S. pullorum* cultures were grown on the simple beef extract medium recommended for preparation of conventional tube agglutination antigen (1) with agar adjusted to 3%. This medium had the following composition: beef extract (Difco), 4 g (0.4%); peptone (Difco), 10 g (1.0%); agar (Difco), 30 g (3.0%); distilled water, 1,000 ml. The medium was dissolved, and the pH was adjusted to 6.8 to 7.2. Medium was dispensed in 150-ml amounts into Roux bottles stoppered with new cotton-gauze plugs each time the medium was made. Bottles were sterilized in the autoclave and laid flat to thoroughly cool and harden. Bottles were incubated at 37°C overnight and observed for sterility before use. The *S. typhimurium* P-10 culture was grown on veal infusion agar (Difco), as recommended for tube agglutination antigen (1), with agar increased to 3%. Dispersion and sterilization were as described above for the *S. pullorum* medium.

**Seed broth.** Transplants of *S. pullorum* strains 17, 19, and 20 and *S. typhimurium* strain P-10 were each made in a separate flask of veal infusion broth (Difco). These were incubated at 37°C overnight. The volume of broth needed of each strain was determined from the number of bottles processed.

**Incubation of Roux bottles.** Six milliliters of the 18- to 24-h veal infusion broth culture was used to flood the surface of each Roux bottle. Equal numbers of Roux bottles were inoculated with each of the three strains of *S. pullorum*. The bottles were allowed to lie flat with the inoculum on their surface for 10 to 15 min. (The agar tends to dislodge when soaked too long.)

**Incubation of Roux bottles.** Bottles were incubated inverted for at least 18 and no more than 24 h at 37°C.
Harvesting growth. The liquid in each bottle was aseptically poured off into a 1% aqueous solution of Formalin. The surface of each bottle was flooded with 20 ml of sterile 0.85% saline. They were picked up and rocked from side to side two separate times to dislodge the bacterial growth. Without excessive flaming, the growth from each flask was rapidly poured into a sterile screw-capped Erlenmeyer flask. Care was taken not to get either the mouths of the Roux bottles or the flasks hot, as there would have been excessive bacterial kill. Since the organisms were living, care was taken to avoid contamination of personnel and work area.

Addition of neo-tetrazolium. To the living bacterial cell suspension, the first of three hourly additions of a predetermined amount of a sterile 1:100 aqueous stock solution of neo-tetrazolium (Nutritional Biochemicals Corp., Cleveland, Ohio) was added by using a sterile pipette. A 1-ml amount of tetrazolium solution multiplied by the number of Roux bottles equals the milliliters of a 1:100 tetrazolium solution to be added to the harvested cell suspension at each of the three hourly intervals. After the first addition, the flask was placed in a 37°C waterbath and left for 1 h, at which time the second addition was aseptically made and the flask was returned to the 37°C water bath. After another hour, the third and last addition of tetrazolium was aseptically made, and the flask was returned to the 37°C water bath for 2 more h of incubation before the bacterial cells were killed.

Preserving and standardizing antigen. Liquefied phenol was added to the stained cells in an amount to provide a final concentration of 0.5%. The antigen flasks were returned to the 37°C water bath for 1 h to allow the phenol to kill the organisms. The antigen suspensions were filtered by gravity flow through one or more sterile milk filter pads in Buchner funnels, depending on the volume. The filtered suspensions were then centrifuged in an International or Sorvall refrigerated centrifuge until cells were well settled. Excessive centrifugation was avoided. The supernatant fluid was removed by decantation. To each clear-plastic centrifuge tube or bottle, a limited amount of 0.5% phenolized saline and sterile glass beads was added, the top was capped, and the cells were thoroughly resuspended by using an electric contact mixer. For resuspending the cells, the liquid was poured from one tube or bottle to the next, and phenolized saline was added as needed. An antigen suspension of density greater than 50 times tube no. 1 of the McFarland scale resulted. The finished S. pullorum or S. typhimurium antigen from all the tubes or bottles was collected into one large screw-capped Erlenmeyer flask. In the case of the S. pullorum antigen, the three strains were combined in equal volume-density.

Adjustment of cell density. The cell density of the stock antigen was adjusted to a value equivalent to a reading of 50 times tube no. 1 of the McFarland scale by using procedures already described (3). Hemocytometer, rather than Hopkins, can also be used for determining and adjusting the cell density of the antigen.

Sera. Sera for titrations were selected from refrigerated stocks of samples from chickens proved to be infected with S. pullorum by bacteriological examination. These sera were from field and experimental cases of S. pullorum infection and were supplied by C. H. Smyser of the University of Massachusetts.

S. typhimurium. One-year-old White Leghorn hens were infected by oral administration of 2.0 ml per bird of a 24-h vein infusion broth (Difco) culture of S. typhimurium having a population count of approximately 250 × 10⁹/ml. Sera from these experimentally infected birds were collected at intervals after infection.

Negative control sera were collected from chickens that had been maintained in isolation since hatching and fed only Salmonella-free feed.

Micro-, microantiglobulin, and macroagglutination tests. Procedures for conducting these tests were described previously (3, 4). All microantigens were used at a final cell density of two times tube no. 1 of the McFarland scale. Sera were titrated at a dilution from 1:20 to 1:2,560. The reciprocal of the highest serum dilution giving complete agglutination was interpreted as the end titer. In Fig. 1, microantiglobulin end titer data for a total of 20 S. typhimurium-exposed chickens were plotted as previously described (4).

RESULTS AND DISCUSSION

Comparative titers of 10 S. pullorum positive sera serially tested with macrotest antigen and three lots of solid and three lots of liquid medium microtest antigen are presented in Table 1. Results with these 10 sera were randomly selected from a total group of 24 that were titrated.

Solid medium microtest antigens revealed titers very comparable to those found with macroscopic tube agglutination antigen. Liquid medium microtest antigens were somewhat more sensitive than either the standard tube agglutination antigen or the solid medium microtest antigens.

End titers with each of the three lots of solid medium microtest antigen agreed with each other very well as did those with the three lots of liquid medium antigen. As noted by Fulton (2) in studies of a microagglutination test for toxo-plasma antibodies, some divergence invariably occurs in serological tests due possibly in this case to slight differences in the quality of different lots of antigen.

In Table 2 are the results of comparative serial titrations with S. typhimurium macro- and solid and liquid media microtest antigens. These sera were randomly selected from a total group of 24. There was good agreement between the different lots of both solid and liquid microtest antigens. As noted with S. pullorum, the
TABLE 1. End titers of sera tested with S. pullorum macroscopic tube agglutination antigen and three different lots of solid and liquid media microtest antigen

| Serum no. | Macro-test | Microagglutination titers |
|-----------|------------|---------------------------|
|           |            | Solid         | Liquid         |
| A | B | C | A | B | C |
| 1 | 160* | 160 | 160 | 160 | 160 | 320 | 320 |
| 2 | 320 | 320 | 320 | 320 | 320 | 320 | 320 |
| 3 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| 4 | 320 | 320 | 160 | 160 | 320 | 320 | 320 |
| 5 | 80 | 80 | 80 | 80 | 160 | 160 | 160 |
| 6 | 320 | 320 | 640 | 640 | 640 | 640 | 640 |
| 7 | 160 | 160 | 160 | 160 | 320 | 320 | 320 |
| 8 | 320 | 320 | 320 | 320 | 640 | 1,280 | 640 |
| 9 | 160 | 160 | 160 | 160 | 320 | 320 | 320 |
| 10 | 160 | 160 | 160 | 160 | 160 | 160 | 160 |
| Neg. control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Neg. control | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Reciprocal of the highest serum dilution revealing complete agglutination.

Microantiglobulin titers were augmented by the use of liquid medium microtest antigen when compared with solid medium antigen.

During this work, experimental use was made of high-yield media such as TG (1) and K (1) for growing production cultures in preparing solid medium microtest antigens. These media were unsatisfactory because cell suspensions had to be harvested in buffered saline prior to staining with tetrazolium, often the stain was poor, and the antigen was hypersensitive. Highly nutritive media should be avoided in preparing microtest antigens.

It was concluded that solid medium antigens gave results more comparable to those observed with both S. pullorum and S. typhimurium macroscopic tube agglutination antigens. For this reason and because of the greater ease with which solid medium microtest antigens can be prepared, the latter were preferred.

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