Microantiglobulin Test for Detecting 
Salmonella typhimurium Agglutinins 

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A sensitive antiglobulin (AG) test procedure for the demonstration and experimental study of the agglutinin response of chickens infected orally with Salmonella typhimurium is described. A tetrazolium-stained S. typhimurium antigen was employed with microagglutination techniques and equipment for the first time in conducting the AG test. Results with the conventional macroscopy tube agglutination test for S. typhimurium and the 24-hr microtest were comparable; however, the AG test enhanced titers as much as 16 times, and these persisted at a significant level for as long as 4 months. This study is being extended to other Salmonella serotypes and possible field applications of the AG test procedure.

During the past 30 years several methods have been studied for the serological detection of carriers of motile salmonellae in poultry flocks and are reviewed elsewhere (19). None has proved entirely satisfactory. Serological testing for Salmonella infections, other than pullorum disease and fowl typhoid, in poultry has been essentially limited to use of the macroscopy tube agglutination test for Salmonella typhimurium.

The ability of the present S. typhimurium tube agglutination test to dependably detect infected chickens and turkeys has frequently been questioned (15, 21). Fecal carriers often reveal no reaction to this test, and the titers of reacting birds may show wide fluctuations. A somatic-type S. typhimurium tube agglutination antigen for several years has been applied in the blood testing programs of the National Poultry Improvement Plan (3).

Because no method reported to date (5, 11, 12, 17) has actually provided an effective and readily applicable test for the detection of agglutinins of motile salmonellae, our interest was stimulated in the possible existence of weak or incomplete antibody in fowl infected with motile salmonellae and recognizable by the antiglobulin (AG) technique of Coombs et al. (6). Persistence of such antibodies for a reasonable period after tissue invasion by salmonellae could provide a means of detecting birds exposed to salmonellosis which pose a threat of spreading the disease to their progeny or other birds.

While used most extensively in blood typing in humans, the AG technique has also been previously applied to bacterial agglutination reactions for typhoid fever, shigellosis, brucellosis, and tuberculosis as already reviewed (7, 9). Coombs and Stoker (8) detected Q fever serologically by using AG. In avian medicine, the technique has been studied for the detection of agglutinins for mycoplasmosis (1, 2) and arizona infections (16). Since additional equally effective means are available for diagnosing most of the above diseases, AG procedures have never been extensively applied for detecting potential carriers of bacterial infections. With the motile salmonellae, an effective procedure unfortunately does not presently exist.

Previous work on microtesting for pullorum disease and fowl typhoid using a tetrazolium-stained antigen (20) has been extended and adapted to these studies of S. typhimurium. This represents, to our knowledge, the first application of microagglutination for the AG test using bacterial antigens. Microtesting, which conserves reagents and time when compared with conventional methods, has greatly facilitated this work. We report in this paper procedures for the application of AG using a microagglutination technique with tetrazolium-stained antigen for the enhancement of agglutinin titers of chickens experimentally infected with S. typhimurium.
MATERIALS AND METHODS

Chickens and housing. Twenty 2-year-old White Leghorn hens (group 1) and ten 12-week-old White Rock chickens (group 2) were obtained from production flocks on the laboratory premises. All groups were housed in tight-isolation buildings with maximum security. Group 1 was kept in a battery of separated wire cages, whereas group 2 was placed together on a salmonella-free deep-litter floor in a separate room of the same isolation building.

Sera. All birds were selected for experimental study on the basis of negative response to all serological procedures applied as well as salmonellae-negative fecal culture. Birds in both groups 1 and 2 were infected by oral administration of 2.0 ml (per bird) of a 24-hour veal infusion broth (Difco) culture of *Salmonella typhimurium* (strain M-24) having a population count of approximately $250 \times 10^8$ per ml. This culture is readily identified by its natural characteristic of weak H$_2$S production on TSI slants.

Sera from these experimentally infected groups were collected at intervals after infection.

Cultural procedures. Cloacal swabs from all birds were cultured at regular intervals following infection. Cultures were enriched in Tetrathionate Brilliant Green medium (BBL) incubated at 37 C. Plates were made on Brilliant Green agar (Difco) after both 24- and 48-hr incubation. At least three suspicious salmonellae colonies were selected from possibly positive plates, identified by standard procedures, and submitted to the USDA Salmonella Serotyping Laboratory in Atlanta, Ga., for final typing.

At the conclusion of the experiment, birds in group 1 were examined bacteriologically by using techniques outlined in the National Poultry Improvement Plan (3).

Tube agglutination test. *Salmonella typhimurium* antigen was prepared and the macroscopy tube agglutination tests were conducted in accordance with standard procedures (4).

Microtest: antigen. The *Salmonella typhimurium* microtest antigen was prepared by using essentially the same procedures as already described for pullorum antigen (20); however, the stained cell suspensions were not subjected to any shaking after collection. A single, naturally nonmotile culture of *Salmonella typhimurium*, strain P-10 (18), was used as seed for antigen preparation. This is the same strain presently used to prepare macroscopy *Salmonella typhimurium* tube agglutination antigen in the United States.

Microplates. Tests were conducted in rigid styrene microplates (no. IS-MRC-96, Linbro Chemical Co., New Haven, Conn.) with 96 rounded (U-shaped) wells having a capacity of 275 $\mu$l per well. In earlier stages of this work, plates were sealed with Linbro's plastic sealers (no. 53) or Scotch tape. Later it was found that no sealers were needed if plates were stacked on metal racks in tightly sealed plastic or stainless-steel boxes with raised bottoms under which there was a water layer containing 1:1,000 Merthiolate as a preservative. These "wet boxes" (Fig. 1) were placed into the incubator and loaded and unloaded without removal, resulting in convenience and cost savings in conducting the tests.

A limited number of 110-well, rigid styrene plates (no. M-220-24A-110, Cooke Engineering Co., Alexandria, Va.) also were employed during these studies.

Titrations. Twofold microtitrations were done in accordance with procedures already described (20). A microdiluter (Cooke) was most frequently used for serum addition. Use was also made of Oxford samplers (Oxford Laboratories, San Mateo, Calif.) and of conventional microcapcity serological pipettes. Dilutions were made by using a 12-place head with 50-$\mu$l microdilutors (Cooke). Any bubbles that formed in the wells during mixing were disrupted by centrifuging the plates up to 1,000 rev/min and immediately cutting power to the centrifuge. Also, this problem can be taken care of at the time the antigen is dispensed into each well with the 12-channel cell rinser (Cooke). In conducting titrations a total final volume of 100 $\mu$liters after antigen addition was used.

Dispensers. Continuous dispensers (96 and 110 channel) constructed according to Middlebrook et al. (13) (Fig. 2) with slight modification were used successfully throughout this work and were an invaluable aid in facilitating and speeding routine serological testing procedures and titrations. The Cooke 12-channel cell rinser was equally useful. These dispensers were employed for distributing varying quantities of both saline and antigen into the microplate wells very quickly and with a minimum of effort. The 110-channel dispenser was used with the Cooke 110-well plate in several experimental trials. It worked quite satisfactorily with this plate.

Incubation and reading. After 18 to 24 hr of incubation at 37 C, microtest results were read and interpreted as already described (20), except that readings were routinely made without tilting the plates.

AG technique: 48 hr. The 24-hr microtest was the first step in the 48-hr AG test. After readings were made and recorded, the plates were centrifuged in 3- or 5-place carriers (Fig. 3) with cardboard padding between plates in an International UV centrifuge (head 976) at a speed of 1,200 to 1,500 rev/min for 10 min. The centrifuge was allowed to stop without braking so that the settled cells would not be disturbed. The 96-well plate carriers are available from Cooke. The 110-well plate carriers were constructed in our laboratory.

After centrifugation the supernant fluid was removed from the plates by using the empty 96- or 110-well dispenser through which was applied a vacuum of 15 inches of mercury. The plates can also be quickly inverted over a sink or pan to remove the fluid. The deposited *Salmonella typhimurium* cells were then resuspended in the small quantity of liquid that remained in each well by stacking the plates on a micro mixer (Cooke) with a setting of 6 for 3 to 5 min. To the resuspended cells were then added 100 $\mu$liters of 0.85% saline by using either the 96-, 110-, or 12-channel dispenser. The plates were then centrifuged again as described above.

The above procedure was repeated so that the cells were subjected to a total of three washings and four centrifugations. Following the fourth centrifugation, the supernatant fluid was discarded and the
Fig. 1. Side-loading stainless-steel "wet box" for the incubation of microplates without sealers.

Fig. 2. Continuous dispensers [110-well (left) and 96-well (right)] for rapid addition of reagents in micro-testing.

Fig. 3. Three- and five-place centrifuge carriers for use with 110- and 96-well microplates in washing bacterial cells in the AG test.
cells resuspended on the mixer, following which 50 μl of 0.85% saline was added per well by using a multiple well dispenser. To each well an equal volume (50 μl) of a 1:100 dilution of rabbit anti-
chicken globulin serum (Sylvana, Millburn, N.J.) was added. The AG serum was diluted immediately prior to use. For a description of procedures for de-
termining the optimal dilution of AG, refer to the report of Coombs and Stoker (8). It is important that the optimal dilution be established for each new lot of AG prior to use.

The plates were incubated in a "wet box" (Fig. 1) overnight at 37 C. In interpreting results, plates were not tilted prior to reading. A bright background and stiff white card placed on the top of each plate facili-
tated interpretation of results. Positive, negative, and suspicious test results were recognized. Fol-
lowing is a description of these as interpreted in the AG test, which requires a somewhat broader range of suspicious reactions. (i) Negative, a well that reveals a rather large round button in the center. (ii) Positive, a well that has no button in the center. (iii) Suspicious, any well that does not meet one of the above two descriptions. Suspicious reactions may tend to be more positive than negative (±) or vice versa (=) and can be so noted if desired.

End titers were expressed as the reciprocal of the highest dilution giving a positive reaction. In plot-
ting data, end titers were converted to log 3 (X/10), summed for each bleeding, and averaged. The av-
verage titer was found by converting the average log 3 (X/10) to its antilog.

AG technique: 24 hr. The 24-hr AG technique was identical to the 48-hr technique except the ini-
tial 24-hr incubation period was eliminated following the mixing of the serum and antigen. The plates were immediately centrifuged and cells washed, and anti-
chicken globulin serum was added prior to a single 24-hr period of incubation. Readings were made as described above.

RESULTS

Group 1. Comparative agglutinin titers de-
tectable by S. typhimurium agglutination and AG tests are presented in Fig. 4.

Both the 24- and 48-hr AG tests provided greatly enhanced titers during the full period of this study. The 48-hr AG test was consist-
ently more sensitive to S. typhimurium agglu-
tinins than was the 24-hr AG test. It was found that the AG test procedure was 5 to 16 times higher in titer than was the conventional tube agglutination test and 5 to 10 times higher than the 24-hr microtest. Similar findings of the enhancement of S. typhi titers in human vaccines with a tube AG technique have been reported by Morgan and Schütze (14).

Titers to both the agglutination and AG tests reached their peak between 9 and 16 days after infection. After 70 days, average S. typhi-
murium titers detectable by both the tube ag-
glutination and the microtest had decreased to very low levels; however, mean AG test titers were still elevated. Titrations of the sera of birds in group 1 were extended to 120 days before termination with little noted decrease in the average titers shown at 70 days (Fig. 4).

Very close agreement was noted in end titers detectable by both the conventional tube ag-
gglutination test and the microtest; however, the microtest in most cases revealed slightly higher titers. Similar findings with Salmonella group D antisera have recently been reported by Gaultney et al. (10).

S. typhimurium was recovered from one or more birds on each test day by cloacal swab-
bning during the full 70-day experimental pe-
riod reported. The percentage of recovery varied from day to day.

The 20 birds in group 1 were bacteriologi-
cally cultured after 120 days. Salmonellae were isolated from three of the birds. A weak H2S-producing S. typhimurium was recovered from the pancreas and ovaries of one bird, and S. bredeney was recovered from the rectum of the same bird. From the cecum and rectum of a second bird and the duodenum, cecum, and rectum of a third bird, S. bredeney was iso-
lated. It was assumed that the S. bredeney cul-
tures isolated from the intestinal tracts of these three birds were introduced with the feed.

Subsequently, fresh poultry feed samples taken from previously unopened sacks were cultured and although S. bredeney was not iso-
lated, S. worthington, S. schwargrund, and S. lexington were. The accidental introduction of multiple Salmonella serotypes through con-
taminated feed during experimental studies is probably much more frequent than is realized or reported.

Group 2. The serological response of the in-
fected 12-week-old chickens was essentially identical to that of the older birds in group 1. As noted in Fig. 5, there was an agglutinin peak at about 13 days after infection to the agglutination test, the microtest, and both the 24- and 48-hr AG test. The agglutination titers had decreased to insignificant levels after 50 days; however, the AG titers remained quite high. Both the 24- and 48-hr AG test considerably enhanced agglutinin titers. The AG test procedure gave titers four to nine times higher than the tube agglutination test and three to seven times higher than the mi-
crotest.

On every sampling date except the last, one or more birds yielded positive recovery of S. typhimurium on cloacal swabbing. The highest
degree of positive recovery was shortly after infection. These birds have not yet been bacteriologically cultured, for the study of their serological response is continuing.

DISCUSSION

In view of the continuing importance of poultry and poultry products in the dissemination of salmonellae to man, the environment, and to the general animal population, there is presently a serious need for a readily applicable procedure to recognize infected chicken and turkey flocks. Prior research on methods to increase the sensitivity of serological test procedures for the detection of agglutinins of motile salmonellae in poultry has been limited. Sieburth (17) studied an indirect hemagglutination test for detection of multiple serotypes in chickens, and Magwood and Annau (12) experimented with salmonellae antigens adsorbed on latex particles in testing turkeys. These procedures have found limited application.

The marked enhancement by use of AG of agglutinin titers of sera of chickens exposed orally to motile salmonellae offers some promising possibilities for the experimental study of the infections as well as their field detection. The clarity with which the agglutinin response was observed using AG during these studies has introduced a means not previously available for observing over an extended time period the antibody titers of birds infected orally with salmonellae. Significant AG titers were detectable as long as 4 months after oral infection whereas conventional agglutination and microtest titers waned rather rapidly.

The S. typhimurium microtest agreed very closely with the conventional agglutination test as previously reported in studies on the serological diagnosis of pullorum and typhoid infections in chickens (20) as well as other bacterial diseases (10). Highest agglutinin titers were demonstrable with the 48-hr AG test.

The adaptation and modification of microtest techniques and equipment for conducting the AG test by using stained salmonellae antigens has greatly simplified and facilitated the procedures described. Both equipment and reagents for conducting the AG test procedures can be acquired at reasonable costs. Use of disposable plastic plates has simplified procedures and eliminated requirements for large numbers of tubes and other specialized equipment. This has resulted in cost savings.

Reactions noted in the AG test have been quite clear and definite without the problems of doubtful and cloudy reactions and prozone phenomena so common with conventional agglutination tests. Results on large numbers of
sera can be read with a minimum of effort.

Test results with sera supplied by several state diagnostic laboratories from flocks of both chickens and turkeys known or suspected to be infected with group B salmonellae have indicated that the technique may have future diagnostic field applications. There is presently a need for a more sensitive serological procedure to establish the exposure status of poultry flocks to salmonellae. Such a procedure would also be useful in accrediting flocks as free of exposure to salmonellae organisms.

Techniques described in this paper are being extended to salmonellae groups C, D, and E. These three groups, along with group B, include most of the Salmonella serotypes of importance in domestic poultry populations in the United States. Possible diagnostic applications of the Salmonella AG test are also being evaluated under field conditions in both chicken and turkey flocks in cooperation with state diagnostic services.

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