Serological Response of Chickens to *Salmonella thompson* and *Salmonella pullorum* Infections

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Chickens were experimentally infected with *Salmonella thompson* (serogroup C, paratyphoid) and *Salmonella pullorum* (serogroup D). Five serological methods and one cultural method were used in detecting the infections. The microantiglobulin test was superior to all other methods for detection of paratyphoid (*S. thompson*) infection and was followed in efficacy by the microagglutination test, rapid serum plate test, cloacal swab culture, macroscopic tube agglutination test, and rapid whole-blood test, in that order. Birds infected with *S. pullorum* showed much higher agglutinin titers than the birds infected with paratyphoid. The microagglutination and microantiglobulin tests were not significantly different for detection of pullorum infection and were followed in efficacy by the rapid serum plate, macroscopic tube agglutination, rapid whole-blood, and cloacal swab culture tests, in that order. The cloacal swab culture test was totally inadequate for the detection of pullorum infection.

About 154 paratyphoid serotypes have been isolated from chickens or turkeys in the United States and are among the most important causes of hatchery-borne diseases that infect all types of poultry. As used here, paratyphoid refers to all salmonellae except *Salmonella pullorum* and *Salmonella gallinarum*. Because paratyphoid infection of poultry is most often an intestinal infection, with chronic invasion of parenteral organs the exception rather than the rule, a low level of agglutinin response is generally stimulated under natural conditions. For this reason, procedures for serological detection of adult carriers of paratyphoid infections have not been accepted or applied on the same scale as that used for the detection of pullorum disease and fowl typhoid.

For the serological detection of paratyphoid infections in both chickens and turkeys, the most frequently applied method has been the macroscopic tube agglutination (T) test (6, 8). Also used, but less extensively, have been the microagglutination (MA; 11), microantiglobulin (MAG; 7, 10, 12, 14), rapid serum plate (SP; 7), rapid whole-blood (WB; 3), and indirect hemagglutination (9) tests.

In the past, the main difficulties in applying serological methods for detection of paratyphoid infections have been the weak stimulation of agglutinins and the inferior sensitivity of test methods used. By far the most sensitive method for the detection of avian paratyphoid infections

has been the MAG test (7, 10, 15, 16). Thain and Cullen (10) reported the MAG test to be positive in 75% of experimentally infected chickens 9 months after infection.

In contrast to paratyphoid infections, *S. pullorum* infection readily establishes a chronic organ carrier state, particularly in the reproductive tract of infected birds, and agglutinin levels stimulated are usually quite high. The T, MA, SP, and WB test methods are all satisfactory for the serological detection of pullorum infection (1, 11) in chickens. Because the agglutinin titers of pullorum-infected birds are so high, the MAG and indirect hemagglutination tests are not needed for their detection. The pullorum MAG test, however, was included in these experiments.

This study was undertaken to further define the serological response of chickens orally infected with salmonella organisms. The responses to *Salmonella thompson* (serogroup C, paratyphoid) and *S. pullorum* (serogroup D) are compared with previous responses (15) to *Salmonella typhimurium* (serogroup B, paratyphoid). An effort was made to compare the serological response of chickens to a paratyphoid infection with that to pullorum infection. The agglutinin levels stimulated by the two types of infection and detected by five serological methods and one cultural method were compared in tests to show and establish the response patterns of the infections.

MATERIALS AND METHODS

Chickens, housing, and feed. White Plymouth Rock chickens from salmonella-free parent stock were hatched at 21-day intervals in sanitized incubators in top-security buildings with filtered air exchange. Immunologically mature birds were used.

Birds were maintained in modified Horsfall units until they were old enough for transfer to batteries or individual wire laying cages. Birds were kept in individual cages in isolation as the experiment progressed. Feed given to the birds was pelleted to minimize possible introduction of salmonellae with the feed.

Bird inoculation. After feed and water withdrawal for 24 h, two groups of 40 birds each were orally administered a 1-ml dose of a 24-h veal infusion (VI) broth (Difco Laboratories) culture of either *S. thompson* or *S. pullorum* with a viable count of about 6.7×10^8 bacteria per ml.

Bleeding and culture schedule. The birds were determined to be free of salmonellae and salmonella serogroup C and D agglutinins by negative preinoculation cloacal swab (C) culture and negative serogroup C and D MAG tests. At the time of primary inoculation and on days 3, 6, 9, 13, 20, 23, 27, 34, 55, 62, 70, 76, 83, 90, and 104 after inoculation, blood samples and C cultures were collected from each bird.

Antigens and serological test methods. All antigens used were of the somatic type. A naturally nonmotile serogroup C culture was used to prepare the T, MA, MAG, SP, and WB serogroup C antigens. *S. pullorum* strains 17, 19, and 20 were used to prepare the pullorum T, MA, MAG, SP, and WB antigens. Serogroup C and D T antigens were prepared according to recommended procedures for typhimurium antigen (1).

Standard procedures were followed in preparing the MA and MAG serogroup C and pullorum antigens (13). These microtest antigens with density adjusted to 20 $\times$ tube 1 of the McFarland scale and treated with heparin and D-mannose (16) were used for the serotype C and pullorum SP and WB tests. Also used in the serological tests for pullorum infection was a commercial crystal-violet-stained, whole-blood polyvalent antigen with 75 $\times$ tube 1 of the McFarland scale bacterial cell density prepared from *S. pullorum* strains 4, 11, 77, 79, and 296.

All serological tests were conducted as previously described (16). Numerical end titers of the MA and MAG tests were read and converted to $\log_2 (x/10)$.

In reading the T test, a diagnostic dilution of 1:20 was selected for data analysis. The results were read after incubation at 37°C for 24 h and recorded as -, $\pm$, 1+, 2+, 3+, and 4+. A - and $\pm$ were designated as negative, 1+ and 2+ as suspicious, and 3+ and 4+ as positive. The SP and WB tests were read, rated, and plotted as indicated for the T test.

In the microtests, a positive (+) reaction at the 1:20 dilution with the MA test and a $\pm$ at the 1:40 dilution with the MAG test were designated as positive. A $\pm$ at the 1:20 dilution with the MA test and a + at the 1:20 dilution with the MAG test were designated as suspicious. Negative samples were read as such in the microtests.

Culture. Cloacal swab cultures were used in the bacteriological examination of each bird at the time of each bleeding. The cloaca of each bird was swabbed, and the swab was placed in a tube of tetrathionate brilliant green (TBG) broth (Baltimore Biological Laboratory). The broths were incubated at 37°C and plated on brilliant green (BG) agar (Baltimore Biological Laboratory) after 24 and 48 h.

Suspicious colonies of salmonella were transferred from BG plates into triple sugar iron agar (Difco) and lysine iron agar (Difco) and incubated for 24 h at 37°C. Colonies giving typical salmonella reactions were inoculated into biochemicals for final identification. Representative cultures were submitted to the National Animal Disease Center, Ames, Iowa, for serological typing.

At the end of the experiment, all living birds that had been exposed to *S. thompson* and *S. pullorum* were bacteriologically cultured according to the standard procedure (4) with TBG broth enrichment and plating on BG agar (TBG/BG agar procedure). Samples from the birds exposed to *S. pullorum* were also cultured in VI broth plated on BG agar and also on VI agar (Difco) plates. The heart, spleen, kidneys, pancreas, liver, and all sections of the reproductive organs were selected for culture. Aseptic techniques were observed in the collection of all samples. Fecal cultures were taken, in the same manner as cloacal swabs, from the duodenum, midgut, both ceca, and rectum and cultured by the TBG/BG agar procedure. Suspicious and typical colonies were selected from the agar plates and identified.

For Fig. 1 we used data obtained in a previous experiment in which *S. typhimurium* was administered to immunologically mature birds.

Data analysis and presentation. At the end of the experiment, several birds had died from various causes. Because unequal numbers would present a problem in statistical validity, 20 birds from each group were selected by generating numbers from 1 to 70 on the Monroe calculator with program 3057NS. The first 20 live bird numbers generated in each group were selected for analysis.

All MA and MAG titers were converted to $\log_2 (x/10)$; thus, equispaced data were provided without loss of the original value and can be readily converted back to the original scale by the use of antilogs (2). Data derived from these calculations are plotted in Fig. 2 and 3.

Each sample was classified as negative, suspect, or positive with results of the T, MA, MAG, SP, and WB tests and as negative or positive with results of the C culture method. The total number in each of these classifications was determined for the two groups of birds used in this study (Tables 1 and 2). The Duncan multiple range test (5) was used to analyze the data for significance and for summary in Tables 3 and 4.

When there were more positive, suspect, or negative reactions with a particular method than with another and a significant difference between that method and another, the method with the greater number was deemed to have significantly more positive, suspect, or negative reactions than the method with the smaller number.

RESULTS AND DISCUSSION

Comparison of serological response of birds exposed to salmonella serogroup B, C, and D infections. Figures 1, 2, and 3 show the serological response curves of birds exposed

to *S. typhimurium* (serogroup B), *S. thompson* (serogroup C), and *S. pullorum* (serogroup D), respectively. The shape of these response curves is similar. The response curves of serogroups B and C (Fig. 1 and 2) are remarkably alike in magnitude. Both differ greatly from the *S. pul-*

FIG. 1. Serological response, MA (●) and MAG (○) tests, of chickens orally inoculated with viable *S. typhimurium* (serogroup B).

FIG. 2. Serological response, MA (●) and MAG (○) tests, of chickens orally inoculated with viable *S. thompson* (serogroup C).

FIG. 3. Serological response, MA and MAG tests, of chickens orally inoculated with viable *S. pullorum* (serogroup D).

lorum curve in magnitude and illustrate the normal agglutinin response resulting from organ invasion (*S. pullorum*) contrasted with an intestinal carrier state that generally occurs in paratyphoid infection. As shown in our previous reports (15, 16), the MA test response curve is considerably lower than that of the MAG test response curve but is similar in shape. The superiority of the MAG test is shown again here.

In Fig. 3 the incomplete antibody titer shown by the MAG curve remains more or less constant after reaching the peak between 15 and 30 days, but the antibody titers shown by the MA curve begin to drop off rapidly after 13 days. Here again is illustrated the value that the MAG test may have in detecting prior exposure to *S. pullorum*. Organ invasion with *S. pullorum*, as noted earlier, obviously elicits a greater response than the intestinal infection that occurs commonly in paratyphoid infections.

Comparison of five serological methods and one cultural method in the detection of salmonella serogroup C and D infections. Tables 1 and 2 show results of six test methods used to detect prior experimental exposure of birds to *S. thompson* (serogroup C) and *S. pullorum* (serogroup D). A total of 320 samples were tested for each method.

As in our previous studies (15, 16), all birds were negative to all tests before oral inoculation with *S. thompson* and *S. pullorum*. Therefore, we assumed that the agglutinins shown by the MAG test method were a direct result of the exposure to these two salmonellae. Also, the MAG test was specifically sensitive for *S. thompson* and *S. pullorum* agglutinins and detected the maximum number of birds previously exposed to these two organisms. These results represent the total number of positive reactions possible over this study period.

Results in Table 1 show that 290 of the 320 samples tested by the MAG test method were positive for serogroup C salmonella agglutinins. The total number of positives by each of the six methods shows that the MA test is the second most reliable method in detection of the serogroup C agglutinins, followed by the SP, C, T, and WB methods. As shown in the previous report (16), the WB test method was the least effective serological procedure. In this study, the fact that the C culture method was more effective

than either the T or WB method differs with results from our previous report (16).

Results in Table 2 show that 280 of the 320 samples tested by the MAG test method were positive for serogroup D salmonella agglutinins. With the exception that the C culture method was the least effective of the six test methods used, the results were the same as those obtained with the serogroup C samples.

A commercially prepared pullorum polyvalent WB antigen and our density-adjusted, tetrazolium-stained microtest antigen were used to conduct comparative WB tests on the serogroup D samples. The results were identical with both these antigens and further illustrate the versatility of the microtest antigen (13).

In Table 1, there are a large number of suspect reactions with all of the serological methods except the MAG test and in Table 2, with all methods except the MA and MAG tests. Under field conditions, these suspect reactions, as well as the positive reactions, would be considered. When the suspect reactors are combined with the positive reactors, effectiveness of the test methods does not change.

Results in Tables 3 and 4 show the test methods that gave statistically significant differences. In detection of positive reactions, the MAG test detected a greater ($P < 0.05$) number of positives than the other methods; however, in Table 4 (*S. pullorum*) differences between the MA and MAG test methods were not significant.

With the birds exposed to *S. thompson*, the MA, MAG, and WB methods were significantly different from all other methods (Table 3). The

TABLE 1. Number of samples classified as positive, suspect, and negative by five serological methods and one cultural method in detection of exposure to *S. thompson* (serogroup C, paratyphoid)

Classification	No. of samples classified by:					
	T	MA	MAG	SP	WB	C
Positive	28	118	290	63	2	59
Suspect	42	42	9	85	42	None ^a
Negative	250	160	21	172	276	261

^a There was no suspect cultural classification.

TABLE 2. Number of samples classified as positive, suspect, and negative by five serological methods and one cultural method in detection of exposure to *S. pullorum* (serogroup D)

Classification	No. of samples classified by:					
	T	MA	MAG	SP	WB	C
Positive	192	269	280	234	166	5
Suspect	56	7	1	42	71	None ^a
Negative	72	44	39	44	83	315

^a There was no suspect cultural classification.

TABLE 3. Summary of methods showing statistically significant differences from other methods in detection of previous exposure to *S. thompson* at the 5% level of significance

Reaction	Method ^a											
	T		MA		MAG		SP		WB		C	
	Less	More	Less	More	Less	More	Less	More	Less	More	Less	More
Positive	MA MAG	WB C	MAG	T SP WB C		T MA SP WB C	MA MAG	WB	T MA MAG SP C		T MA MAG	WB
Suspect	SP	MAG	SP	MAG	T MA SP WB			T MA MAG WB	SP	MA	None ^b	
Negative	WB C	MA MAG SP	T WB C	MAG	T SP WB C		T WB C	MAG		T MA MAG SP		MA MAG SP

^a "Less" implies that this method had fewer reactions classified as positive, suspect, or negative than the methods below and that a significant difference exists. "More" implies that this method had more reactions classified as positive, suspect, or negative than the methods below and that a significant difference exists.

^b There was no suspect cultural classification.

TABLE 4. Summary of methods showing statistically significant differences from other methods in detection of previous exposure to *S. pullorum* at the 5% level of significance

Reaction	Method ^a											
	T		MA		MAG		SP		WB		C	
	Less	More	Less	More	Less	More	Less	More	Less	More	Less	More
Positive	MA MAG SP	WB C		T SP WB C		T SP WB C	MA MAG	T WB C	T MA MAG SP	C	T MA MAG SP WB	
Suspect		MA MAG	T WB		T SP WB		WB	MAG		MA MAG SP		None ^b
Negative	C	MA MAG SP	WB C		T WB C		WB C		C	MA MAG SP		T MA MAG SP WB

^a "Less" implies that this method had fewer reactions classified as positive, suspect, or negative than methods below and that a significant difference exists. "More" implies that this method had more reactions classified as positive, suspect, or negative than the methods below and that a significant difference exists.

^b There was no suspect cultural classification.

T method was significantly different from the MA, MAG, WB, and C culture methods, and the SP method was significantly different from the MA, MAG, and WB methods. The C culture method was significantly different from the T, MA, MAG, and WB methods.

The MA and MAG test methods with the *S. pullorum* samples were not significantly different from each other but were significantly different from the T, SP, WB, and C culture methods. The T, SP, WB, and C culture methods individually were significantly different from the other five methods.

As in our previous report (16), there were more negatives with the C culture method than with the five serological methods (Tables 1 and 2 combined). With the *S. pullorum* birds, the fact that only 5 of the 320 samples cultured positive shows the ineffectiveness of the C culture method in detecting pullorum infection. The chronic fecal excretion of *S. pullorum* is not common. When the C culture method was used, 59 positives in the 320 samples taken from the *S. thompson* birds were detected, a finding similar to that obtained in our earlier studies (16).

We can again conclude that the C culture method was the most unreliable method studied under the conditions of these experiments. The T, MA, SP, and WB test methods fall short in their ability to detect previous exposure to a serogroup C paratyphoid infection in chicken flocks; and the T, WB, and C culture methods are not as effective as the other methods studied in the detection of pullorum infections. The MA and MAG tests were both reliable methods in

detection of *S. pullorum* exposure, but the MAG test alone was reliable in detection of serogroup C paratyphoid exposure and was superior to all other methods studied.

Terminal culture. At the end of the experiment, 31 birds that had been exposed to *S. thompson* remained, and terminal culture of these birds with the TBG/BG agar procedure revealed no serogroup C salmonellae. Of 33 birds that had been exposed to *S. pullorum* and remained at the end, 13 (39%) were positive on culture for *S. pullorum* with the TBG/BG agar procedure. The VI broth cultures revealed six (18%) positives when plated on BG agar and three (9%) positives when plated on VI agar. These three positives plated on VI agar were also isolated on the BG agar plated from VI broth and had been identified by the TBG/BG agar procedure. All birds infected with *S. pullorum* which had a positive culture test also had positive serological tests.

Through aseptic technique used in these methods, contaminants were kept at a minimum on the noninhibitory media and did not in any way interfere with the isolation of *S. pullorum*. *S. pullorum* was isolated from one or more organs cultured, but not from the liver. The organism was not isolated from any intestinal tract cultures.

Nine birds exposed to *S. thompson* and seven birds exposed to *S. pullorum* died during this experiment. Three of the nine birds exposed to *S. thompson* cultured positive for *S. thompson*. Three of the seven birds exposed to *S. pullorum* cultured positive for *S. pullorum*.

LITERATURE CITED

1. **Anonymous.** 1971. Methods for examining poultry biology and for identifying and quantifying avian pathogens, p. 190-214. National Academy of Science, National Research Council, Washington, D.C.
2. **Brugh, M., Jr.** 1978. A simple method for recording and analyzing serological data. *Avian Dis.* **22**:362-365.
3. **El-Agroud, M. A., and I. M. Sadek.** 1966. A rapid screening test for detecting *Salmonella typhimurium* infection in poultry. *J. Arab. Vet. Med. Assoc.* **26**: 235-240.
4. **Ellis, E. M., J. E. Williams, E. T. Mallinson, G. H. Snoeyenbos, and W. J. Martin.** 1976. Culture methods for the detection of animal salmonellosis and arizonosis, p. 9-87. Iowa State University Press, Ames.
5. **Hicks, C. R.** 1964. Fundamental concepts in the design of experiments, p. 31-33. Holt, Rinehart, and Winston, New York.
6. **Kumar, M. C., H. R. Olson, L. T. Ausherman, W. B. Thurber, M. Field, W. H. Hohlstein, and B. S. Pomeroy.** 1972. Evaluation of monitoring programs for salmonella infection in turkey breeding flocks. *Avian Dis.* **16**:644-648.
7. **Kumar, M. C., M. D. York, J. E. Williams, A. D. Whittemore, B. S. Pomeroy, and L. T. Ausherman.** 1974. Development of a serum-plate antigen for detection of salmonella infections in turkeys, p. 19-32. *In* Proceedings, 17th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians. American Association of Veterinary Laboratory Diagnosticians, Roanoke, Va.
8. **Olesiuk, O. M., V. L. Carlson, G. H. Snoeyenbos, and C. F. Smyser.** 1969. Experimental *Salmonella typhimurium* infection in two chicken flocks. *Avian Dis.* **13**: 500-508.
9. **Sieburth, J. M.** 1957. Indirect hemagglutination studies on salmonellosis of chickens. *J. Immunol.* **78**:380-386.
10. **Thain, J. A., and G. A. Cullen.** 1978. Detection of *Salmonella typhimurium* infection of chickens. *Vet. Rec.* **102**:143-145.
11. **Williams, J. E., and A. D. Whittemore.** 1971. Serological diagnosis of pullorum disease with the microagglutination system. *Appl. Microbiol.* **21**:394-399.
12. **Williams, J. E., and A. D. Whittemore.** 1972. Microantiglobulin test for detecting *Salmonella typhimurium* agglutinins. *Appl. Microbiol.* **23**:931-937.
13. **Williams, J. E., and A. D. Whittemore.** 1973. Avian salmonella-stained microtest antigens produced on solid media. *Appl. Microbiol.* **26**:1-3.
14. **Williams, J. E., and A. D. Whittemore.** 1973. Microtesting for avian salmonellosis, p. 607-613. *In* Proceedings, 77th Annual Meeting of the U.S. Animal Health Association. U.S. Animal Health Association, Minneapolis, Minn.
15. **Williams, J. E., and A. D. Whittemore.** 1975. Influence of age on the serological response of chickens to *Salmonella typhimurium* infection. *Avian Dis.* **19**:745-760.
16. **Williams, J. E., and A. D. Whittemore.** 1976. Comparison of six methods of detecting *Salmonella typhimurium* infection of chickens. *Avian Dis.* **20**:728-734.