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EVALUATION OF SEROLOGICAL AND CULTURAL METHODS FOR THE DIAGNOSIS OF CHRONIC SALMONELLOSIS IN MICE

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ABSTRACT

MORELLO, JOSEPHINE A. (Boston University School of Medicine, Boston, Mass.), TERESA A. DIGENIO, AND EDGAR E. BAKER. Evaluation of serological and cultural methods for the diagnosis of chronic salmonellosis in mice. J. Bacteriol. **88**:1277-1282. 1964.—Mice were infected by inoculation of varying doses of a virulent culture of *Salmonella typhimurium* directly into the stomach. Feces were cultured at intervals on Brilliant Green and Eosin Methylene Blue Agar. Most survivors shed organisms consistently during the early stage of infection, but, after 1 month, a large number had become intermittent shedders. These animals would have been considered salmonella-free if cultured at a time when they were not shedding the organism. Serological tests to detect antibodies in their sera, therefore, were investigated. Standard agglutination, and hemagglutination and hemolytic tests with the use of sheep erythrocytes modified with extracts of *S. typhimurium*, were performed with sera from infected mice. It was possible to detect smaller amounts of antibody in the sera by means of the hemolytic test, and this test was often positive when the others were negative. Antibodies detectable by the hemolytic test appeared within 21 days after inoculation, and were present in the sera of surviving animals for 3 months or longer. All animals known to be infected gave positive serological reactions. Cultural techniques were most satisfactory for detection of salmonellosis during early stages of infection, and serological tests were of primary value at later intervals. Concurrent use of both techniques ensured discovery of the largest number of infected animals.

Salmonellosis is one of the most commonly occurring infections in mouse colonies. Carriers of this disease serve an important role in maintaining the infection. To eliminate salmonellosis from a colony, these carriers must be detected and destroyed (Slanetz, 1948). Currently, the most commonly used technique for the detection of salmonella carriers is periodic culturing of mouse feces (Margard et al., 1963). However, infected mice shed salmonellae intermittently, and, on any specific day set aside to culture a group of mice, it is certain that some infected animals will not excrete the organisms, and will be considered healthy. An attempt was made therefore, to devise a serological test to detect salmonella antibodies in the sera of infected mice and to compare this with cultural techniques.

MATERIALS AND METHODS

Infection of mice with salmonella. To have a constant source of infected mice for cultural and serological investigations, animals were artificially infected with a virulent *Salmonella typhimurium*, strain W (TmW), obtained from C. V. Seastone, University of Wisconsin. Swiss mice, strain CD 1, were obtained from Charles River Breeding Laboratories, Inc.

Tenfold serial dilutions of an overnight culture of the organism were prepared in Trypticase Soy Broth (BBL), and 0.5 ml was inoculated directly into the stomach of each mouse by means of a 2-ml syringe fitted with an 18-gauge hypodermic needle with the point cut off. The number of organisms inoculated was varied from approximately 10^4 to 10^9. Each animal was housed individually in a glass Mason jar (Douglas and Wheeler, 1941) to eliminate problems arising from cross-infection. As a standard procedure, preliminary fecal cultures and serological tests were performed on each group of mice before infection.

Cultural technique. Cultural examination of uninoculated mice, or those known to be salmonella nonshedders, was performed on a pool of four fecal pellets collected from each animal. Four was the most practicable number of pellets

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that could be obtained in a reasonable period of time. When an animal was known to be a salmonella shedder, only one pellet was cultured because experience showed that, with few exceptions, whenever a pooled sample was positive, a single pellet sample was also positive.

Once obtained, the pellets were enriched in Selenite-F broth (BBL) for 18 to 24 hr at 37 C. At the end of this time, one loopful from the selenite was streaked onto Levine Eosin Methylene Blue Agar (EMB), and three loopfuls onto Brilliant Green Agar (BGA). The two commercially prepared (BBL) media were altered in the following way: 2 ml of 1 N NaOH were added to each liter of EMB to enhance its selectivity for salmonellae (Taft and Daly, 1946), and the agar concentration of the BGA was increased from 2 to 2.5% in an attempt to check the spreading of *Proteus* spp. After incubation at 37 C for 18 to 24 hr, the plates were read with a 3× magnifier. Suspicious colonies were confirmed or eliminated as TmW by slide agglutination with specific antiserum.

Cultures were frequently made from the livers, spleens, intestines, and ceca of animals which died or were sacrificed. The same cultural technique was used.

Preparation of hyperimmune mouse serum. Because mice are reputed to be poor O-antibody producers, a preliminary series of tests was conducted in which two groups of mice were hyperimmunized with either O- or H-antigen preparations of *S. typhimurium* (Edwards and Ewing, 1962). The mice received a total of 10^9 organisms in four weekly intraperitoneal injections. The mice were exsanguinated by decapitation 1 week after the last injection, and the sera from animals within each group were pooled.

Collection of blood from mice. The orbital bleeding technique of Riley (1960) was found to be very satisfactory for obtaining small amounts of blood from the mice. One end of a capillary melting point tube (Kimax; Kimble Glass Co., Toledo, Ohio) was fire-polished, and was used to rupture the capillaries of the ophthalmic venous plexus; 0.1 ml of blood (0.05 ml of cells and 0.05 ml of serum) was collected in the tube and was allowed to clot. The contents were then expelled into a serological tube containing sufficient Veronal buffer to make a first serum dilution of 1:10 or 1:20. The erythrocytes were removed by centrifugation, and the supernatant fluid was inactivated at 56 C for 10 min. Twofold serial dilutions were then prepared from the supernatant fluid, and serological tests were carried out as described below.

Agglutination. The classical tube agglutination test was used. Antigen was prepared according to the method of Edwards and Ewing (1962). Twofold serial dilutions of serum were made, and 0.5 ml of antigen suspension was added to 0.5 ml of each serum dilution. The tubes were read for agglutination after incubation at 37 C for 2 hr, and after standing overnight at room temperature.

Hemagglutination. For use in the hemagglutination test, sheep erythrocytes were modified with alkali-treated extracts of antigen (Neter et al., 1956). A 100-mg portion of a water extract of acetone-dried *S. typhimurium* (Whiteside and Baker, 1962) was dissolved in 5 ml of 0.1 N NaOH, and was incubated at 56 C for 0.5 hr. The solution then was neutralized by titration to pH 7.1 with 0.1 N HCl. The treated extract was stored frozen in a concentration of 5 mg/ml, but was diluted to 1 mg/ml for use. Sheep erythrocytes were washed three times with Veronal buffer, and 1 mg of antigen extract was added to each 0.1 ml of packed erythrocytes. The suspension was incubated for 1 hr at 37 C with occasional mixing. The cells were again washed three times to remove excess antigen, and sufficient Veronal buffer was added to make a 0.5% suspension of cells. Twofold serial dilutions of sera were prepared, and 0.25 ml each of serum dilution and antigen were mixed and incubated at 37 C for 2 hr. At the end of this time, and after 24 hr at 4 C, the tubes were examined for hemagglutination.

Hemolysis. The hemolytic test was identical to the hemagglutination test, except that complement was added to each tube containing serum and treated erythrocytes before incubation. A single large pool of guinea pig serum tested for the absence of salmonella antibodies was preserved by the addition of NaCl to 8.5% and was stored frozen at −20 C. The thawed serum was diluted 1:10 with distilled water, and an amount was used to give a final concentration of 1:100. This amount was found to be in excess 10- to 20-fold, and was used to avoid the possibility of any anticomplementary effect of other reagents. Included in each series of tests was a known positive serum as a control for sensitization of erythrocytes, as well as a test in

which the serum was replaced with saline. All series of tests included negative sera. All the mice were bled prior to use. These sera were tested to preclude the pre-existence of antibodies that might give a positive reaction in the hemolysis test.

RESULTS

Effectiveness of cultural technique. The cultural technique outlined in the preceding section was found to give the highest recovery of salmonellae from infected mouse specimens. The use of two media not only increased the size of the inoculum plated but also made it possible at times, by their different selectivities, to isolate the pathogen on one plate when it could not be distinguished on the other. This was most often the case when a spreading *Proteus* sp. was part of the normal intestinal flora of the mice. Certain strains had a tendency to swarm over the entire surface of a BGA plate, and thus masked the growth underneath, whereas on EMB the same organism spread only a short distance from the edge of the colonies. On the other hand, because BGA is a more inhibitory medium, the organism was well-isolated on BGA in instances where the normal flora overcrowded the pathogen on EMB. The size of the inoculum plated onto BGA was an important factor, because, if only a small number of salmonellae were present in a heavy inoculum containing many lactose-fermenting organisms, the biochemical effect which permitted their identification could not be observed on the plate. With experience, however, BGA is an excellent medium for salmonella isolation.

Comparison of agglutination, hemagglutination, and hemolytic tests. The titers of the hyperimmune sera and sera from several infected animals were determined by means of the three serological tests. Higher titers were consistently obtained with the hemolytic test than with either the agglutination or hemagglutination test, and it was often positive when the others were negative (Table 1). Because small quantities of antibody could be detected in sera with the hemolytic test, it alone was used in all subsequent experiments with infected animals.

Cultural and serological tests with infected mice. In contrast to other strains of mice which rapidly succumbed to infection with small numbers of TmW, strain CD 1 animals fell into several categories, including those which died as a result

TABLE 1. *Titers of O antibody in mouse sera as determined by agglutination, hemagglutination, and hemolytic tests*

Serum	Serological test		
	Agglutination	Hemagglutination	Hemolysis
Hyperimmune			
O	320	320	1,280
H	320	320	2,560
Infected mouse			
A-B 7	—*	—	160
A-B 12	40	—	320
A-B 14	80	160	640
A-C 1A	—	—	160
A-C 2E	—	—	320

* Since the serum dilution in the first tube was 1:40, negative titers may have been between 0 and 40.

of the infection, those which did not become infected, and those which became infected and shed the pathogen for variable periods of time. With few exceptions, mice in the first group shed continuously until death. Pathogens were always recovered from fecal specimens, and organs were obtained at autopsy. Mice which did not become infected never shed the pathogen, and no salmonellae were isolated from the organs of those which were sacrificed. The third group includes both mice from which pathogens were consistently recovered, and mice which shed intermittently during the experimental period. The shedding pattern of a typical group of animals is given in detail in Table 2.

Another group of animals similarly inoculated with TmW was subjected to a series of serological as well as cultural tests. The cultural results were similar to those of the animals listed in Table 2. During early stages of the disease, infected mice shed regularly, but a majority of them soon became intermittent shedders.

In these animals, antibody was first detectable by the hemolytic test approximately 21 days after inoculation. After 132 days, antibody was still present in most of them (Table 3). Comparison between the cultural and serological results obtained on the same day showed that all animals known to be infected gave positive serological reactions despite the fact that, at the time of testing, they may not have been shedding

TABLE 2. *Recovery of Salmonella typhimurium from feces of infected mice*

Mouse no.	No. of *S. typhimurium* received	Day after infection[a]										
		2	3	4	7	8	9	14	17	23	30	37
1	6×10^4	+	+	+	+	+	+	+	+	+	+	+
2		+	+	+	+	+	+	+	+	+	+	−
3[b]		+	−	+								
4		+	+	+	+	+	+	+	+	+	+	−
5		+	+	+	+	+	+	+	+	+	+	−
6[c]	6×10^5	+	+	+	+							
7		−	−	−	−	+	−	+	−	−	−	−
8		−	−	−	−	−	−	−	−	−	−	−
9		+	+	+	+	+	+	+	+	−	+	−
10		+	+	+	+	+	+	+	+	+	+	+
11	6×10^6	+	+	+	+	+	+	+	+	+	+	+
12		+	+	+	+	+	+	+	+	+	−	−
13		+	+	+	+	+	+	+	+	+	+	+
14		+	+	+	−	−	+	+	−	+	−	−
15		−	−	+	+	+	+	+	+	−	+	−
16	6×10^7	+	+	+	+	+	+	+	+	+	+	+
17[d]		+	+	+		+	+	+	+			
18[e]		+	+	+								
19		+	+	+	+	+	+	+	+	+	+	+
20		+	+	+	+	+	+	+	+	+	+	+
21	6×10^8	+	+	+	+	+	+	+	+	+	+	+
22		+	+	+	+	+	+	+	+	+	+	+
23		+	+	+	+	+	+	+	+	+	+	+
24		+	+	+	+	+	+	+	+	+	+	+
25		+	+	+	+	+	+	+	+	+	+	+

[a] Symbols: + = cultures positive; − = cultures negative.

[b] Died on 7th day.

[c] Died on 8th day.

[d] Died on 21st day.

[e] Died on 4th day.

the infecting organism. Animals which never had a positive fecal culture never gave a positive serological test, and were presumed to have resisted infection completely. The last row in Table 3 indicates the shedding pattern of each animal listed.

DISCUSSION

During an acute outbreak of salmonellosis in a mouse colony, diagnosis is not a major problem. A large number of animals succumb to the disease, and other infected mice shed large numbers of the causative agent which can be detected upon fecal culture. After the acute stage of the disease, however, many survivors retain foci of infection, and shed only intermittently for long periods of time. The detection of these carriers is of prime importance, because they serve as the potential source of another epidemic whenever a new group of susceptible mice is collected.

In the past, most emphasis has been placed on the detection of carriers by means of periodic fecal culturing of a random sample of mice. An extensive study of some important factors involved in such procedures was reported recently by Margard et al. (1963). The modification of their technique used in this work was found to be highly satisfactory for isolation of salmonellae. However, even the most efficient cultural technique cannot identify infected animals which are not shedding on the day they are sampled. A more consistent indication of infection would be detection of antibodies in the sera of these animals.

Several earlier investigators of salmonellosis in mice were able to demonstrate low titers of O antibody in their infected animals by means of bacterial agglutination (Topley and Ayrton, 1924; Hobson, 1957). However, a positive titer was not always present in culturally positive animals. This was found to be true also by Sacquet (1958), who accepted both O and H agglutination titers as low as 1:10 as an indication of infection. On the basis of such studies, mice were thought to produce poor titers of O antibody, and serological tests for diagnosis of salmonellosis were not widely used.

The results obtained in these studies with hyperimmune sera indicate that it is possible for mice to produce significant quantities of O antibody detectable by the agglutination test. However, because lower titers of antibody undoubtedly occur during natural infection, a more sensitive serological test than bacterial agglutination was sought. Hemagglutination tests employing erythrocytes modified with extracts of antigens, in general, are more sensitive in detecting antibody than are standard agglutination tests. Yet, when tested with the hyperimmune sera and sera from infected mice, hemagglutination was not found to be superior to standard agglutination in detecting small amounts of antibody in the sera. A similar situation was encountered by Neter et al. (1952), in studies

TABLE 3. *Hemolysis titers of mice infected with Salmonella typhimurium*

No. of days after infection	10^{3} [a]		10^4					10^5			10^6		10^7			10^8		10^9
	18[b]	20	21	22	23	24	25	27	28	30	32	33	36	39	40	41	42	47
21	0	0	40	0	0	80	0											
28	0		80	0	40	80	0	40	40	640[c]	160	160	640	0	0	160	160	80
34			640	0	320	320	160	640	320	640	640	640	640	640	640	640	640	320
46	0	0	320	0	320	320	320		320	640	400	640	640	640	640	400	640	640
62	0	0	640	0	640	640	640	640	320	640	320	640	640	640	640	640	640	640
87			320		320	160	320	640	160	640	80	320	640	640	640	320	640	80
132			40		80	0	160	640	160	640	160	640	640	640	640	80	160	40
140										640		640	320	320				
Shed[d]	0	0	I	0	I	I	I	I	I	I	I	+	I	I	I	+	I	I

[a] Infecting dose of organism.

[b] Figures indicate animal number.

[c] In most instances, the titration was carried out only to a dilution of 1:640. Titers indicated as 640, therefore, may be considerably higher. For example, on the 46th day, serum no. 30 was found to have a titer of >6,400, and 33, 36, 39, 40, 42 had titers of 3,200.

[d] Symbols: 0 = salmonella never recovered; + = salmonella consistently recovered; I = salmonella recovered intermittently.

with rabbit antisera prepared against *Escherichia coli* strains O111 and O55. Hemagglutination and bacterial agglutination titers of the sera were alike. However, by adding complement to the hemagglutinating system which contained sheep erythrocytes coated with an extract of *E. coli*, Neter et al. (1952) obtained titers far exceeding those of the other tests. The superiority of this hemolytic test was also shown in our studies for, as a rule, at least a fourfold greater difference in titer was obtained when complement was present in the system, and the hemolytic test was often positive when the hemagglutination test was negative.

The usefulness of the hemolytic test, when applied to detection of salmonella carriers, is illustrated in the work performed with strain CD 1 mice. These animals proved to be more resistant to the lethal effects of virulent *S. typhimurium* than were other strains of mice studied. Even though they were inoculated with far greater numbers of bacteria than would be encountered in a natural situation, after 1 month a number of survivors had become intermittent shedders. No predictable pattern of shedding could be discerned. It is evident, therefore, that, on the basis of cultural results alone, many infected animals might have been declared salmonella-free. The hemolytic test performed at the same time, however, indicated that good titers of antibody were present in the sera of these mice. Although the serum titration was usually carried out to a dilution of 1:640, in several instances, when it was continued to the end point, titers of 3,200 or greater were found. These figures are far in excess of those obtained by others using agglutination tests, and obviate the question of whether a titer as low as ten is truly significant of infection.

Because of the time necessary for the development of antibody, the hemolytic test is of greatest value during chronic stages of infection. Because cultural results are most satisfactory early in the disease, cultural and serological tests used in conjunction will uncover the greatest number of infected animals. Familiarity with the dissemination of infection through a colony, however, should enable one to apply one or the other technique with maximal effect.

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