Improved Serodiagnosis of *Salmonella* Enteric Fevers by an Enzyme-Linked Immunosorbertent Assay

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The value of the enzyme-linked immunosorbent assay (ELISA) for the serological diagnosis of typhoid or paratyphoid fevers was tested with a collection of five sets of sera from 23 individuals who had no history of *Salmonella* infection from a nonendemic area (set 1) and from 143 patients from a highly endemic area (sets 2 to 5). The test was performed with a crude cell envelope fraction derived from *Salmonella typhi*. On the basis of results with sera in set 1, titers >160 (inverse of serum dilution) for immunoglobulin M (IgM) or >500 for IgG were regarded as signifying a specific antibody response. These titers were occasionally exceeded in sera in set 2, derived from patients with no clinical or laboratory evidence of *Salmonella* infection. In presumptive cases of typhoid or paratyphoid fevers (culture positive and agglutination negative or vice versa; 13 patients, set 3, and 26 patients, set 4, respectively), IgG antibodies were encountered more frequently than IgM, but some sera were negative in each group. In 81 confirmed cases (culture and agglutination positive, set 5) IgG antibodies were detected in at least one of the paired sera from all patients, and the IgG titers were higher (median 3,500) than those of the IgM antibodies (median 600). There was no evidence of a significant difference in IgM or IgG titers between acute- and convalescent-phase sera. The titers of 10 paired sera from patients from whom *S. enteritidis*, bioserotype paratyphi A, was isolated were somewhat lower than those of 84 patients with *S. typhi* isolation (serogroup D). There was some correlation between O antigen agglutination and IgM enzyme-linked immunosorbent assay, but otherwise the titers in the two serological tests varied independently. Of 11 patients in set 3 (2 patients with serogroup B agglutinins omitted), 1 belonged to blood group O, in contrast to 54 group O among 107 agglutination-positive patients, a distribution not necessarily reflected by the enzyme-linked immunosorbent assay. This study has provided good evidence that the enzyme-linked immunosorbent assay can serve as a valuable aid to the diagnosis of *Salmonella* infection in an endemic area, as a substitute for the agglutination test.

The value of the Widal test (bacterial agglutination) for the diagnosis of febrile illnesses due to *Salmonella typhi* and other *Salmonella* has been analyzed by several investigators. In some endemic regions where laboratory facilities are quite limited, bacterial agglutination tests are among the few available to clinicians who must differentiate enteric infection from febrile illnesses due to other bacteria, viruses, or animal parasites (1, 14). However, it is recognized that agglutination tests have serious shortcomings. Discrepancies in results obtained in different laboratories or in the same laboratory with preparations from different sources have been uncomfortably high (8, 9). In a significant number of patients with otherwise well-documented typhoid fever, development of agglutinins has not been demonstrated (14, 25, 28). On the other hand, agglutinins against *S. typhi* are often obtained in response to nontyphoid *Salmonella* infections (21, 24, 25) and sometimes in diseases not attributable to *Salmonella* (20). The most important shortcoming, however, as the test is usually performed in the clinical laboratories, is that it is primarily a test of immunoglobulin M (IgM) antibodies (5, 6, 13). Hemagglutination tests, which offer several advantages over bacterial agglutinations in terms of sensitivity and versatility (19), often depend on IgM antibodies (2, 31).

Carlsson et al. (3, 4) and Svensonsson et al. (29) applied the enzyme-linked immunosorbent assay (ELISA) to the titration of antibodies to *Salmonella* O antigens. These authors tested purified lipopolysaccharide antigens derived from several serogroups against specific rabbit

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antiseras and human sera with O agglutination titers >160 (inverse of serum dilution) for serogroups D or B. Their results indicated that the ELISA correlated well with the agglutination reaction, was approximately 100-fold more sensitive, was highly reproducible, and displayed some serogroup specificity. In a more limited study of the ELISA with Salmonella antigens, Sippel et al. (26) demonstrated that a cell envelope fraction (CE) derived from S. typhi was a satisfactory antigen. This antigen (12) is relatively crude and contains lipopolysaccharide and protein, but relatively low levels of flagellar components. Encouraging results were obtained with sera from proven typhoid and paratyphoid A cases that had reacted weakly in the agglutination test.

The availability of a serum collection from patients from a highly endemic area provided us with an opportunity to determine the value of the ELISA with a single crude antigen, prepared as described by Sippel et al. (26). The results clearly indicate that the ELISA eliminates most of the shortcomings often encountered in the agglutination test.

MATERIALS AND METHODS

Organisms. The source and maintenance of S. typhi strain T68, a human isolate, used in this study have been described elsewhere (26). Identity of this organism was confirmed by the Center for Disease Control, Atlanta, Ga.

Antigens. S. typhi CE was prepared by the modified Schnaitman technique of Hill and Weiss (12) with discontinuous sucrose gradients to separate CE from cytoplasmic membrane, as described earlier (26). As coating antigen in the ELISA, 12 lots were prepared, but only one was used to titrate the human sera. Rabbit anti-S. typhi serum (26) was used to determine the optimal antigen concentration for use in the ELISA.

Antigens for slide agglutinations, typhoid H, typhoid O, and paratyphoid A and B, were obtained from commercial sources (BBL Microbiological Systems, Cockeysville, Md.).

Human sera. Five sets of human sera were selected for this study (Fig. 2). The first set comprised 23 single serum specimens from 11 United States resident laboratory personnel and from 12 United States Marine recruits (the latter kindly furnished by E. Edwards, U.S. Naval Health Science Research Center, San Diego, Calif.) with no known history of Salmonella infection or recent vaccination. Sets 2 through 5 were paired sera collected from hospitalized Indonesian patients who participated in a fever study conducted at the Jakarta Detachment, U.S. Naval Medical Research Unit No. 2 (1). The first serum collected at the hospital was designated acute-phase serum, although the interval between serum collection and day 1 of recognized illness was quite variable. The sera collected 10 to 14 days later were designated convalescent-phase sera. Set 2 was derived from 23 patients for whom there was no clinical or laboratory evidence of a Salmonella infection. Set 3 was obtained from 13 patients from whom S. typhi serogroup D (12 patients) or S. enteritidis bioserotype paratyphi A (1 patient) was isolated, but agglutination tests were negative (inverse of serum dilution ≤ 20) with serogroup D or A antigens. Two of these patients had titers of 80 with serogroup B antigen. Convalescent sera were obtained from only 5 of these 13 patients. Set 4 was from 26 blood culture-negative patients presumed to have had a Salmonella infection on the basis of agglutination titers with serogroup D, O, or H antigens ≥ 80. Set 5 was derived from Salmonella enteric fever cases diagnosed by both positive culture and agglutination. S. typhi was isolated from 72 of these patients, and S. enteritidis bioserotype paratyphi A was isolated from 9 patients. Included in the definition of agglutination positive were all sets of paired sera in which at least one serum had a titer ≥ 40 against serogroup D or A antigens. However, most of these paired sera had at least one titer ≥ 80 against serogroup D antigens. All sera were maintained at −70°C until used.

Microplate ELISA procedure. All antigen and serum titrations presented in this communication were performed by the ELISA using the alkaline phosphatase reaction exactly as described by Halle et al. (11) and Dasch et al. (7). Serum IgG and IgM activities were titrated by using four to six half-log10 dilution steps beginning with 1:160. Both members of a given serum pair were titrated in the same assay, each assay included the same positive and negative reference human sera, and all tests were done in duplicate. The ELISA titer was defined as the reciprocal of the serum dilution (obtained from the titer curve by interpolation) that produced an optical density reading of 0.30 (approximately 0.25 optical density unit above controls) at 400 nm after 60 min incubation at 37°C (7).

Ethanethiol treatment. Undiluted human sera were treated with 0.03 M ethanethiol (Eastman Organic Chemical Div., Eastman Kodak Co., Rochester, N.Y.) for 3 h according to a modification of the procedure of Murray et al. (16) described by Halle et al. (11) and used immediately in the bacterial agglutination test employing Salmonella O and H agglutinogens (serogroup D) (Difco Laboratories, Detroit, Mich.). Samples were subsequently incubated uncapped for 30 min at 37°C to allow residual reagent to evaporate (11) before use in the ELISA. Also, with each examination of the test specimens, appropriate negative and positive control sera treated in the same fashion were included as a quality control.

Isoagglutinin determinations. We are indebted to W. P. Monaghan and N. Olenyik, Blood Bank, National Naval Medical Center, Bethesda, Md., for the performance of isoaagglutinin tests against A and B blood group antigens, as described by Miller (16).

RESULTS

Reproducibility of the ELISA. The binding activity of S. typhi CE antigen was tested against homologous rabbit antisera. The results (Fig. 1) indicate that 10 μg of protein per ml was the
smallest concentration that optimally coated the ELISA plates and that variation among 12 preparations was quite small. Subsequent experiments with human sera were done with just one of these preparations which did not change in serological activity during 20 months of storage at -20°C.

The human immunoglobulin titers against the CE antigen were also reproducible. In 34 assays, performed during a 9-month period, of the same positive and negative reference sera maintained at 4°C, the mean titers ± standard deviations were, respectively, 5,230 ± 670 and 220 ± 55 for IgG, and 1,040 ± 230 and invariably <160 for IgM. Comparable titers were obtained when antigen-coated plates were stored for 6 months at -20°C before completion of the tests.

**Titration of human sera by ELISA.** The results of a total of 301 assays for IgM and IgG antibodies are illustrated in Fig. 2.

Of 23 sera (set 1) from individuals from a nonendemic area with no known history of disease none had IgM titers >160, whereas 4 had IgG titers >160 but <500. On the basis of these results and the results with the reference sera, the tentative assumption was made that only IgM titers >160 and IgG titers >500 may indicate a significant response to the test antigen. The validity of this assumption can be examined by analysis of the other sets in Fig. 2.

Results similar to those described above were obtained with 23 paired acute and convalescent sera obtained from Indonesian patients with illnesses unrelated to *S. typhi* (set 2). Only one IgM titer was >160, and the same serum had a moderately high IgG titer (2,300). The significance of these titers is not known, since both IgM and IgG titers from the convalescent phase serum from the same patient were <160. Another convalescent serum IgG titer slightly >500 was accompanied by an acute-phase serum IgG titer slightly <500. Most of the other IgG titers >160 but <500 were obtained in both specimens from the same patient.

Greater variation in ELISA antibody response was encountered in the sera from 13 patients who were culture positive (*S. typhi*, 12 isolations; *S. enteritidis*, 1 isolation) but agglutination negative with serogroup D or A antigens (set 3). Two of these patients (*S. typhi* isolations) with agglutinins against serogroup B antigen had moderately high acute-phase IgM and IgG ELISA antibodies. Unfortunately, the convalescent-phase serum from the patient with the highest acute-phase IgG titer (12,500) was not available. Among the remaining 11 patients there were 2 acute-phase sera positive for IgM (>160) and 5 (including the 2 IgM-positive sera) positive for IgG (>500). The five convalescent phase sera that were available (including one from the patient with serogroup B antibodies) had titers comparable to their acute phase counterparts. The sera from the *S. enteritidis* patient were negative by ELISA.

Even greater variation was seen in the sera from patients who were culture negative and agglutination positive (set 4). Somewhat less than half of the ELISA IgM titers were positive, and about three-fourths of the IgG titers were >500. There was an increase in the number of IgM-positive reactions in the convalescent sera, and a greater number of IgG than IgM reactions were positive. Otherwise, there was good correlation between IgG and IgM antibodies and antibodies in the acute- and convalescent-phase sera. There was no obvious difference in the agglutination titers of the ELISA-positive and -negative reactors, with the possible exception of slightly greater incidence of agglutination positive with only H or O antigen in the negative ELISA reactors.

The great majority of acute and convalescent sera derived from patients who were both culture (*72 S. typhi* and 9 *S. enteritidis* isolations) and agglutination positive (set 5) had IgM and IgG antibodies that ranged in titer from 160 to 6,000 and from 360 to 45,000, respectively. The IgG titers were generally higher (median, 3,500) than the IgM titers (median, 600). There was no obvious difference between titers obtained from acute- and convalescent-phase sera. The latter observation was confirmed by plotting the acute-phase serum titer against the convalescent-phase serum titer of each culture- and agglutination-positive patient (Fig. 3). The results in-

![Fig. 1. Determination of optimal concentration of S. typhi CE antigen for coating of wells in the ELISA. The closed circles represent the mean values for 12 CE preparations; the vertical segments indicate the standard deviations.](http://jcm.asm.org/)
The patterns are very similar to those of the other sera, except that the median titers are somewhat lower (400 and 1,500 for IgM and IgG, respectively) even when the sera from the agglutination- and ELISA-negative patient are excluded. Because of the high degree of variation in this small sample, the significance of this reduced reactivity is not apparent.

**Ethamethiol treatment of the sera.** Ethamethiol has been used to selectively destroy IgM to demonstrate the presence of this immunoglobulin class in immune sera (18), and this treatment has been shown to be compatible with the ELISA procedure (11). Serum specimens from seven proven _Salmonella_ infections were treated with the reagent, and residual antibody activities were then measured with both the ELISA and agglutination tests. Effects of ethamethiol on the serological activities of these sera are shown in Table 1. The ELISA IgG titers were not appreciably affected by this treatment, but IgM titers were drastically reduced. The concomitant reductions in O and H agglutinin activities confirm the finding (5, 6, 13) that the
agglutination reaction is brought about primarily by IgM antibodies.

Correlation between ELISA and agglutination titers. The sera from set 5 of confirmed *Salmonella* patients were divided into subsets of identical H or O antigen agglutination titers. The geometric means ± standard deviations of the ELISA IgM titers of these subsets of sera are shown in Fig. 5. There is some correlation between ELISA IgM and O agglutination titers, but not necessarily H agglutination titers, in the acute-phase sera (A and B). No correlation can be detected in convalescent-phase sera (C and D) or between H or O agglutination and ELISA IgG titers (data not shown).

**Blood group distribution of patients in relation to the antibody response.** Intestinal bacteria have been implicated in the stimulation of isoagglutinins, but this stimulation is limited by the presence of the corresponding blood group antigens (27). It is reasonable to expect, therefore, that the antibody responses of typhoid and paratyphoid patients might be influenced in some way by their blood group. This possibility was investigated by determining the presence or absence of anti-A and anti-B isoagglutinins (and thus, indirectly, the blood group) of 118 patients with confirmed or presumptive diagnosis of ty-
phase sera; geometric mean ELISA titers; the vertical segments indicate the standard deviations. (A and B) acute-phase sera; (C and D) convalescent phase sera; (A and C) H agglutination; (B and D) O agglutination.

**Table 2. Antibody response as determined by agglutination in confirmed and presumptive cases of typhoid and paratyphoid fever in relation to blood group**

<table>
<thead>
<tr>
<th>Set</th>
<th>Culture</th>
<th>Agglutination (antigen)</th>
<th>D and/or A</th>
<th>B only</th>
<th>Total patients</th>
<th>Blood group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>11</td>
<td>1 6 3 1</td>
</tr>
<tr>
<td>3b</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>2</td>
<td>1 1 1</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>25°</td>
<td>17 5 2 1</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>80°</td>
<td>36</td>
<td>21 19 4</td>
</tr>
</tbody>
</table>

*a Determined by the presence of isoaagglutinins in serum.  
*b Isolation of S. typhi or S. enteritidis.  
*c As defined in Materials and Methods.  
*d Probability of random distribution by chi square <5% in the following comparisons: blood groups A/O set 3a versus set 4 or 5; A + B + AB/O set 3a versus set 4 or sets 3b + 4 + 5.  
*e One patient omitted because of insufficient serum.

**Table 3. Antibody response as determined by ELISA in confirmed and presumptive cases of typhoid and paratyphoid fever in relationship to blood group**

<table>
<thead>
<tr>
<th>Bacterium isolated</th>
<th>ELISA</th>
<th>Total patients</th>
<th>Blood group</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhi</td>
<td>IgM−</td>
<td>12</td>
<td>3 6 2 1</td>
</tr>
<tr>
<td></td>
<td>IgM+</td>
<td>71</td>
<td>31 20 16 4</td>
</tr>
<tr>
<td></td>
<td>IgG−</td>
<td>5</td>
<td>3 1 1</td>
</tr>
<tr>
<td></td>
<td>IgG+</td>
<td>78</td>
<td>34 23 17 4</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>IgM−</td>
<td>3</td>
<td>2 1</td>
</tr>
<tr>
<td></td>
<td>IgM+</td>
<td>7</td>
<td>3 2 2</td>
</tr>
<tr>
<td></td>
<td>IgG−</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IgG+</td>
<td>8</td>
<td>4 2 2</td>
</tr>
<tr>
<td>None</td>
<td>IgM−</td>
<td>13</td>
<td>10 2 1</td>
</tr>
<tr>
<td></td>
<td>IgM+</td>
<td>12</td>
<td>7 3 1 1</td>
</tr>
<tr>
<td></td>
<td>IgG−</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IgG+</td>
<td>20</td>
<td>13 4 2 1</td>
</tr>
</tbody>
</table>

*a Determined by the presence of isoaagglutinins in serum.  
*b IgM+ is defined as serum titer >160; IgG+ is defined as a titer of >500 in at least one of two sera.  
*c Agglutination positive.

demonstrated that blood group O patients are the most likely to develop antibodies in response to Salmonella antigens. There is a slight preponderance of blood group O patients among those who were agglutination positive but culture negative (Table 2, set 4), but the difference in distribution between this series and the patients who were both culture and agglutination positive (set 5) is not significant.

Table 3 illustrates the division of the sera from the same 118 patients on the basis of the ELISA results. Although most of the 11 patients in set 3a (Table 2) were IgM negative by ELISA, a few sera from the other sets were IgM negative as well. The net result is a blood group distribution among IgM-negative patients that does not differ significantly from that of the majority. The same is true of the distribution among IgG-positive patients, possibly because their number was small (Table 3, S. typhi isolations) or Salmonella infection had not been confirmed (Table 3, no bacterium isolated).

**DISCUSSION**

The ELISA, as performed in this study, meets the requirements of objectivity, reproducibility, sensitivity, and simplicity. Some of the earlier difficulties encountered by Sippel et al. (26), resulting in a relatively low span of difference in optical density readings between positive and negative sera, were eliminated by strict adherence to the protocols of Halle et al. (11) and Dasch et al. (7) (Fig. 1). The amount of antigen required for the test (10 μg/ml) is comparable to
that used by the investigators cited above and by others (3, 4, 29). It was shown by Halle et al. (11) that the reaction is improved by the removal of extraneous components from the antigen preparation. We selected, however, a crude antigen, containing O antigen 12, common to three Salmonella serotypes frequently pathogenic in humans, which could be expected to be broadly reactive at the expense, perhaps, of the serological specificity achieved by Svennungsson et al. (29). The results shown in Fig. 2 are endpoint titrations, but, as in the case of other studies (7, 11, 29), entirely satisfactory results can be obtained within limits with appropriate single serum dilutions, thereby permitting an estimation of titers directly from optical density values (not shown). Although a spectrophotometer is convenient, under field conditions a semiquantitative estimation of serum titers can be obtained by visual inspection (7, 11). The usefulness of the precoated plate, demonstrated in this study, may further contribute to the simplicity and reproducibility of the test under field conditions.

The sera used in this study come from a variety of clinical situations. If the criterion is adopted that IgM titers ≤160 and IgG titers ≤500 are negative, all sera from individuals from a nonendemic area with no known history of disease were negative. These results compare favorably with those of Carlsson et al. (4), who obtained somewhat higher titers against serogroup D and B antigens in his control sera. As expected, in a comparable examination of control sera from an endemic area, an occasional serum was positive. It is not known whether this was due to previous exposure to Salmonella antigens or to unrelated causes. When the diagnosis of typhoid or paratyphoid fevers was presumptive, i.e., when either the agglutination test or blood culture was negative, the ELISA confirmed the presence of IgM or IgG antibodies in some patients, but five patients in each group remained negative (Table 3). It appears, therefore, that additional blood cultures (only one attempt was made per patient) or more extensive serology would have narrowed the number of unconfirmed Salmonella diagnoses, but some still would have remained unconfirmed. These results contrast with the confirmation of antibodies by ELISA in that at least one serum specimen in all culture- and agglutination-positive patients was positive. The value of determining IgG as well as IgM antibodies with the ELISA, as previously illustrated by Svennungsson et al. (29), is obvious, since both the frequency of occurrence and titer of IgG antibodies were higher in our study.

The well-established criterion for serological diagnosis by rising antibody titers in paired sera was not valid in this study. It was not possible to extract from the data shown in Fig. 2 even a small but significant subset of paired sera in which a clear rise in titers did occur. This finding can be attributed to several causes. Most of the patients who sought admission to the hospital had been febrile for several days and might have achieved peak antibody titers at the time the first blood specimen was withdrawn. Some patients might have been antigenically stimulated through inapparent infection before illness and might have responded anamnestically. In addition to serotype D, serotype A strains are apparently quite common in Indonesia (22, 23), and serotype B strains have been isolated from surface waters in Jakarta (10). There is some indication that anamnestic responses have occurred in some of the patients of this study, because, at least in the agglutination test, the patients did not always respond preferentially to the serogroup antigen of the Salmonella species that was isolated from them. This aspect was not studied in the ELISA, but it is obvious (Fig. 4) that the patients reacted reasonably well with heterologous antigens. The effect of chloramphenicol treatment on the serological response is not known, but the ELISA titers of 32 patients who claimed to have received chloramphenicol prior to hospital admission were not different from those of 28 patients who claimed not to have been so treated (data not shown).

The chemical composition of the CE antigen used in the ELISA most closely resembles the O antigen of the agglutination test. It is not surprising, therefore, that there was some correlation between the IgM response demonstrated by ELISA and O agglutination in the acute-phase sera (Fig. 5), and in this respect the results agree with those of Carlsson et al. (4). However, other correlations in titer could not be made, even though the overall correlation between positive ELISA and positive agglutination is good (Fig. 2). These results indicate that the ELISA, even with a crude antigen, measures antigen-antibody reactions not detected by agglutination (Table 1) which is heavily biased for IgM detection. In our limited experience, an Enterobacteriaceae common antigen or rheumatoid factor did not influence the ELISA reaction.

The lack of agglutinin development in some patients with well-documented typhoid fever, including human volunteers, has surprised a number of investigators (14, 25, 28). The results shown in Table 2 which suggest some correlation between blood group and diminished agglutinin response against Salmonella antigens, not necessarily reflected in the ELISA (Table 3), should
be viewed with caution, since a relatively small number of patients was involved and the serological studies have not been extensive. However, this observation cannot be disregarded since a somewhat similar association involving blood group B individuals has been suggested for the Weil-Felix Proteus OX-19 reaction for typhus fevers (30). If such investigations are pursued or extended to reported instances of association between certain human blood group or leucocyte antigens and some types of Salmonella infections (reviewed in 15, 17), the ELISA would be the technique of choice because of its objectivity and versatility.

In conclusion, this study has provided good evidence that the ELISA performed with a single CE antigen derived from S. typhi is a valuable aid to the diagnosis of Salmonella infection in an endemic area and is a suitable alternative to the agglutination test as it is performed in many laboratories.

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