Detection of *Salmonella* Infections by Polyvalent Enzyme-Linked Immunosorbent Assay

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Serum immunoglobulin G (IgG) and IgM were measured for individual *Salmonella* species by the enzyme-linked immunosorbent assay (ELISA). F344 rats were experimentally infected with *Salmonella typhimurium* (serogroup B), *S. enteritidis* (serogroup D), and *S. rubislaw* (serogroup G). Endotoxin extracted from each serogroup served as the antigen in a classical indirect ELISA. Antibody specific for each *Salmonella* serogroup was detected by ELISA. Normal gut flora from control animals appeared not to cause cross-reactions in the ELISA. Specificity and sensitivity of the IgG ELISA were determined by statistically evaluating false-positives and false-negatives. Ideal values of 90% or better were achieved in nearly all instances. Each antigen was also tested with heterologous antisera in an effort to develop a polyvalent assay for *Salmonella* species. No single antigen detected all positive heterologous antisera. Therefore, a polyvalent antigen composed of the three serogroup antigens was tested. The results suggested that *Salmonella* infections can be detected by measuring serum IgG levels with a polyvalent ELISA 6 to 9 days postinfection.

In vitro culture is the predominant means for isolating and identifying *Salmonella* species from fecal samples. This is time consuming, usually requiring 72 to 96 h for the organism to be identified by its cultural, biochemical, and serological properties. An alternative method is to detect serum antibodies formed against infecting *Salmonella* species. The antibodies can be detected by the Widal (tube agglutination) (16) or passive hemagglutination test (10). These tests are not used routinely due to technique-related insensitivity and nonspecific cross-reactions. However, detection of serum antibodies to *Salmonella* infections is still theoretically more desirable for mass screening than in vitro culture. The enzyme-linked immunosorbent assay (ELISA) has been developed for a wide variety of antigen-antibody reactions (4, 6, 15), indicating promise for determination of antibody titers to *Salmonella* infections.

An ELISA test for detection of *Salmonella* antibodies was described by Carlsson et al. (5). Antibody levels were compared both quantitatively and qualitatively by ELISA, Widal agglutination, passive agglutination, and quantitative precipitation tests. ELISA was the most sensitive test. Later, group-specific antigens were used to stimulate an immune response in rabbits (4), and serum antibody was measured by the ELISA method. Recently, *Salmonella enteritidis* and *S. typhimurium* outbreaks have been diagnosed by ELISA (8, 14). As yet, researchers and diagnosticians in general have not used the ELISA as a clinical polyvalent test to efficiently screen large numbers of animals for *Salmonella* antibody titers.

The objective of this investigation was to develop an ELISA for detection of *Salmonella* species serum antibodies in rodents and evaluate a polyvalent antigen for use in the ELISA. An in vitro serological test of this nature would be highly desirable for diagnostic screening of large numbers of rats and mice in commercial colonies or in research facilities. Since *Salmonella* serogroups B and D represent a high percentage of *Salmonella* strains isolated from humans (14) and these serogroups contain nearly all of the strains causing disease in mice and rats (9), the test was directed against these serogroups. In addition, these studies were intended to examine the possibility of detecting, by ELISA, subclinical infections or carrier states. Furthermore, the potential for false-positives due to cross-reactions with other gut flora was explored and shown to be negligible.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* LT2 wild type, O:4,5,12 (ATCC 15277), *S. enteritidis*, O:1,9,12 (ATCC 13076), and *S. rubislaw*, O:11 (ATCC 10717), were used. An isolate of *S. enteritidis* from a porcine source was used to infect experimental animals.
Antigen and endotoxin preparation. Each strain was grown in Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.) at 37°C overnight. The cells for antigen preparation were harvested by centrifugation at 10,000 \(\times g\). Endotoxin was extracted by the method of Roberts (12). Two grams of dried cells was suspended in 200 ml of distilled water heated to 80°C for 60 min. The mixture was allowed to cool to room temperature and then centrifuged at 6,000 \(\times g\) for 30 min. The residue was extracted once more with 100 ml of distilled water. The pooled supernatants were filtered through a Whatman no. 42 filter and concentrated to approximately \(\frac{1}{2}\) the original volume. The concentrated solution was dialyzed overnight at 4°C against distilled water. It was then concentrated to approximately 25 ml and dialyzed overnight against running tap water. This was followed by overnight dialysis against distilled water at 4°C. The endotoxin solutions were then lyophilized and stored at room temperature.

Sera. (i) Hyperimmune sera. Hyperimmune sera were prepared for each of the three serotypes of *Salmonella*. Each of three groups of five 3-month-old, conventionally housed male F344 rats received a different serotype intramuscularly. Formalinized cells were prepared from 12-h cultures in TSB. The 12-h cultures were centrifuged and washed twice in phosphate-buffered saline. The packed cells were then resuspended in Formalin. A 0.2-m1 suspension of formalinized cells in Freund incomplete adjuvant (1:1) was used. This was followed by an intraperitoneal injection of 0.2 ml of heat-killed organisms (from 12-h cultures) in TSB 16 and 30 days later. Blood was drawn on day 37. Uninoculated cohort animals were bled for use as reference negatives.

(ii) Experimental infection. To simulate a natural infection, three groups of nine 4-week-old male F344 rats were inoculated by gastric gavage with 0.5 ml of TSB containing \(1.0 \times 10^5\) to \(3.5 \times 10^6\) live organisms. Each group was inoculated with a different *Salmonella* serogroup. Additionally, there was a cohort group of nine uninoculated rats. Blood was drawn by cardiac puncture from two animals in each group three times weekly through 28 days post-inoculation. Just before initial inoculation and on days animals were bled, fecal pellets were cultured in gram-negative broth. Uninoculated control animals were bled and cultured on the same schedule as inoculated animals.

ELISA. The ELISA test was performed essentially as described by Carlsson et al. (5). However, microtiter plates (MA Bioproducts, Walkersville, Md.) were used instead of polystyrene tubes. The plates were coated with endotoxin (10 \(\mu g/ml\)) for 4 hr at 37°C. Sera were diluted 1:20 and 1:100, and all tests were done in duplicate. Anti-rat immunoglobulin (IgG) and anti-rat IgM conjugates were prepared according to the procedure outlined by Horowitz and Cassell (7). Substrate (p-nitrophenyl phosphate) was incubated at 37°C for 30 min in IgG assays and for 60 min in IgM assays. The reaction was stopped by the addition of 1 N NaOH followed by refrigeration for at least 20 min before reading at 400 nm on a Beckman DB spectrophotometer. Each sample was transferred from the microtiter plate with a Rainin P200 (Rainin, Woburn, Mass.) to a microcuvette (Hellma Cells, Forest Hills, N.Y.). As previously described (7), substrate, conjugate, and antiserum controls, plus positive (hyperimmune) and negative sera, were used.

Analysis of data. The ELISA value was determined by subtracting the conjugate control absorbance value from the average absorbance value of each test serum dilution. Initial minimal positive ELISA values (MPEV) for each *Salmonella* assay were calculated by adding 2 standard deviations to the mean of tested reference negative sera. A positive ELISA value was defined as greater than or equal to the MPEV, and a negative ELISA value was defined as less than the MPEV. Sera from infected animals could be divided into two groups: those which gave a positive ELISA value (true-positive) and those which gave a negative ELISA value (false-negative). Sera from uninfected control animals could also be divided into two groups: those with a negative ELISA value (true-negative) and those with a positive ELISA value (false-positive). The sensitivity and specificity (13) of each assay were calculated with initial MPEV and several higher minimal absorbance values. Sensitivity was calculated by the following formula: sensitivity = \(\sum\text{EV}\) of true-positives/\(\sum\text{EV}\) of true-positives + \(\sum\text{EV}\) of false-positives, where \(\text{EV}\) is ELISA value. Specificity equaled \(\sum\text{EV}\) of true-positives/\(\sum\text{EV}\) of true-positives + \(\sum\text{EV}\) of false-positives. Table 1 gives the resultant sensitivities and specificities for optimal minimal positive absorbance values.

RESULTS

Experimental infection. Animals gavaged with live *S. typhimurium* had positive IgG ELISA tests at 7 days and positive IgM tests at 9 days post-inoculation (p.i.) (Fig. 1). Between days 19 and 26, all antisera were positive on the IgG and IgM ELISA. On the final bleeding day (day 28 p.i.), negative titers were obtained from some infected animals: one of eight IgG and five of eight IgM. Animals infected with *S. typhimurium* were cultured positive on day 2, and many remained so throughout the experiment.

*S. enteritidis* IgG antibody was detected 6 days p.i. (Fig. 2). All sera collected from days 6 through 28 p.i. had positive IgG ELISA titers for *S. enteritidis*. IgM titers were detected in 12 of 19 sera tested on days 11 through 28 p.i. (Fig. 2). *S. enteritidis* was recovered from the feces of most animals on days 6 through 28.

Titers to both IgG and IgM were detected on days 9, 14, and 19 through 28 for *S. rubislaw* infected animals (Fig. 3). *S. rubislaw* was cultured from the feces of most animals on days 2 through 28.

Table 2 is a comparison of the IgG ELISA and culture results for each *Salmonella*-infected group of animals. As expected, numerous positive culture results and few positive ELISA results were observed in the 1st week for all three groups. Thereafter, the weekly trend was toward
more ELISA positives. In the final week, nearly all infected animals were ELISA positive and only a small fraction were culture positive for Salmonella.

Antisera-antigen cross-reactions. (i) Antisera versus heterologous antigens. Positive sera for each Salmonella species from experimentally infected animals were reacted with the other two heterologous antigens. Hyperimmune sera for each agent were similarly tested. Different degrees of cross-reactivity were observed among the three antigens, with S. typhimurium being the most cross-reactive (Table 3). S. enteritidis was the least cross-reactive. No single antigen detected all of the positives in the heterologous system.

(ii) Antisera versus polyvalent antigen. Ten positive serum samples from rats experimentally infected with each of the three Salmonella strains were incubated in microtiter plates coated with a mixture of the three antigens (10 μg/ml per antigen). The polyvalent ELISA antigen detected every positive serum as determined by that serum’s homologous reaction. Negative control sera were also tested with the polyvalent antigen. In three cases, the polyvalent antigen detected positive titers in sera which were negative by the ELISA using the homologous antigen (Table 4). Two of these sera were collected on day 28 from animals inoculated with a Salmonella species.

**DISCUSSION**

An ELISA for Salmonella infections of rodents was described before this study (8, 14). However, the test was not designed as a clinical assay for mass screening of rodents for Salmonella species. The study reported herein presents data on the development of a polyvalent ELISA test for detecting clinical infections and possibly for screening rodent colonies for latent infections. S. typhimurium and S. enteritidis were selected to represent serogroups B and D, which are the most common serogroups isolated in humans (14) and the most important Salmonella pathogens in rats and mice. Including an antigenically unrelated (not cross-reactive) S. rubislaw (serogroup F) provides evidence for
Fig. 2. Serum ELISA values for *S. enteritidis*-infected rats at 1:20 dilution. Base lines were calculated from 53 reference negative sera. All points above the base lines are considered positive. Points may represent more than one animal. (A) Serum IgG values. The base line is 0.072, which is 2 standard deviations above the mean of reference negative sera plus 0.010. (B) Serum IgM values. The base line is 0.172, which is 2 standard deviations above the mean of reference negative sera.

Fig. 3. Serum ELISA values for *S. rubislaw*-infected rats at 1:20 dilution. Base lines were calculated with 25 reference negative sera. All points above the base lines are considered positive. Points may represent more than one animal. (A) Serum IgG values. The base line is 0.151, which is 2 standard deviations above the mean of reference negative sera. (B) Serum IgM values. The base line is 0.000.
DETECTION OF SALMONELLA SPECIES BY ELISA

TABLE 2. Culture and ELISA results

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>S. typhimurium</th>
<th>S. enteritidis</th>
<th>S. rubislaw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture</td>
<td>ELISA</td>
<td>Culture</td>
</tr>
<tr>
<td>7</td>
<td>4/8</td>
<td>1/8</td>
<td>3/6</td>
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<tr>
<td>14</td>
<td>3/6</td>
<td>5/6</td>
<td>4/6</td>
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<td>0/6</td>
<td>5/6</td>
<td>2/6</td>
</tr>
<tr>
<td>28</td>
<td>3/12</td>
<td>11/12</td>
<td>3/9</td>
</tr>
</tbody>
</table>

* IgG ELISA results at 1:20 serum dilution.

TABLE 3. Positive Salmonella antiserum* versus homologous and heterologous antigens

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>S. typhimurium</th>
<th>S. enteritidis</th>
<th>S. rubislaw</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:20</td>
<td>1:100</td>
<td>1:20</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>6/6</td>
<td>6/6</td>
<td>4/6</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>1/2</td>
<td>1/2</td>
<td>2/2</td>
</tr>
<tr>
<td>S. rubislaw</td>
<td>5/7</td>
<td>5/7</td>
<td>2/7</td>
</tr>
</tbody>
</table>

* Serum was assayed for IgG antibody.

TABLE 4. Polyvalent ELISA* for three Salmonella species

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Dilution</th>
<th>Polyvalent antigen</th>
<th>Homologous antigen</th>
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</thead>
<tbody>
<tr>
<td>S. antimurium</td>
<td>1:20</td>
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<td>8</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>S. enteritidis</td>
<td>1:20</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>S. rubislaw</td>
<td>1:20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

* Plates coated with a mix of the three serogroup antigens. Ten animals were tested per assay.

monitoring experimental infections (Fig. 1–3). In all three groups, titers to the organisms were observed between 6 and 11 days for both IgM and IgG, as would be expected.

A simple explanation does not exist for the simultaneous rise of IgG and IgM. Typically, the IgM rise should occur 3 to 5 days p.i. However, the introduction of live Salmonella by gastric gavage into normal rats may not invoke a classical immune response. Rats seem to be more resistant than mice to Salmonella infections and generally do not develop clinical disease unless under stress. They frequently become carriers. Data presented in Table 2 substantiate a carrier state or subclinical disease. Many animals continued to shed Salmonella throughout the study without clinical disease, also suggesting that ELISA has the potential to detect Salmonella carriers. The immune response provoked by normal gut flora and carrier state should be similar. Researchers (1, 2, 11) investigating the humoral response to normal flora admit that the immune response in the gut is not well understood. Attempts have yet to be made in a complete study determining the antibody response of a noninvasive organism. Although the researchers agree that IgM, IgG, and IgA responses are involved (1, 2, 11), the degree to which each immunoglobulin class asserts itself is controversial. Furthermore, Svenungsson et al. (14) used a polyspecific antiserum and reported very low relative titers to an active infection of S. enteritidis up to 11 days in duration. It is difficult to compare results from another laboratory, but this finding supports our data. These researchers did not specifically determine the initial IgM response, making it difficult to correlate their results with ours. One other possibility for a late IgM response is that the enzyme conjugate was less sensitive. Although possible, this is unlikely since the anti-IgM conjugate was prepared similarly and from the same source as the IgG conjugate; in addition, high absorbance values (>1.0) were obtained from sera of infected animals, suggesting a highly sensitive conjugate.

A very important part of developing a clinical test is setting the minimal value to be considered a positive test. ELISA values for negative serum, 25 or more for each Salmonella species, were accumulated, and 2 standard deviations from the mean of these values was at first determined to be our base line. To ascertain the success of the test and the base line, sensitivity and specificity values were calculated. Sensitivity considers the number of false-negatives, and specificity...
were tested for S. enteritidis and S. rubislaw achieved near the ideal values. Fewer sera were tested in the S. rubislaw system. As more sera are examined, the specificity and sensitivity values for S. rubislaw should rise. Furthermore, the strictest guidelines were maintained for determining the number of true-positives. All sera were considered positive if the animal had been inoculated with the organism, even though the animal at no time had symptoms of disease or shed Salmonella. These animals probably will not seroconvert, yet by our definition they would be considered false-negatives. Due to the expected and observed decline of IgM titers to near zero, specificity and sensitivity values would be of little value and were not determined.

In reference to Table 2, a strong case can be made for ELISA as more sensitive than culture for detection of Salmonella-infected animals. For screening purposes, once the animals seroconvert, the ELISA seems to be superior to culture. However, one must keep in mind that the ELISA is an indirect technique, and confirmation of positives by other methods is suggested.

Antigen was extracted by the water method reported by Roberts (12). This method is not one of the techniques commonly used to extract lipopolysaccharide. Roberts did an extensive study demonstrating the recovery of the lipopolysaccharide fraction. At any rate, the extract in our work served very well as the antigen and did not seem to cross-react with antibodies formed against normal flora.

Next, a polyvalent antigen was evaluated for use in the ELISA. Each Salmonella antigen was tested against two other Salmonella species antisera to determine if one antigen could provide adequate cross-reactivity and thus avoid the need for a polyvalent antigen (Table 3). Although all three antigens were recognized in high frequency by heterologous antisera, no antigen was 100% effective. The polyvalent antigen provided a higher degree of sensitivity than any of the three homologous test systems (Table 4), probably as a result of the cross-reactivity of heterologous antisera (Table 3). Considering this information, we are assuming that with the correct combination of serogroup antigens, a truly polyvalent Salmonella ELISA will result.

In conclusion, the ELISA described herein provides a high level of sensitivity and specificity and has the potential to become a valuable clinical test. Intestinal flora other than Salmonella species appear not to have interfered with the assay. Our results indicated that an ELISA for quantitating IgG would be superior to measuring IgM levels. Measurable IgG titers occurred as early as IgM titers and would not decrease in long-term infections. Finally, these initial results provide strong evidence that a single polyvalent ELISA test may be capable of identifying natural infections or carrier states produced by a variety of Salmonella species. A polyvalent test would be of great significance for long-term research projects or commercial suppliers of research animals. The test would reduce expenses for shipping of animals or media to diagnostic laboratories and would greatly reduce the time necessary to establish a diagnosis.

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LITERATURE CITED

