Enzyme-Linked Immunosorbent Assay for Detection of *Salmonella typhi* Protein Antigen

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A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was designed for the detection of *Salmonella typhi* protein antigen. The optimal concentration of antibody for coating the plate was found to be 50 μg/ml. The optimum conditions for antibody coating and antigen and conjugate incubation were 37°C for 3 h, 37°C for 2 h, and 4°C overnight, respectively. The enzyme-substrate reaction was allowed to take place at 30°C for 1 h. The established ELISA was found to be reproducible, with an inter-run coefficient of variation of less than 12% for the detection of an *S. typhi* protein antigen concentration of 0.5 to 50 μg/ml. The minimal detectable level of the antigen was 0.5 μg/ml. Cross-reactions were observed with the high level (50 μg/ml) of protein antigens obtained from *Salmonella typhimurium*, *Escherichia coli*, *Salmonella paratyphi* A, and *Salmonella enteritidis*. The ELISA established was used for the detection of *S. typhi* protein antigen in serum from 62 patients with typhoid, 30 patients with clinically diagnosed typhoid fever, 21 patients with paratyphoid, 17 patients with pyrexia caused by other bacteria, and 160 normal, healthy individuals. It was found that the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of this assay were 83.87, 89.04, 87.93, 67.53, and 95.31%, respectively.

The diagnosis of typhoid fever in endemic areas mainly depends on the isolation of *Salmonella typhi* from hemoculture. It takes several days before the results can be obtained however. In addition, hemoculture can be falsely negative due to prior antibiotic treatment and inappropriate timing of specimen collection (8, 13). In some instances, the diagnosis must be based on the clinical picture in association with a positive test for antibody detection (17). Among the various immunological tests for the detection of antibody to *S. typhi* that have been developed for the diagnosis of typhoid fever (10, 14, 16, 20, 22), the most widely used is the Widal test, which is an inexpensive and simple method. It is of limited value, however, because of false-positive and -negative results (13).

An alternative method is the detection of *S. typhi* antigen in body fluids of patients with typhoid. Although tests for the detection of various types of *S. typhi* antigens have been developed (5, 10, 15, 18, 19), none of them has been confirmed as being useful for the diagnosis of typhoid fever in endemic areas. Moreover, one of them has been reported to be useless in such situations (19).

Therefore, we considered the establishment of an enzyme-linked immunosorbent assay (ELISA) for the detection of an *S. typhi* protein antigen which has been shown to induce antibody responses in patients with typhoid (1, 6, 21) and protective immunity in animals (3), with the hope that it might be useful for the diagnosis of typhoid fever in endemic areas.

MATERIALS AND METHODS

Buffers and reagents. The following buffers were prepared by the method of Gomori (9): 0.05 M carbonate buffer (pH 9.8) and 0.05 M Tris hydrochloride (pH 8.0), and phosphate-buffered saline–Tween 20 (PBST), which was 0.01 M phosphate-buffered saline (pH 7.1), containing 0.15% Tween 20.

Protein concentrations. Protein concentrations were determined by the Hartree (11) modification of the method described by Lowry with bovine serum albumin used as a reference standard.

Antigen. *S. typhi* protein antigen (BP) was prepared from *S. typhi* 0-901 by the method described by Barber and co-workers (2, 4). Briefly, washed and acetone-dried cells were extracted with Veronal buffer (pH 8.4), and BP was purified by precipitation with trichloroacetic acid.

The other protein antigens were also prepared from related enteric bacteria, i.e., *Salmonella paratyphi* A, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Salmonella enteritidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Shigella flexneri*, by the same method described above for BP. These organisms were obtained by isolation from clinical specimens and were kindly supplied by the Clinical Pathology Division, Department of Medical Sciences, Ministry of Public Health and Bacteriology Division, Department of Microbiology, Siriraj Hospital.

Preparation of rabbit anti-BP immunoglobulins. Rabbit anti-BP antisera were raised in five adult rabbits by subcutaneous administration (5 times at 3-day intervals) of 0.2 mg of BP prepared from *S. typhi*. The antisera were tested against specific antigens by the double immunodiffusion method. Only high-titer antisera were pooled and used for the preparation of anti-BP immunoglobulins by ammonium sulfate precipitation. This pooled rabbit antisera was used throughout the study and stored at −20°C until use. Such pooled antisera had no antilipopolysaccharide activity, as tested by immunoelectrophoresis against lipopolysaccharide of *S. typhi* 0-901.

Conjugate. The conjugate used was rabbit anti-BP immunoglobulins labeled with alkaline phosphatase (Sigma Chem-

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ical Co., St. Louis, Mo.) by the two-step glutaraldehyde method (7). It was stored in fractions at 4°C.

Conjugate substrate. The conjugate substrate was p-nitrophenyl phosphate and was obtained in tablet form from Sigma. The substrate was stored in the dark at −20°C. For use in the assay, p-nitrophenyl phosphate was dissolved in 0.05 M carbonate buffer (pH 9.8), containing 0.005 M $MgCl_2 \cdot 6H_2O$, to a concentration of 1 mg/ml. This solution was freshly prepared immediately before use.

Serum samples. Serum samples used in this study were single specimens, obtained from five groups of subjects and stored at −20°C until use. The following subjects were included in this study: (i) 62 patients with typhoid with positive hemoculture for *S. typhi*; (ii) 30 patients with clinically diagnosed typhoid fever, as symptoms and signs were compatible with this disease but hemoculture was negative; (iii) 21 patients with paratyphoid with hemoculture positive for *S. paratyphi* A (17 patients) and *S. paratyphi* B (4 patients); (iv) 17 patients with pyrexia caused by other bacteria, as diagnosed by hemoculture results (these included one patient with *E. coli* and *Enterobacter* spp., two patients with *Salmonella* spp. group C, three patients with *K. pneumoniae*, five patients with *E. coli*, one patient with *P. aeruginosa*, one patient with *Aeromonas hydrophila*, one patient with *Acinetobacter* spp., one patient with *Proteus mirabilis*, one patient with *Proteus vulgaris*, and one patient with *Staphylococcus aureus*); (v) 160 normal healthy individuals, whose past history of typhoid vaccination and natural infections with either *Salmonella* spp. or other related organisms were not excluded.

Established ELISA for the detection of BP. A double-antibody sandwich ELISA for the detection of BP was established by the standardization of various conditions. Optimal conditions chosen were then used to assay clinical specimens. The following methods were used. (i) Either rabbit anti-BP immunoglobulin or preimmune immunoglobulin was diluted in 0.05 M carbonate buffer (pH 9.8), containing 0.1% sodium azide, to a protein concentration of 50 μg/ml. A total of 100 μl of the solution was added to each well of a Microelisa Immulon plate (Dynatech Produkta AG, Klonten, Switzerland), which was then incubated at 37°C for 3 h. (ii) The immunoglobulin-coated plates were washed 3 times, for 3 min each time, with PBST. Excess fluid was removed by shaking them dry. (iii) A total of 100 μl of either test serum diluted 1:10 in PBST or standard BP was then added to each well. Each specimen was assayed in duplicate and incubated at 37°C for 2 h. (iv) After 3 washes with PBST, 100 μl of conjugate suitably diluted with PBST was added to each well. Then, the plate was incubated overnight at 4°C. (v) Excess conjugate was washed out, and 100 μl of the substrate was added. The plate was then incubated at 30°C for 1 h. The reaction was stopped by the addition of 25 μl of 3 N NaOH. (vi) The absorbances of the color developed were read at 405 nm (Titertek Multiskan; Flow Laboratories Ltd., Ayrshire, Scotland). In each assay, a direct conjugate control and a substrate blank were included, 100 μl of PBST was substituted for test specimens in the direct conjugate control, and 100 μl of PBST was substituted for both test specimens and conjugate in the substrate blank.

To evaluate the results, the mean absorbance value of the direct conjugate control was always subtracted from the mean absorbance value of the test samples. In addition, for some samples that had nonspecific binding with preimmune immunoglobulins, the mean absorbance value of this nonspecific binding was also subtracted from that of the test sample. Such corrected absorbance values of test samples

were then used for the evaluation of the results. The amount of the antigen present in each sample was determined by relating the corrected absorbance value of the test sample with that of the BP antigen concentration (0.5 μg/ml) and expressed as the absorbance index (AI).

Statistical methods. The statistical method used was the Mann-Whitney U test for comparison of the mean value between two independent samples.

The indices of sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of the established ELISA were calculated as follows: sensitivity $[a/(a + c)] \times 100$; specificity, $[d/(b + d)] \times 100$; accuracy, $[(a + d)/(a + b + c + d)] \times 100$; positive predictive value, $[a/(a + b)] \times 100$; negative predictive value, $[d/(c + d)] \times 100$; where a is the number of true-positive samples, b is the number of false-positive samples, c is the number of false-negative samples, d is the number of true-negative samples. Only positive results obtained from the group of patients with typhoid with positive hemoculture for *S. typhi* were considered as true positive. The positive results obtained from the other four groups of subjects were considered as false-positive for the calculation of the validities of the established ELISA.

RESULTS

Standardization of ELISA. Standardization of conditions for each step of the double-antibody sandwich ELISA was performed by checkerboard titration. Optimal conditions were selected, based on the maximum sensitivity of the test, which could be performed within a period of time suitable for diagnostic purposes. For coating the plates, the rabbit anti-BP immunoglobulins or rabbit preimmune immunoglobulins, at a concentration 50 μg/ml, were incubated at 37°C for 3 h. Incubation conditions for antigen and conjugate were 37°C for 2 h and 4°C overnight, respectively. The substrate was incubated at 30°C for 1 h.

In addition, it was found that the plates could be coated with rabbit anti-BP immunoglobulin and stored at 4°C for up to 4 weeks before use in this ELISA without any deterioration of the coating antibody.

Sensitivity and reproducibility. A standard curve was established by assaying the BP preparation over a concentration range of 0.1 to 50 μg/ml. The sensitivity and reproducibility of the established ELISA were estimated from the dose-response curve, which was constructed 25 different times over 7-month periods. By using statistical analysis (Mann-Whitney U test), the established ELISA was found to be able to detect as little as 0.5 μg/ml or 50 ng of BP antigen at the 0.1% probability level of statistical significance (α = 0.001). The reproducibility of the established ELISA is shown in Table 1. The inter-run coefficient of variation of absorbance values were lower than 12% for all antigen concentrations, except that at 0.1 μg/ml, which was 29.4%.

Specificity. To evaluate the specificity of the established ELISA for the detection of BP, the protein antigens prepared from some other related enteric bacteria, i.e., *S. paratyphi* A, *S. typhimurium*, *S. choleraesuis*, *S. enteritidis*, *E. coli*, *K. pneumoniae*, *C. freundii*, *P. aeruginosa*, and *Shigella flexneri*, were then subjected to this assay. The specificity of the established ELISA was evaluated from dose-response curves of the various antigens described above over a range of 0.1 to 50 μg/ml. All the antigens were assayed simultaneously in the same experiment.

It can be seen that the protein antigen prepared from *S. typhi* (BP) has a stronger reactivity than those from the other

TABLE 1. Inter-run reproducibility of established ELISA

Antigen concn (µg/ml)	Absorbance values (n = 25)[a]	
	χ ± SD	% CV
0.1	0.051 ± 0.015	29.4
0.5	0.102 ± 0.012	11.8
1	0.155 ± 0.017	10.9
2	0.226 ± 0.020	8.8
2.5	0.250 ± 0.021	8.4
4	0.293 ± 0.024	8.2
5	0.320 ± 0.027	8.4
7.5	0.361 ± 0.028	7.9
10	0.390 ± 0.032	8.2
12.5	0.418 ± 0.035	8.4
20	0.471 ± 0.039	8.3
25	0.492 ± 0.043	8.7
30	0.513 ± 0.036	7.0
40	0.533 ± 0.046	8.6
50	0.566 ± 0.049	8.4

[a] Abbreviations: χ, arithmetic mean of absorbance values; CV, coefficient of variation of absorbance values.

FIG. 2. Dose-response curves of antigenemia assay. Symbols: —, serum from patients with typhoid; –··–, serum from patient with paratyphoid; – –, serum from a normal healthy individual.

members of the family *Enterobacteriaceae* that were tested (Fig. 1). By using statistical analysis (Mann-Whitney U test), the difference between the reactivity of *S. typhi* protein antigen and that of *S. typhimurium*, which gave the strongest reactivity among the heterologous antigens, was found to be highly significant ($P < 0.001$) for every concentration of antigen tested.

Because the detection limit of the established ELISA was 50 ng of *S. typhi* protein antigen and results of 25 experiments, performed on different occasions, have shown that the corrected absorbance value of this antigen concentration ranges from 0.09 to 0.114, this established ELISA also
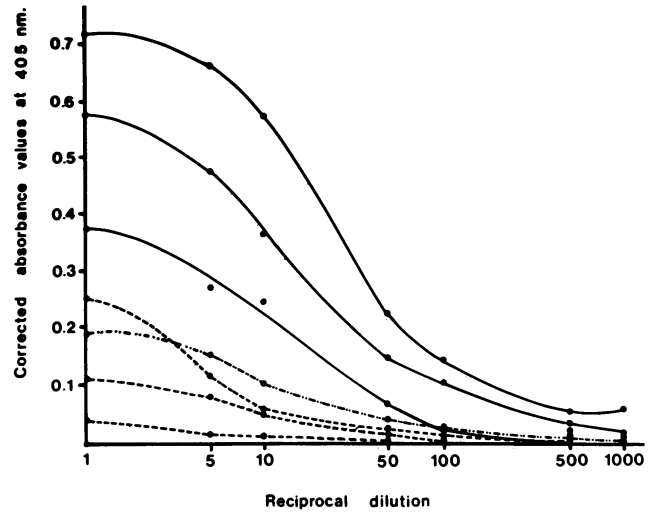
appears to detect some of those heterologous antigens if they are present at high quantities that give absorbance values higher than those that are detectable limit, such as 50 µg of *S. typhimurium*, *E. coli*, *S. paratyphi* A, and *S. enteritidis* per ml. The absorbance value of the heterologous antigens detected by the ELISA, however, were much lower than that of *S. typhi* at the corresponding concentration (Fig. 1).

Assay of test specimens. For the assay of antigen in a single dilution of serum sample, the dilution that would be optimum for use in the assay was investigated over a wide range of dilutions, starting with the undiluted sample: 1:10 to 1:1,000.
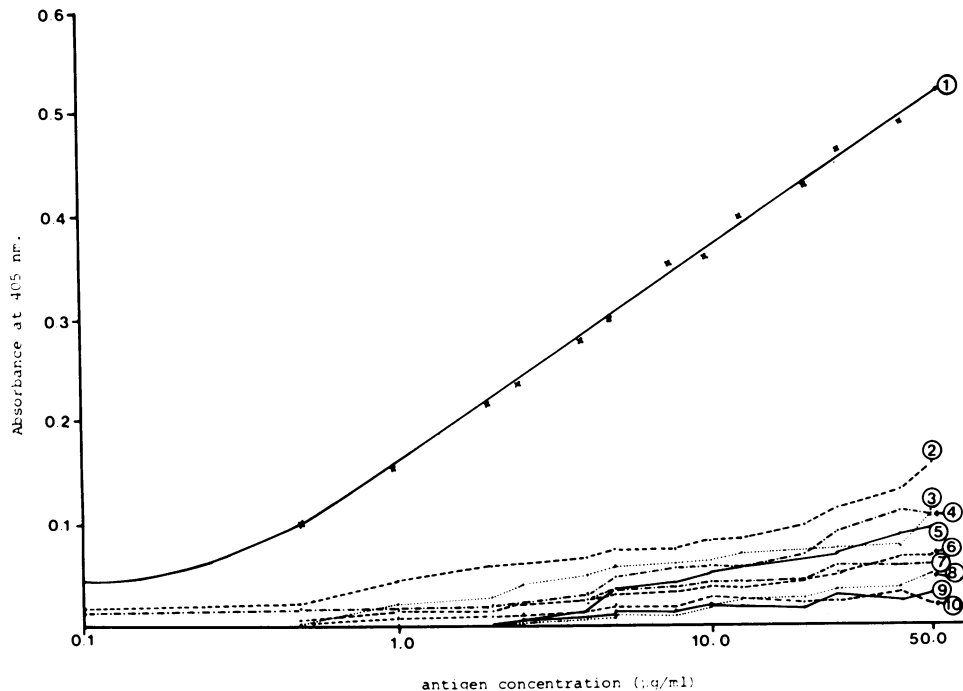

FIG. 1. Dose-response curves of protein antigens, prepared from various bacteria, as assayed by the established ELISA. The antigens were prepared from the organisms as follows: (1) *S. typhi* 0-901, (2) *S. typhimurium*, (3) *E. coli*, (4) *S. paratyphi* A, (5) *S. enteritidis*, (6) *S. choleraesuis*, (7) *P. aeruginosa*, (8) *C. freundii*, (9) *Shigella flexneri*, (10) *K. pneumoniae*.
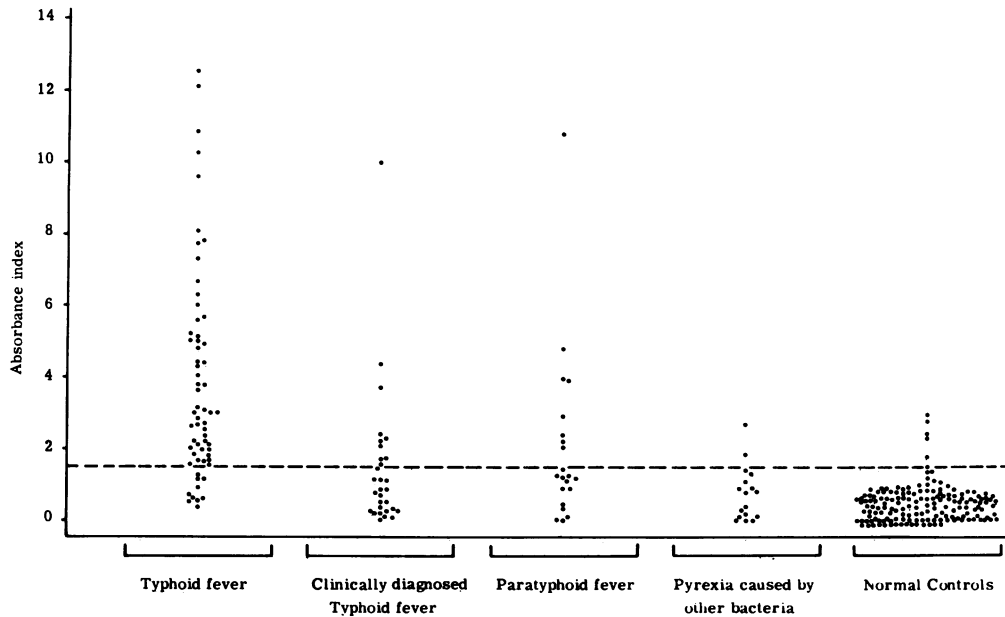
FIG. 3. Scatter diagram of AIs of antigen determined in serum samples obtained from five groups of subjects.

Dose-response curves of typhoid, paratyphoid, and normal control sera were constructed (Fig. 2). The serum dilution of 1:10 was selected for use in the assay.

For the screening test, serum samples were assayed against rabbit anti-BP immunoglobulins. Samples with an AI of <1.0 were considered to have no reaction with anti-BP immunoglobulins or have a reaction at less than the detectable limit. Only samples with an AI of ≥1.0 were subjected to further analysis by assaying them against rabbit preimmune immunoglobulins while simultaneously assaying them against rabbit anti-BP immunoglobulin, to test for the specificity of the reaction observed in the screening test. For the samples that showed nonspecific binding of substances in serum to rabbit preimmune immunoglobulins, the absorbance value of this nonspecific binding was subtracted from that of the test sample assayed against rabbit anti-BP immunoglobulins, before the absorbance value was used for the calculation of the AI of that sample.

The AI determined in serum samples obtained from the five groups of subjects studied are shown in Fig. 3. Because the AIs determined were overlapping, the cutoff level for positive antigenemia was investigated, at various AIs, for the one that provided the assay with the highest accuracy and sensitivity, based on hemoculture as the gold standard. It was found that an AI of 1.5 met this requirement.

By using the chosen cutoff level, positive results were found in 52 of 62 patients with typhoid, 10 of 30 patients with clinically diagnosed typhoid fever, 8 of 21 patients with paratyphoid, 2 of 17 patients with pyrexia caused by other bacteria, and 5 of 160 normal healthy individuals. Therefore, the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of this assay were 83.87, 89.04, 87.93, 67.53, and 95.31%, respectively.

DISCUSSION

Because hemoculture and the Widal test, which are currently used as standard methods for the diagnosis of typhoid fever, still possess some disadvantages, many alternative methods have been developed to achieve quick, sensitive, and reliable results (5, 10, 14–16, 18–20). Among them the detection of S. typhi antigens appears to have some advantages, as its result can be obtained more quickly than by hemoculture and there is no need for testing paired sera. Results of none of those previously reported tests, however, has been confirmed for usefulness in endemic areas.

Therefore, we decided to develop an ELISA for the detection of BP, which has not been reported by other investigators. We found that the established ELISA can detect BP at concentration as low as 0.5 µg/ml. This assay appeared to have good reproducibility, as the inter-run coefficient of variation was less than 12% for the detection of the antigen within the range of detectable limit. Although the cross-reaction with a high concentration (50 µg/ml) of protein antigens obtained from some other related enteric bacteria, i.e., S. typhimurium, E. coli, S. paratyphi A, and S. enteritidis, could be observed (this result was not unexpected), this did not cause many problems for the application of this assay as a diagnostic test. The validities of this assay were still satisfactory, even though this study was carried out in Thailand, which is endemic for typhoid fever, and sufficient numbers of patients with bacteremia due to other related gram-negative bacteria were included in this study. It was found that the sensitivity, specificity, and accuracy of the test were 83.87, 89.04, and 87.93%, respectively. These values are comparable to the validities of a passive bacterial agglutination test for the detection of S. typhi somatic antigens, as reported recently by Jacob John and co-workers (12), if they used the same criteria that we did for the calculation of validities of their test. This possibly could be due to the similarity of the antigens detected by the two methods, as the protein antigen extracted by the method used in this study was reported to be a component of somatic antigens (2). The ELISA reported in this study, however, appears to be superior to the detection of Vi antigen carried out in another area endemic for typhoid fever (19). The false-positive results obtained in normal healthy individuals and patients with pyrexia caused by other bacteria were only

3.1 and 11.76%, respectively. Because the treatment for patients with paratyphoid is the same as that for patients with typhoid, the false-positive results in the former (38.10%) should not be considered a great disadvantage of this assay, with regard to the effect of the test on patient treatment. In this study, although the positive results (33.3%) in the group of patients with clinically diagnosed typhoid fever was considered false-positive, this might be a true positive result because these patients might really be infected with *S. typhi*, but the organisms may not have been able to grow in hemoculture because of some other factors. Therefore, the ELISA for the detection of BP appears to be one that is valid for use as a diagnostic test of typhoid fever in endemic areas.

Apart from its good validity, the ELISA established here also has other advantages. It can be performed within 24 h, and the antibody-precoated plates can be stored at 4°C for up to 4 weeks before use. Thus, this test should be considered for use in conjunction with hemoculture to obtain an early diagnosis of typhoid fever, particularly in patients that have been treated with antibiotics prior to the collection of blood specimens, which might result in a negative hemoculture test result.

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