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Detection of *Salmonella typhi* Protein Antigen in Serum and Urine: A Value for Diagnosis of Typhoid Fever in an Endemic Area

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SUMMARY Using haemoculture as the gold standard, a double antibody sandwich ELISA for the detection of *Salmonella typhi* Barber protein antigen (BP) was compared with the Widal test. Specimens used were serum and urine obtained from normal healthy individuals and from patients with typhoid fever, paratyphoid fever, pyrexia caused by other bacteria and pyrexia with negative haemoculture. The ELISA for antigenuria gave a significantly higher sensitivity, specificity, accuracy and positive predictive value than the Widal test ($p < 0.05$). The ELISA for antigenaemia gave a significantly higher sensitivity and positive predictive value only. All other values were not significantly different. The timing of specimen collection was critical for sensitivity in the ELISA for antigenaemia and antigenuria, and the best results could be obtained by carrying out both assays simultaneously. The clearance of BP from serum into urine occurred around 16 days after the onset of fever in one patient. In two patients, BP could be detected in sera up to 3 weeks after the onset of fever. In two patients, serum BP could still be detected although haemoculture was negative.

It has been accepted that a definite diagnosis of typhoid fever can only be obtained by the presence of *Salmonella typhi* in biological fluids, especially the blood of the patients. However, haemoculture may be less valuable because of ongoing antibiotic treatment.[2,4] The Widal test is the serological test which has been widely used for the diagnosis of typhoid fever. However, in areas with endemic typhoid, this test poses some disadvantages, *e.g.* raised antibody levels may be detected in normal healthy individuals. Thus, in order to obtain definite diagnosis, paired sera must be tested to demonstrate a 4-fold rise of antibody titre.[7,12]

Apart from the detection of specific antibody, other immunological methods have been reported for the detection of *S. typhi* antigen in biological fluids.[1,2,4,7,9,11] Recently, we reported an ELISA for the detection of *S. typhi* Barber protein antigen (BP), which appeared to have good possibilities for the practical assay of sera.[1] In this study, we have evaluated the usefulness of the method for the diagnosis of typhoid fever in an endemic area. Both sera and urine specimens were assayed from each individual and the results were compared to those of the Widal test.

MATERIALS AND METHODS

Serum and urine samples

Serum and urine samples used in this study were divided into 5 groups as follows:

1. Typhoid fever Both serum and urine samples were obtained from 9 patients who had haemocultures positive for *S. typhi.* A total of twelve serum samples were obtained from these patients. Three were obtained serially from 1 patient, 2 were paired sera of 1 patient and the remainder were single sera. A total of ten urine samples were obtained from the same patients, including one paired and eight single samples.

In addition, 10 serum samples were obtained from four other typhoid patients. Six were serial samples from 2 patients and the remainder were paired samples.

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2. Pyrexia with negative haemoculture Single samples of both serum and urine were obtained from each of thirty patients who had fever for approximately one week. Some of them also had nausea, vomitting, abdominal pain and diarrhoea. All of them had negative haemocultures for bacteria.

3. Paratyphoid fever A single serum and urine sample was obtained from a patient who had a haemoculture positive for *S. paratyphi A*.

4. Pyrexia caused by other bacteria Six single serum and urine samples were obtained from patients who had haemocultures positive for *Escherichia coli* (4 cases), *Klebsiella pneumoniae* (1 case) and *Proteus vulgaris* (1 case).

5. Normal control group The normal control samples comprised 46 single serum and urine samples obtained from healthy individuals. Those with past histories of typhoid vaccination and natural infection with either *Salmonellae* or other related organisms were not excluded.

All serum samples were stored at -20°C until used.

All urine samples were centrifuged at 1500 rpm for 10 minutes at room temperature (Dynac II centrifuge: Clay Adams, Division of Becton, Dickinson and Company, U.S.A.). The supernatant was stored at -20°C until used.

Widal test

The standard Widal agglutination test was performed for the determination of antibodies to O and H antigens of *S. typhi,* using suspensions of organisms obtained from Gamma Diagnostic (Division Gamma Biologicals, Houston, Texas).

The diagnostic titre of the Widal test was ≥1:80 and ≥1:100 for O and H agglutinins respectively.[10]

ELISA for the detection of *S. typhi* protein antigen

A previously described[1] double antibody sandwich ELISA for the detection of *S. typhi* protein antigen (BP), was used in this study. Briefly, either rabbit anti-BP immunoglobulins or rabbit preimmune immunoglobulins were used at a concentration of 50 μg/ml, in 0.05 M carbonate buffer pH 9.8 containing 0.1% sodium azide, for coating each well of a microelisa Immulon® plate (Dynatech Produkta AG, Klonten, Switzerland) at 37°C for 3 hours. Sera were assayed at a dilution of 1:10 in 0.01 M phosphate buffered saline pH 7.1 containing 0.15% Tween 20 (PBST). Urine samples were assayed neat. Standard BP, at various concentrations, were used as positive controls. The incubation at this step was at 37°C for 2 hours. Each specimen was assayed in duplicate. The conjugate used was alkaline phosphatase labelled rabbit anti-BP immunoglobulin and it was incubated overnight at 4°C. The substrate used was p-nitrophenyl phosphate at a concentration of 1 mg/ml (Sigma Chemical Company, St. Louis, U.S.A.) dissolved in 0.05 M carbonate buffer pH 9.8, containing 0.005 M $MgCl_2 \cdot 6H_2O$. The substrate incubation was at 30°C for 1 hour.

The amount of the antigen detected was expressed as an absorbance index (AI) by relating the absorbance value of the sample tested to that of the standard BP concentration of 0.5 μg/ml, which was the lowest detectable limit of antigen as assayed by this method.

Positive results for antigenaemia and antigenuria assays were considered to be AI ≥ 1.5 and ≥ 1.0, respectively. The criteria used in selecting these values were previously described.[1]

Statistical methods

The indices of sensitivity, specificity, accuracy, positive predictive value and negative predictive value were calculated as follows: sensitivity, $[a/(a+c)] \times 100$; specificity, $[d/(b+d)] \times 100$; positive predictive value $[a/(a+b)] \times 100$; negative predictive value $[d/(c+d)] \times 100$; where a was the number of true-positive samples, b was the number of false positive samples, c was the number of false negative samples and d was the number of true-negative samples.[3]

These indices for each test were calculated from data obtained from the same population. When more than one sample was collected from one subject, only the result obtained with the first sample was used for the calculation of such indices. Only positive results obtained from the group of typhoid patients with haemocultures positive for *S. typhi* were considered as true positives. The positive results obtained from all other groups of subjects were considered false positive results.

Each index of all the tests performed was compared statistically, by using the test of hypothesis on population proportion.[8]

RESULTS

Comparison of antigenaemia and antigenuria detection by the ELISA and Widal test

The results of antigenaemia and antigenuria detection by the ELISA and of H and O agglutinin detection by the Widal test obtained from the same subject are shown in Table 1. It can be seen that more patients gave positive ELISA (antigenaemia and antigenuria) than

Table 1 Positive results obtained from ELISA for the detection of *S. typhi* protein antigen and from the Widal test in typhoid patients and non – typhoid controls

Method	Number positive/total number of subjects				
	gr. I	gr. II	gr. III	gr. IV	gr. V
ELISA for *S. typhi* Antigen detection					
in serum	7/9	10/30	1/1	0/6	3/46
in urine	7/9	4/30	0/1	0/6	0/46
Widal test					
O agglutinin	3/9	3/30	1/1	0/6	11/46
H agglutinin	4/9	3/30	1/1	0/6	24/46

gr. I = Typhoid patients
gr. II = Patients who had pyrexia with negative haemoculture
gr. III = Paratypnoid patients
gr. IV = Patients with pyrexia caused by other bacteria
gr. V = Normal controls

positive Widal tests in group 1 (typhoid patients) and group 2 (patients who had pyrexia with negative haemoculture). The reverse relationship was found with the normal controls.

By using the results of haemoculture as the gold standard, various indices of each test were calculated and they are shown in Table 2. It can be seen that the ELISA for detection of *S. typhi* protein antigen in urine had a significantly higher sensitivity, specificity, accuracy and positive predictive value than the Widal test for both O and H agglutinins ($p<0.05$). The ELISA for antigenaemia detection, however, had significantly higher sensitivity and positive predictive value only ($p<0.05$).

Presence of BP antigen in sera and urine samples of typhoid patients

The results of antigenaemia and antigenuria tests by ELISA with samples obtained from the same typhoid patient, on the same day, are shown in Table 3. It can be seen that the detection of antigenaemia was positive in patients 1-3, 5, 7-9 while the detection of antigenuria was positive in patients 2-6, 8 and 9. However, when antigenaemia and antigenuria assays were considered together, all 9 patients gave positive results for ELISA with at least one or the other specimen.

In addition, a follow up study in one patient indicated that the detectable BP antigen decreased in serum while it increased in urine. In this patient, the clearance of the antigen from the blood occurred approximately 16 days after the onset of fever (Table 4).

Persistence of BP antigen detected in the sera of typhoid patients

A follow up study carried out with sera obtained from 6 typhoid patients showed that the persistence of BP antigen in sera varied among individuals (Table 5). In two patients (10 and 12), it persisted up to approximately 3 weeks after the onset of fever. In addition, the BP antigen was still detectable in sera by the time when the haemoculture had become negative (patients 11 and 12).

DISCUSSION

Several immunological methods have been reported with different validities[1,2,5,6,9,11] for the detection of various antigens of *S. typhi*.

Table 2 Validities of the Widal test and of ELISA for the detection of *S. typhi* protein antigen

Method	Typhoid		Non typhoid		Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)
	No.true positive	No.false negative	No.false positive	No.true negative					
ELISA for the Detection of									
antigenaemia	7	2	14	69	77.78	83.13	82.61	33.33	97.18
antigenuria	7	2	4	79	77.78	95.18	93.48	63.64	97.53
Widal test for									
O agglutinin	3	6	15	68	33.33	81.92	77.17	16.67	91.89
H agglutinin	4	6	28	55	44.44	66.27	64.13	12.50	91.67

Table 3 The presence of *S. typhi* protein antigen in sera and urine samples of typhoid patients

Patient no.	Antigen in serum		Antigen in urine	
	AI	Interpretation	AI	Interpretation
1	9.582	+	0.342	−
2	1.802	+	1.216	+
3	2.552	+	3.370	+
4	0.510	−	4.205	+
5	6.286	+	1.189	+
6	0.684	−	2.696	+
7	3.650	+	0.432	−
8	4.413	+	1.600	+
9	1.788	+	1.635	+

\+ = Positive result
− = Negative result
AI = Absorbance Index

Table 4 Follow up study of antigenaemia and antigenuria in a typhoid patient with haemoculture positive for *S. typhi*

Days after onset of fever	Antigenaemia (AI)	Antigenuria (AI)
13	1.802	1.216
16	0.153	ND
20	ND	7.800

ND = Not determined

Table 5 Serial assessment of antigenaemia in 6 typhoid patients with haemoculture initially positive for *S. typhi*

Patient no.	Age (years)	Days after onset of fever	Antigenemia	
			AI	Interpretation
1	19	8	9.582	+
		11	10.080	+
		14	12.300	+
2	68	14	1.802	+
		16	0.153	−
10	43	10	2.007	+
		14	2.016	+
		24	2.778	+
11	2.5	12	2.829	+
		18*	1.810	+
12	6	14	2.680	+
		22*	4.440	+
13	19	11	2.989	+
		14	3.143	+
		16	2.269	+

* = Negative haemoculture
\+ = Positive result
− = Negative result

Recently, we established an ELISA for the serum detection of BP, which is the *S. typhi* protein antigen.[1] In this study, we have evaluated the usefulness of the assay for the diagnosis of typhoid fever in an endemic area (Thailand) using urine and serum as test specimens.

It was found that ELISA for the detection of BP in urine had a significantly higher sensitivity, specificity, accuracy and positive predictive value than the Widal test for both O and H agglutinins ($p<0.05$). For detection of BP in serum it had a significantly higher sensitivity and positive predictive value only ($p<0.05$). Because of the higher positive predictive value, it can be concluded that the ELISA for *S. typhi* protein antigen detection is more reliable for the diagnosis of typhoid fever than the Widal test.

Subjects with a history of typhoid vaccination and infection with either *Salmonellae* or other related organisms were not excluded from the normal control group and such subjects could have yielded positive antigenaemia results found in 3/46 of them. However, in contrasting the ELISA and Widal results in groups 5 and 2, it is important that ELISA positive individuals were relatively lower in group 5 (normals) and relatively higher in group 2 (pyrexia with negative haemoculture). In group 2, the patients who gave a positive Widal test and/or ELISA could possibly have been typhoid fever patients whose haemocultures were negative due to some unknown factor. If this were the case, then ELISA for the detection of *S. typhi* protein antigen would be a more valuable diagnostic tool than the Widal test or haemoculture. Further investigation is required before any definite conclusion can be drawn.

The time of each specimen

collection appeared to be a critical factor for the sensitivity of the ELISA. A similar finding was reported by Gupta and Rao[5] and Jacob and co-workers[6] who found that *S. typhi* antigen could be detected more frequently in acute sera than in convalescent sera of typhoid patients. We found that protein antigen detection in serum and urine varied according to the time of specimen collection and that assay of both serum and urine obtained on the same day improved the sensitivity of the ELISA for the diagnosis of typhoid fever (Table 3).

The clearance of *S. typhi* protein antigen from serum can occur around 16 days after onset of fever. This is in agreement with the report by Jacob and co-workers (around 18 days).[6] However, we also found that *S. typhi* protein antigen could persist in the sera of some patients for at least 3 weeks after the onset of fever. Furthermore, positive antigenaemia could still be observed although the haemoculture was negative (Table 5). The persistence of antigen varied from individual to individual.

The results obtained from this study support our previous proposal[1] on the usefulness of ELISA detection of *S. typhi* protein antigen as a rapid diagnosis of typhoid fever in endemic areas. They also showed that the assay was more useful when both serum and urine of each patient were obtained on the same day and were tested simultaneously.

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