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Determination of Antibody from Typhoid Patients against Lipopolysaccharide and Protein Antigens of *Salmonella typhi*

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SUMMARY **Although the Widal test is simple, inexpensive and the most widely used for serodiagnosis of typhoid fever, the sensitivity and specificity of the test is sometimes doubtful. In this study, an enzyme-linked immunosorbent assay (ELISA) was developed for the detection of serum IgG and IgM antibodies to protein and lipopolysaccharide (LPS) antigens of *Salmonella typhi* which was compared with the Widal test in various groups of subjects. In typhoid patients with hemocultures positive for *S.typhi* (TP group), ELISA positivity was found on 100% for IgG anti-protein, 94.44% for IgG anti-LPS and 88.89% for IgM to both the protein and LPS antigens. In contrast, the Widal test was positive in only 61.11% for anti-O and 83.33% for anti-H antibodies. In healthy control subjects (HC group), only 5% of serum samples were positive for IgG anti-protein and none was positive for IgG anti-LPS or IgM to either the protein or LPS. In contrast, the Widal test was positive in 7.5% of HC group for anti-O and 17.5% for anti-H antibodies. In blood bank donors (BB group), both ELISA and Widal tests were positive in 23-40% of sera. Since the hospital records of BB group were incomplete. It might be possible that some of these subjects had recently been infected with *S.typhi*. Our data indicate that the standard Widal test was associated with false negative reactions in 16-39% of blood culture positive subjects. In non-typhoid patients with blood culture positive for bacteria other than *S.typhi* (OP group), most of the sera were negative by ELISA; 5% were positive for IgG anti-protein and 2.5% for IgM anti-protein and IgG anti-LPS, whereas 15% were positive on the Widal test for anti-O and 40% for anti-H antibodies. The final two groups of patients included in this study were those with a diagnosis of typhoid fever based on clinical signs and symptoms, in whom hemocultures were not done (WP group) or were negative (NP group). All of these subjects were positive for anti-O antibody by Widal test since this was the antigen for their diagnosis. The NP group had about 70% positive tests for IgG and IgM anti-protein and anti-LPS antigens by ELISA. The WP group showed lower percentage (28-44%) of sera positive for antibodies against protein and LPS antigens. Our data suggest that ELISA assays with *S.typhi* protein or LPS antigens have superior sensitivity and specificity to the Widal test in the diagnosis of typhoid fever and that the majority of individuals who are Widal seropositive but blood culture negative may not have typhoid fever. Further evaluation of the ELISA test is indicated.**

A definitive diagnosis of typhoid fever can be made only by the cultivation of *Salmonella typhi* from blood or from other biological fluids. However, only 80% of typhoid patients are positive by blood culture during the first week of fever, and the percentages of positivity gradually decrease thereafter.[1] In addition, antibiotic treatment and/or inappropriate timing of the specimen collection may make the hemoculture less helpful. Therefore, the routine diagnosis of typhoid fever is based mainly on the clinical picture in combination with a serological test.[2] The validity of the Widal agglutination test, the most common test routinely used for the serological diagnosis of typhoid fever, is sometimes doubtful. In spite of the fact that a four fold rise in antibody titer or high titer of antibody to O-antigen in single serum specimen detected by Widal test may indicate typhoid fever, the agglutination is also frequently observed in normal and febrile non-typhoid sera, particularly in endemic areas.[3]

The indirect ELISA method has proved to be valuable for the measurement of antibody levels in several diseases.[4,5] This method was applied for the detection of antibodies in typhoid patient sera against various antigens of *S.typhi*,[6,7] and it was found that ELISA is approximately 100 times more sensitive than the agglutination reaction. In this study, we have targetted the protein and LPS from *S.typhi* as the antigens for ELISA test. The LPS antigen of Salmonella is regarded as the major

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antigenic determinant of the somatic-0 antigen.[8] It has also been shown that Salmonella organisms, like other gram-negative bacteria, contain protein antigens in addition to LPS[9] and that these protein antigens could induce protective immunity in experimental animals.[10,11] The accumulated evidence appears to single out the importance of protein and LPS antigens from *S.typhi* in the serodiagnosis of typhoid patients. We therefore developed an ELISA for the detection of IgG and IgM antibodies to protein and LPS antigens of *S. typhi* in various groups of typhoid patients, non-typhoid septic patients, blood bank donors and healthy control subjects. We also compared the diagnostic value of the ELISA with that of the Widal test.

MATERIALS AND METHODS

Subjects

Six different groups of subjects were included in this study.

Group 1 Healthy controls (HC) : This group consisted of 40 healthy laboratory personnel and graduate students.

Group 2 Blood bank donors (BB) : This group consisted of 30 apparently healthy male blood donors at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand.

Group 3 Patients with positive hemoculture for bacteria other than *S.typhi* (OP) (n = 40).

Group 4 Typhoid patients with positive hemocultures for *S.typhi* (TP) (n = 18).

Group 5 Typhoid patients with negative *S.typhi* hemoculture (NP) : This group consisted of 57 patients with a clinical diagnosis of typhoid fever, whose hemocultures were negative for *S.typhi* and other bacteria. The anti-0 antibody titers by Widal test of these patients were ≥ 160.

Group 6 Typhoid patients without hemoculture record (WP) : This group consisted of 25 patients with clinical diagnosis of typhoid fever who did not have blood culture obtained. The criteria for diagnosis of typhoid fever in this group was the same as those of group 5.

Preparation of protein and LPS antigens.

S.typhi isolated from typhoid patients of Maharaj Nakorn Chiang Mai Hospital, biochemically and serologically proven, was grown overnight on Trypticase soy agar (Difco) at 37° C. The bacteria were harvested in normal saline solution and poured into three volumes of acetone for inactivation. These bacteria were centrifuged and the pellet further washed with acetone. The LPS antigen of *S.typhi* was extracted from acetone-dried cells by the hot phenol-water method,[12] while the protein antigen was obtained from the acetone dried cells by Veronal buffer extraction and purified by repeated precipitation with trichloroacetic acid.[13] The carbohydrate and protein content of these preparations were determined by the method of Anthrone and Lowry.[14]

ELISA

Polystyrene microtiter plates were coated with protein and LPS antigens of *S.typhi* at concentrations of 4 and 40 μg/ml (which were determined to be optimal concentrations) respectively in carbonate-bicarbonate buffer (*p*H 9.6) and the plates were incubated at 37° C for 3 hours. After three washings with phosphate buffered saline containing 0.05% tween 20 (PBS-tween), the plates were incubated for 1 hour with optimal dilutions of serum samples (1:40 for the detection of IgG and 1:20 for the detection of IgM against protein antigen and 1:20 for the detection of both IgG and IgM against LPS antigen), washed again and then filled with peroxidase conjugated anti-human IgG (γ-chain specific, Cappel Co., Dowington, PA.) or peroxidase conjugated anti-human IgM (μ-chain specific, Nordic, Netherland), diluted with PBS-tween to 1:5000 and 1:2000, respectively. After one hour incubation, non-reactive conjugate was washed away and 2-2′ azino-di (3-ethylbenzothiazoline sulfonate, diammonium salt, ABTS) substrate was added. The reaction was allowed to proceed for 30 minutes at room temperature, and the color that developed was immediately read by a spectrophotometer at 405 nm (Titertek multiskan, Flow Laboratories, Helsinki, Finland), using ABTS solution as a blank. Optical density of samples were corrected by reference to the positive control serum. The reference positive serum sample was included in each plate tested and the OD value of the test serum was calculated according to the following formula.

$$\text{Corrected OD of test serum} = \frac{\text{OD of test serum x mean OD of reference serum}}{\text{OD of reference serum of each experiment}}$$

In this study, the positive cut off OD values for IgG antibody to protein and LPS antigens were 0.278 and 0.277 respectively while IgM antibody to protein and LPS antigens were 0.259 and 0.250 respectively.

Preparation of O and H antigens.

The same strain of *S.typhi* used for the preparation of protein and LPS antigens for ELISA was also used for the preparation of O and H antigens for the Widal test. For the preparation of O antigen, *S.typhi* was grown on Trypticase Soy agar (Difco) at 37° C overnight and harvested by adding normal saline solution (NSS) to each plate and then the bacterial cells were brushed off from the agar surface by cotton swab. The bacterial suspension was centrifuged at 5000 × g for 30 minutes and the supernatant fluid was decanted. The bacterial cell pellet was washed three times and resuspended in NSS. Bacterial cells were killed by heating at 100° C for 30 minutes,

diluted with NSS at appropriate dilution and kept at 4° C until used. For the preparation of H antigen, Trypticase Soy broth (Difco) was heavily inoculated with *S.typhi* and incubated at 37° C overnight. After incubation the bacterial cells were killed with formalin at a final concentration of 0.5% and harvested by centrifugation at 5000 × g for 30 minutes. The bacterial cell pellet was washed three times before being resuspended in NSS. It was kept at 4° C until used.

Widal test

Anti-O and anti-H antibodies to *S.typhi* were measured by the conventional microtiter method of the Widal test. Sera were diluted in serial two fold dilutions to 1:5, 1:10, 1:20, upto a dilution of 1:1280 with normal saline solution in microtiter plates. O or H antigen was added to each well and the contents of the wells were mixed by shaking and the plate incubated at 37° C overnight. In a positive agglutination test, the bacterial cells form a continuous carpet like sheet on the bottom of the well, while in a negative agglutination test the bacterial cells form a tight button on the bottom of the well when sedimented. The highest dilution of serum that gave a positive agglutination was considered as the end point. Serum was considered positive for Widal test when anti-O or anti-H titer was $\geqslant 160$.

RESULTS

Among individuals with typhoid fever who had positive blood cultures (TP group) ELISA tests were positive in 89-100%, whereas the Widal test were positive in only 61-83% as shown in Table 1. Among individuals with positive blood cultures for bacteria other than *S.typhi* (OP group) the Widal test was positive in 15-40%, whereas the ELISA was only positive in 2.5-5.0% (Table 1). Among healthy controls (HC group) 7.5-17.5% had positive Widal tests but only 0-5% were reactive in the ELISA assays. On the other hand, among apparently healthy blood blank donors (BB group) seroreactivity in both the Widal and the ELISA tests was more frequent (23-40%, Table 1).

We also studied 57 patients who had negative blood cultures for *S.typhi* but were diagnosed and treated for typhoid fever based on a positive Widal test and clinical symptoms (NP). Among these subjects 68-70% were positive on the ELISA assay. Among 25 subjects in whom blood cultures were not obtained (WP), who were diagnosed having typhoid fever on the basis of a positive Widal test and clinical symptoms, 44% were

Table 1. Percentage of serum positive for antibody to O and H antigens by Widal test and positive for IgG and IgM antibodies to protein and LPS antigens of *S. typhi* by indirect ELISA in various groups of subjects.

Group of subjects	Number of subjects	Number (%) of subjects positive for antibody to					
		O antigen	H antigen	Protein antigen		LPS antigen	
				IgG	IgM	IgG	IgM
HC	40	3 (7.5)	7 (17.5)	2 (5)	0 (0)	0 (0)	0 (0)
BB	30	7 (23.33)	11 (36.67)	11 (36.67)	8 (26.66)	9 (30)	12 (40)
OP	40	6 (15)	16 (40)	2 (5)	1 (2.5)	1 (2.5)	0 (0)
TP	18	11 (61.11)	15 (83.33)	18 (100)	16 (88.89)	17 (94.44)	16 (88.89)
NP	57	57 (100)*	57 (100)*	40 (70.18)	40 (70.18)	39 (68.42)	40 (70.18)
WP	25	25 (100)*	25 (100)*	7 (28)	11 (44)	8 (32)	11 (44)

HC = healthy control group; BB = blood bank group; OP = patient with hemoculture positive for bacteria other than *S. typhi*; TP = typhoid patient with hemoculture positive for *S. typhi*; NP = typhoid patient with hemoculture negative for *S. typhi*; WP = typhoid patient without hemoculture record;

*only patients positive by Widal test (anti–O titer > 160) were selected and tested for IgG and IgM antibody to protein and LPS antigens from *S. typhi*.

positive to either the protein or LPS antigen on ELISA testing.

The mean ± SD of anti-O (Fig. 1) and anti-H (Fig. 2) antibody titers by Widal test in the TP group were 274 ± 318 and 448 ± 451 respectively while the mean ± SD of the OD values of IgG (Fig. 3,5) and IgM (Fig. 4,6) antibodies to protein and LPS antigens by ELISA were 0.750 ± 0.214, 0.685 ± 0.238 and 0.534 ± 0.156, 0.530 ± 0.180, respectively. The mean ± SD of anti-O and anti-H antibody titers in OP group were 58 ± 51 and 112 ± 76, respectively while the mean ± SD of OD values of IgG and IgM antibodies to protein and LPS antigens were 0.218 ± 0.056, 0.225 ± 0.042 and 0.218 ± 0.045, 0.212 ± 0.039, respectively. The mean ± SD of anti-O and anti-H titers in the HC group were 50 ± 38 and 77 ± 43, while ELISA gave mean ± SD OD values of IgG and IgM anti-protein and anti-LPS of 0.214 ± 0.032, 0.209 ± 0.034 and 0.207 ± 0.027, 0.196 ± 0.027, respectively. The mean ± SD of anti-O and anti-H titers in the BB group were 74 ± 51 and 105 ± 63 and mean ± SD OD values of IgG and IgM anti-protein and anti-LPS were 0.289 ± 0.107, 0.252 ± 0.084 and 0.245 ± 0.078, 0.250 ± 0.063, respectively.

In NP group, the mean ± SD of anti-O and anti-H titer were 390 ± 282 and 755 ± 419, while the mean ± SD OD values of IgG and IgM anti-protein and anti-LPS were 0.598 ± 0.309, 0.558 ± 0.274 and 0.474 ± 0.207, 0.440 ± 0.202, respectively. The mean ± SD of anti-O and anti-H titers in the WP group were 220 ± 114 and 398 ± 300 and the mean ± SD OD values of IgG and IgM anti-protein and anti-LPS were 0.339 ± 0.211, 0.329 ± 0.161 and 0.311 ± 0.149, 0.304 ± 0.135, respectively.

DISCUSSION

In this study, an ELISA assay was developed for the determination of antibodies against the protein and LPS antigens of *S.typhi* in various groups of subjects. Most sera from

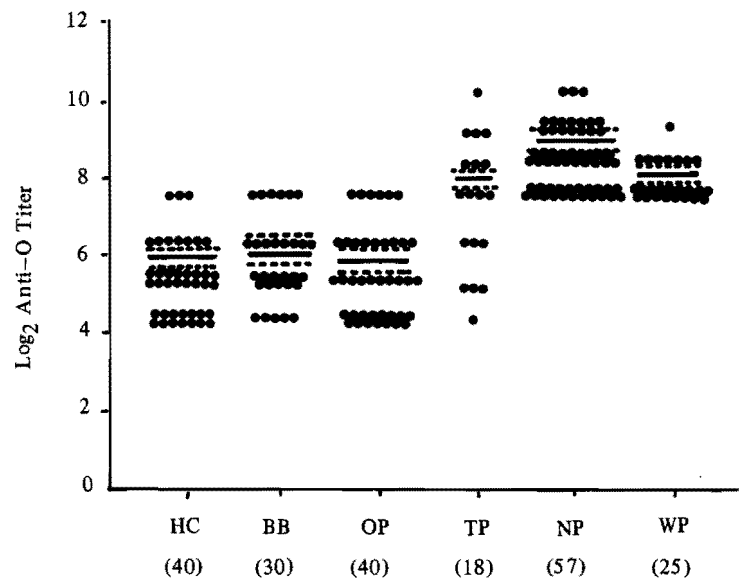

Fig. 1 Distribution of anti–O antibody titers in various groups of subjects. Mean ± SD of HC = 50 ± 38; BB = 74 ± 51; OP = 58 ± 51; TP = 274 ± 318; NP = 390 ± 282 and WP = 220 ± 114.

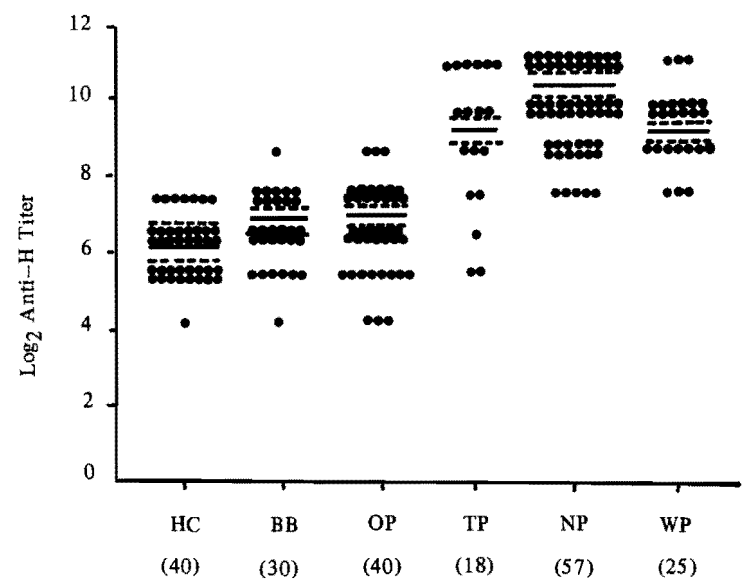

Fig. 2 Distribution of anti–H antibody titers in various groups of subjects. Mean ± SD of HC = 77 ± 43; BB = 105 ± 63; OP = 112 ± 76; TP = 448 ±451, NP = 755 ± 419 and WP = 398 ± 300.

a healthy control (HC) group were negative for IgG and IgM antibodies to both the protein and LPS antigens of *S.typhi* by ELISA. Only 2 of 40 healthy individuals had positive ELISA test for IgG anti-protein. However, the OD values of these two cases were only slightly above the cut off value. It is possible that these two cases might have had an *S.typhi* infection in the past and IgG antibody to *S. typhi* protein remained at a low level. When sera from healthy controls were assayed by Widal test, 7.5% and 17.5% of the sera were positive for anti-O and anti-H antibodies respectively. This observation suggests that background seropositivity of the Widal test may be quite common. Positive Widal reactions may result from a subclinical infection with *S.typhi* or a cross reaction of *S.typhi* antigen with the antigen of another serologically related Enterobacteriacae.[13] In the blood donors (BB) group both ELISA and Widal tests were more frequently positive than in the HC group. The Widal test was positive in 23.33% for anti-O and 36.67% for anti-H antibodies; ELISA tests were positive in 27-40%. No medical records were available from these subjects. However, since the blood samples were drawn primarily from the donors in the lower socioeconomic class with poor hygiene they may have higher rates of infection with *S.typhi*. Although previous immunization with typhoid vaccine can not be ruled out in this population, typhoid vaccine has not been used recently or frequently. The data indicated that professional blood donors are not suitable to be included as normal control subjects.

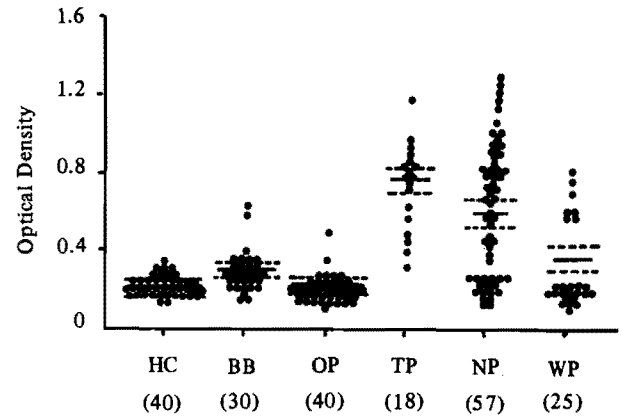

Fig. 3 Distribution of optical density values of IgG antibody to *S. typhi* protein antigen. Mean ± SD of HC = 0.214 ± 0.032; BB = 0.289 ± 0.107; OP = 0.218 ± 0.056; TP = 0.750 ± 0.214; NP = 0.598 ± 0.309 and WP = 0.339 ± 0.211.

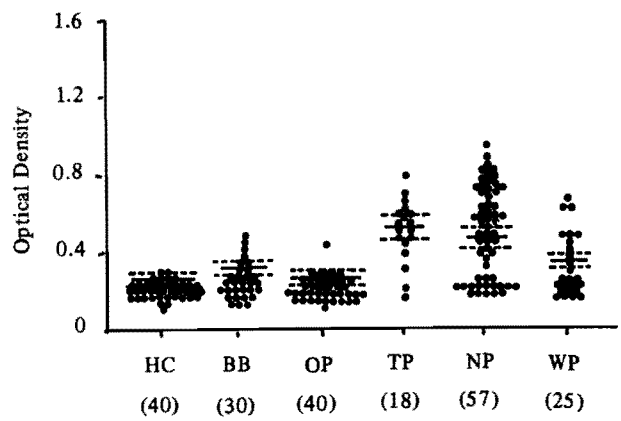

Fig. 4 Distribution of optical density values of IgM antibody to *S. typhi* protein antigen. Mean ± SD of HC = 0.207 ± 0.027; BB = 0.245 ± 0.078; OP = 0.218 ± 0.045; TP = 0.534 ± 0.156; NP = 0.474 ± 0.207 and WP = 0.311 ± 0.149.

It is important to evaluate the sensitivity and specificity of the ELISA which we have developed in this study for the serodiagnosis of typhoid fever. Our data suggest that the ELISA may have superior sensitivity to the Widal test since patients with hemocultures positive for *S.typhi* were almost all positive. The sera from typhoid patients were 100% positive for IgG anti-protein, 94.44% positive for IgG anti-LPS and 88.89% positive for IgM anti-protein as well as anti-LPS. In contrast, only 61.11% and 83.33% of these sera gave positive Widal tests for anti-O and anti-H antibodies, respectively. In the TP group, 3 out of 18 (16.67%) were positive by ELISA but negative by Widal test while 15 out of 18 (83.33%) were positive by both ELISA and Widal tests. Our data indicate that the Widal test may have given false negative results in 16-39% of cases. In comparison, there were no false negative ELISA tests for IgG anti-protein and only 6% for IgG anti-LPS. Nevertheless, IgM anti-protein and anti-LPS may be associated with

a false negative rate of about 11%. Because of the high prevalence of positive Widal tests, diagnostic tests are generally based on a four-fold increase of titer using paired sera or the diagnosis is confirmed by hemoculture positive for *S.typhi*. In the non-typhoid patients with other infections (OP group) ELISA was positive in 0-5% to both the protein and LPS antigen of *S.typhi*, whereas 15% of the Widal tests were positive for anti-O and 40% for anti-H antibodies. Taken together, the data imply that ELISA we have developed and evaluated in this study is more highly sensitive and specific for serodiagnosis of typhoid fever than the Widal test.

The last two groups of patients included in this study were those with a clinical diagnosis of typhoid fever but with negative (NP group) hemocultures or those without cultures done (WP group). However, these two groups of patients were positive for anti-O antibody by Widal test. When their sera were tested by ELISA, 70% of the patients of the NP group were positive for IgG and IgM antiprotein and anti-LPS antigens. Furthermore, only 28% and 32% of the patients of the WP group were positive for IgG anti-protein and anti-LPS, respectively and 44% were positive for IgM antibodies against both antigens. These two groups of patients had discrepant results be tween their ELISA and Widal tests. As has been shown in this study and previously by others [15,16] the ELISA using protein or LPS as an antigen is much more sensitive and specific for detecting antibodies to *S.typhi* than the Widal test. This may be explained in part by the discrepancy in the data resulting from false positive results obtained by the Widal test.

In conclusion, the ELISA assay we developed and reported in this study is highly sensitive and specific for the serodiagnosis of typhoid fever. It has been shown to be more reliable than the Widal test and could be an alternative method for routine use in the hospital laboratory. Nevertheless, the protein and LPS antigens of *S.typhi* used in this study are relatively crude. The protein and LPS antigens from *S.typhi* should be further fractionated and purified in order to improve the sensitivity and specificity of ELISA for a serodiagnosis of typhoid fever.

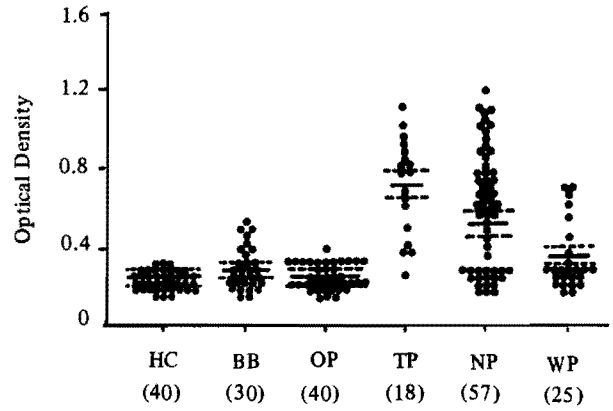

Fig. 5 Distribution of optical density values of IgG antibody to *S. typhi* LPS antigen. Mean ± SD of HC = 0.209 ± 0.034; BB = 0.252 ± 0.084; OP = 0.225 ± 0.042; TP = 0.685 ± 0.236; NP = 0.558 ± 0.274 and WP = 0.329 ± 0.161.

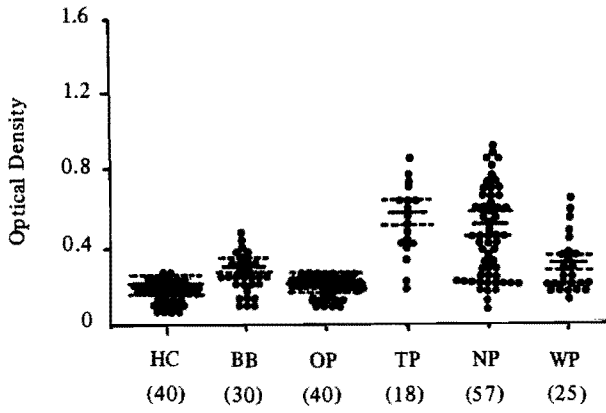

Fig. 6 Distribution of optical density values of IgM antibody to *S. typhi* LPS antigen. Mean ± SD of HC = 0.196 ± 0.027; BB = 0.250 ± 0.063; OP = 0.212 ± 0.039; TP = 0.530 ± 0.180; NP = 0.440 ± 0.202 and WP = 0.304 ± 0.135.

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