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Characterization of a Phase 1-d Epitope on *Salmonella typhi* Flagellin and Its Role in the Serodiagnosis of Typhoid Fever

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SUMMARY A monoclonal antibody (MAb) directed against *Salmonella typhi* 52 kDa flagellin protein has been previously produced by our group. In this study, we have demonstrated that the epitope specific to the MAb is unique to phase 1-d. To map the epitope, plasmids encoding different regions of *S. typhi* flagellin gene were constructed. Analysis of protein produced from each recombinant plasmid indicated that the epitope specific to the MAb resided within amino acids 171-303 (region IV) of *S. typhi* flagellin protein. The recombinant region IV flagellin was used to develop an ELISA for the detection of IgM antibody to *S. typhi* in serum. In the hemoculture-positive typhoid group, the developed ELISA was positive in 77 of 92 cases. In patients with non-typhoidal *Salmonella*, gram-positive and gram-negative bacteria or dengue virus, the ELISA was negative in all 78 cases. Two from 116 healthy control subjects had positive reactions with the assay. The calculated sensitivity, specificity, positive and negative predictive values of the test were 83.7%, 99.0%, 97.5% and 92.8%, respectively. With such high validity together with the requirement of only a single serum specimen and one day for performing the test, the developed ELISA should become a valuable diagnostic test for typhoid fever.

Typhoid fever is a systemic disease due to *Salmonella typhi* infection. The disease remains an important public health problem in many developing countries. *S. typhi* present in most parts of the world typically has only phase 1-d or H 1-d flagellar antigen (monophasic). This has a molecular weight of 52 kDa and is encoded by a 1,521 base-pairs (bp) DNA sequence.[1] This property is different from most *Salmonella* that can alternatively express two distinctive antigenic types, phase 1 and phase 2, which are determined by two flagellin genes, called *fli*C and *fli*B. For example, *S. muenchen* can produce flagellar antigen identified as "d" (phase 1) or "1, 2" (phase 2).

The conventional laboratory diagnosis of typhoid fever is based on hemoculture and detection of anti-H and anti-O antibodies against flagellar (H 1-d) and somatic (O = 9, 12) antigens, respectively (the Widal test). This method has several limitations. The isolation of *S. typhi* from hemoculture provides a conclusive diagnosis, but it is a time consuming process and sometimes can give rise to false negative results owing to prior antibiotic therapy. The Widal test is known to be unreliable because of its cross reactivity with other bacteria, especially because of the antigenic preparations used,[2-4] and the high prevalence of antibodies in normal populations.[5] The confirmation of typhoid fever with the Widal test requires both acute and convalescent phase sera, which are seldom collected in hospital.

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The need for a rapid, sensitive and reliable test to detect *S. typhi* infection has stimulated many investigators to develop various diagnostic tests including antigen,[6,7] antibody[8-12] and DNA-based[13,14] detection methods. In antibody detection methods, different *S. typhi* antigens were used as specific antigens such as lipopolysaccharide,[8,9] flagellin[8] and outer membrane proteins.[10-12] However, all of these antigens were purified from *Salmonella* cultures. None of them were prepared using recombinant DNA technology to generate well-defined antigen which can be produced in large quantities in the absence of culturing bacterial *Salmonella*. To achieve this aim, characterization and expression of *S. typhi* phase 1 flagellin or H 1-d antigen were undertaken. We were interested in molecular cloning of flagellin protein, monomer of bacterial flagellar filament, because epitope specific to our previously produced monoclonal antibody (MAb) is located on this protein.[1] This MAb was highly specific when tested with protein antigens of 11 enterobacteria causing enteric fever and enteric fever-like illness *i.e. S. paratyphi* A, B and C, *S. enteritidis*, *S. typhimurium*, *S. panama*, *S. cholerae-suis*, *S. krefeld*, *Burkholderia pseudomallei*, *Yersinia enterocolitica* and *Escherichia coli*.[15] In addition, the presence of IgM antibody against 52 kDa *S. typhi* flagellin in typhoid sera was observed.[15] These studies suggested the potential usefulness of *S. typhi* flagellin for diagnostic purposes.

In the study described here, we demonstrated that the epitope specific to the MAb is unique to phase 1-d and located on the variable region IV of the flagellin protein. This variable region IV is one of eight regions on *Salmonella* flagellins according to Wei and Joys classification.[16] In addition, the *S. typhi* region IV flagellin protein, expressed from recombinant *E. coli*, has been used to develop a microtiter plate ELISA for IgM antibody detection. Sera from patients who were hemoculture positive for *S. typhi* as well as sera from other groups of patients and normal subjects were used for the evaluation of this serodiagnostic test.

MATERIALS AND METHODS

Bacterial strains

Salmonella typhi and other *Salmonella* possessing phase 1-d flagellar antigen including *S. muenchen* (d:1,2), *S. stanley* (d:1,2), *S. manhattan* (d:1,5), *S. berlin* (d:1,5), *S. verginia* (d:1,2), *S. gamminara* (d:1,7) and *S. liverpool* (d:e,n,z_{15}) were isolated from patients. They were serotyped by the WHO National Salmonella and Shigella Center, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. The standard *Escherichia coli* strain TG1 was used for the cloning and production of flagellin-GST (glutathione S-transferase) fusion protein.

Serum samples

Serum specimens used in this study were obtained from 4 groups of patients and 116 healthy normal subjects. The 4 groups of patients included 92 patients with *S. typhi*, 31 with non-typhoidal *Salmonella*, 13 patients who had serologically confirmed dengue virus infection, a febrile illness which might be confused with typhoid fever, and 34 with either gram-positive cocci (*Staphylococcus aureus*, *Streptococcus pneumoniae*) or gram-negative bacilli (*Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *E. coli*).

Western blot of phase 1-d flagellin

Biphasic *Salmonella* were rendered to produce only phase 1-d flagellar antigen according to the Gard technique.[17] In brief, highly motile isolates of each *Salmonella* were obtained after several passages on 0.7% soft agar in petri plates which had been incubated at 37°C for 16 hours. Unwanted phase 2 flagellar antigens were eliminated by stabbing the active motile *Salmonella* at the center of the soft agar plate containing homologous phase 2 agglutinating sera (H&A Reagents Lab., Nonthaburi, Thailand). After overnight incubation, the phase1-d producing *Salmonella*, which had migrated from the site of inoculation, were harvested and subjected to Western blot analysis. Essentially, 3 ml of overnight bacterial cultures were pelleted and mixed with 600 µl sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heated for 2 minutes in a boiling water bath. The crude whole bacterial cell antigens were loaded onto 12% polyacrylamide gels[18] before being electroblotted onto nitrocellulose membranes with the buffers described by Towbin *et al.*[19] The blotted flagellin proteins on nitrocellulose membrane were detected using MAb specific to *S. typhi* 52 kDa antigen and alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins.[1,15]

Plasmid construction and localization of phase 1-d specific epitope

The hypervariable regions IV-VI and V-VI of *S. typhi* flagellin gene were amplified by PCR technique. All the PCR primers had been designed to contain *Bam*HI and *Eco*RI sites at 5' and 3' ends, respectively. The modified primer sequences for region IV-VI (nucleotide 514-1,110) are 5'CTT GGA TCC CAA GAT GCC TAC ACC3 and 5'TCC GAA TTC AGC AAC GCC AGT ACC3' whereas those for region V-VI (nucleotide 925-1,110) are 5'GCC GGA TCC GAC AAT ACT AGC3' and 5'TCC GAA TTC AGC AAC GCC AGT ACC3'. Amplification was done for 35 cycles on a DNA Thermal Cycler (Perkin-Elmer Corp). Each cycle consisted of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. After digestion with *Bam*HI and *Eco*RI, the amplified DNA fragments which were 596 and 185 bp in length, were cloned inframe into expression vector pGEX-2T[20] and were designated pPT1 and pPT2, respectively. For cloning of hypervariable region IV, the amplified region IV-VI fragment was digested with restriction enzyme *Pst*I to remove the region IV DNA fragment from region V-VI. The 3' cohesive end of region IV DNA was manipulated to be blunt-ended DNA by T4 DNA polymerase. Thereafter, the modified region IV flagellin DNA was digested with *Bam*HI and ligated to *Bam*HI-*Sma*I digested pGEX-2T. This constructed plasmid was designated pYT. All the constructed plasmids were transformed into TG1 *E. coli*. Whole bacterial cell lysates from these recombinant *E. coli* were subjected to Western blotting and probed with the MAb specific to *S. typhi* 52 kDa flagellin antigen.[15]

Preparation of GST and flagellin-GST fusion protein

Overnight culture of *E. coli* carrying plasmid pGEX-2T or pGEX-2T with the flagellin DNA insert was diluted 1/100 in Luria-Bertani broth and grown at 37°C with vigorous shaking to an optical density (OD) of 0.5 at 600 nm. Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.2 mM to induce the expression of GST or GST-flagellin fusion protein and the culture was incubated for a further 3 hours. Cells were harvested by centrifugation at 3,000 x *g* and resuspended in 1/50 of the original volume of phosphate-buffered saline (PBS) pH 7.4. The cells were then lysed by sonication, and debris discarded after centrifugation at 10,000 x *g*. The culture supernatant was applied to a glutathione sepharose 4B column (Pharmacia). The column was washed with PBS prior to the elution of the protein. GST or GST-flagellin fusion protein was eluted by competition with 5 mM reduced glutathione (Sigma Chemical Co., St Louis, MO, USA) in 50 mM Tris-HCl buffer pH 8.0. The purity of the protein sample was checked by observation of the protein pattern after SDS-PAGE. The protein concentration was calculated from the absorbance at 280 nm.

ELISA for IgM antibody detection

The detection of IgM antibody in the serum samples by an indirect-ELISA was carried out in duplicate under optimal conditions. In brief, microtiter plates (96-well, flat-bottom, Corning Laboratory Science Company, New York, USA) were coated overnight at 4°C with 100 µl of 13 µg/ml of GST or 20 µg/ml of region IV flagellin-GST fusion protein. Plates were washed 3 times on the next day with PBS pH 7.4 containing 0.1% Tween-20 (PBS-Tween). Blocking was then carried out by the addition of 100 µl PBS containing 3% skim milk and 0.1% Tween-20, incubation for 1 hour at 37°C, followed by three washes with PBS-Tween. Each diluted serum sample (1:200 with 1% skim milk in PBS) was added to both GST and flagellin-GST coated wells, then the plate was incubated for 1 hour at 37°C. After 3 washes with PBS-Tween, 100 µl of alkaline phosphatase conjugated anti-human IgM $F(ab)'_2$ fragment (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:1,000, was added to each well and the plate was incubated for a further 3 hours at 37°C. The plate was then washed 3 times with PBS-Tween before the addition of 100 µl of 1 mg/ml p-nitrophenyl phosphate (Sigma). The reaction was allowed to develop for 30 minutes at 37°C and the absorbance at 405 nm was measured by a Titertek Multiskan (Flow Laboratories Ltd., Ayrshire, Scotland). The level of IgM in the serum sample is the absorbance obtained from the well coated with flagellin-GST fusion protein minus that from GST. The samples were considered positive when the delta absorbance was greater than 0.15.

Statistical method

The method of Galen[21] was used for calculating the diagnostic sensitivity, specificity, positive predictive and negative predictive values of the established ELISA in comparison with the conventional

bacterial culture method. Only those positive results obtained from the group of typhoid patients with hemoculture positive for *S. typhi* were considered as true positives.

RESULTS

MAb specific to phase 1-d flagellin

Seven *Salmonella* serotypes which have phase 1 antigen defined as "d" similar to *S. typhi*, but with different phase 2 flagella, including *S. muenchen*, *S. stanley*, *S. manhattan*, *S. berlin*, *S. liverpool*, *S. virginia* and *S. gamminara*, were rendered monophasic before Western blot analysis with the MAb and compared with *S. typhi*. Fig. 1 shows that the MAb not only reacts with the 52 kDa flagellin from *S. typhi* but also with all other phase 1-d *Salmonella* at the same molecular weight of 52 kDa.

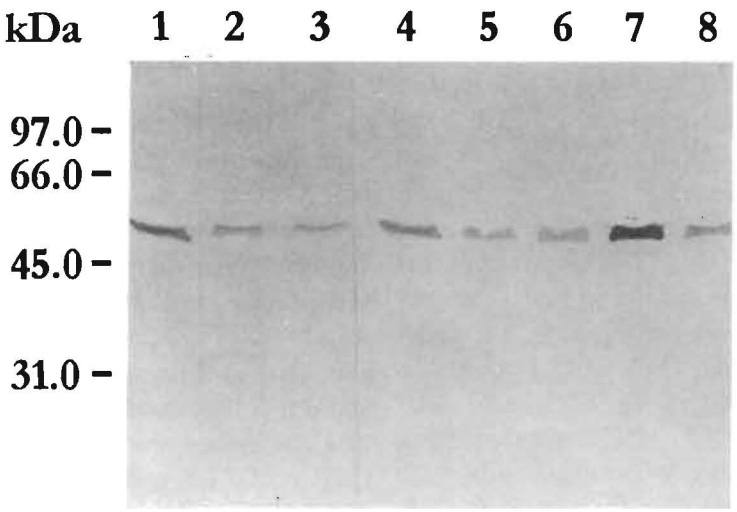

Fig. 1 Western blot of whole bacterial cell proteins extracted from phase 1-d *Salmonella* spp. probed with the MAb, previously identified as specific to *S. typhi* 52 kDa protein.[15] *S. verginia* (lane 1), *S. gamminara* (lane 2), *S. liverpool* (lane 3), *S. manhattan* (lane 4), *S. berlin* (lane 5), *S. muenchen* (lane 6), *S. stanley* (lane 7) and *S. typhi* (lane 8). Numbers at left indicate molecular weight markers in kDa.

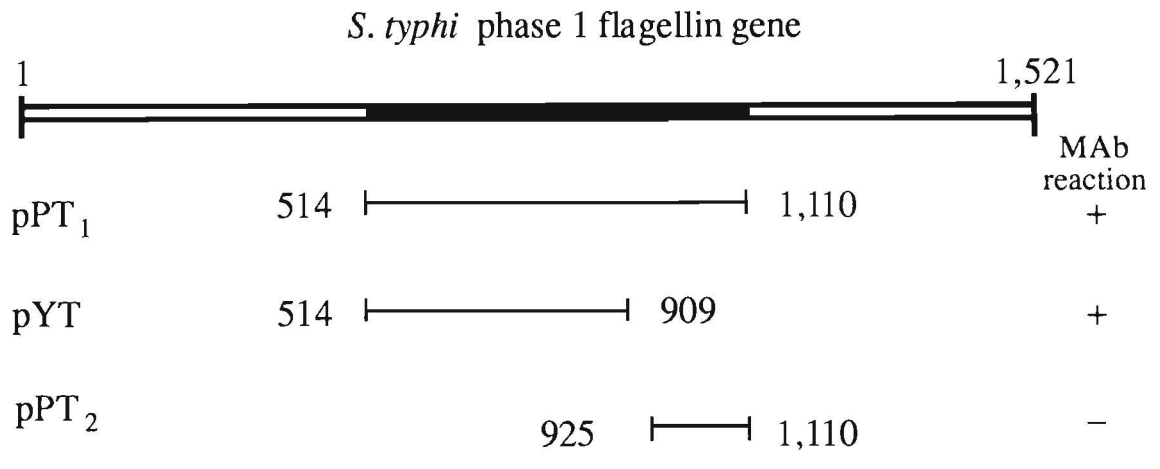

Fig. 2 Plasmids pPT1, pYT and pPT2 encoding *S. typhi* flagellin regions IV-VI, IV and V-VI were constructed. Solid bar indicates the location of hypervariable region IV-VI on *S. typhi* phase 1 flagellin gene. The Western blot analysis of proteins produced from each constructed plasmid reacted with the MAb are also given.

Specific phase 1-d epitope located on hypervariable region IV

To localize epitope on flagellin which is specific to the MAb, whole bacterial cell proteins extracted from 3 different *E. coli* clones carrying either pPT1, pYT or pPT2 (Fig. 2) were subjected to Western blot analysis. Fig. 3A, lanes 3, 4 and 5 depicted the SDS-PAGE of those 3 recombinant *E. coli* clones. When the electrophoresed proteins were allowed to react with the MAb, it was revealed that plasmids pPT1 and pYT harbouring the *S. typhi* flagellin nucleotides 514-1,110 and 514-909, respectively, showed positive reaction with the MAb (Fig. 3B, lanes 3 and 4). In contrast, plasmid clone pPT2 encoding the *S. typhi* flagellin nucleotide 925-1,110 showed negative reaction. (Fig. 3B, lane 5). It can be concluded that the antigen-determining part of phase 1-d flagellin is encoded by nucleotide 514-909 which corresponds to hypervariable region IV on flagellin protein.

Application of flagellin fragment with phase 1-d epitope

An indirect ELISA was developed to detect IgM antibody against purified recombinant flagellin-GST fusion protein isolated from plasmid clone pYT (Fig. 3A and B, lane 7). All of the serum specimens were composed of 116 healthy normal subjects and 4 groups of patients including 92 cases with hemoculture positive for *S. typhi*, 31 cases with non-typhoidal *Salmonella*, 34 cases with either gram-positive cocci or gram-negative bacilli and 13 patients who had serologically confirmed dengue virus infection. The distribution of delta absorbance values of IgM to *S. typhi* region IV flagellin protein among these serum samples was demonstrated in Fig. 4. The mean ± SD of the delta absorbance values in typhoid group was 0.980 ± 74 while the mean ± SD in patients infected with non-typhoidal *Salmonella*, other gram-positive and gram-negative bacteria,

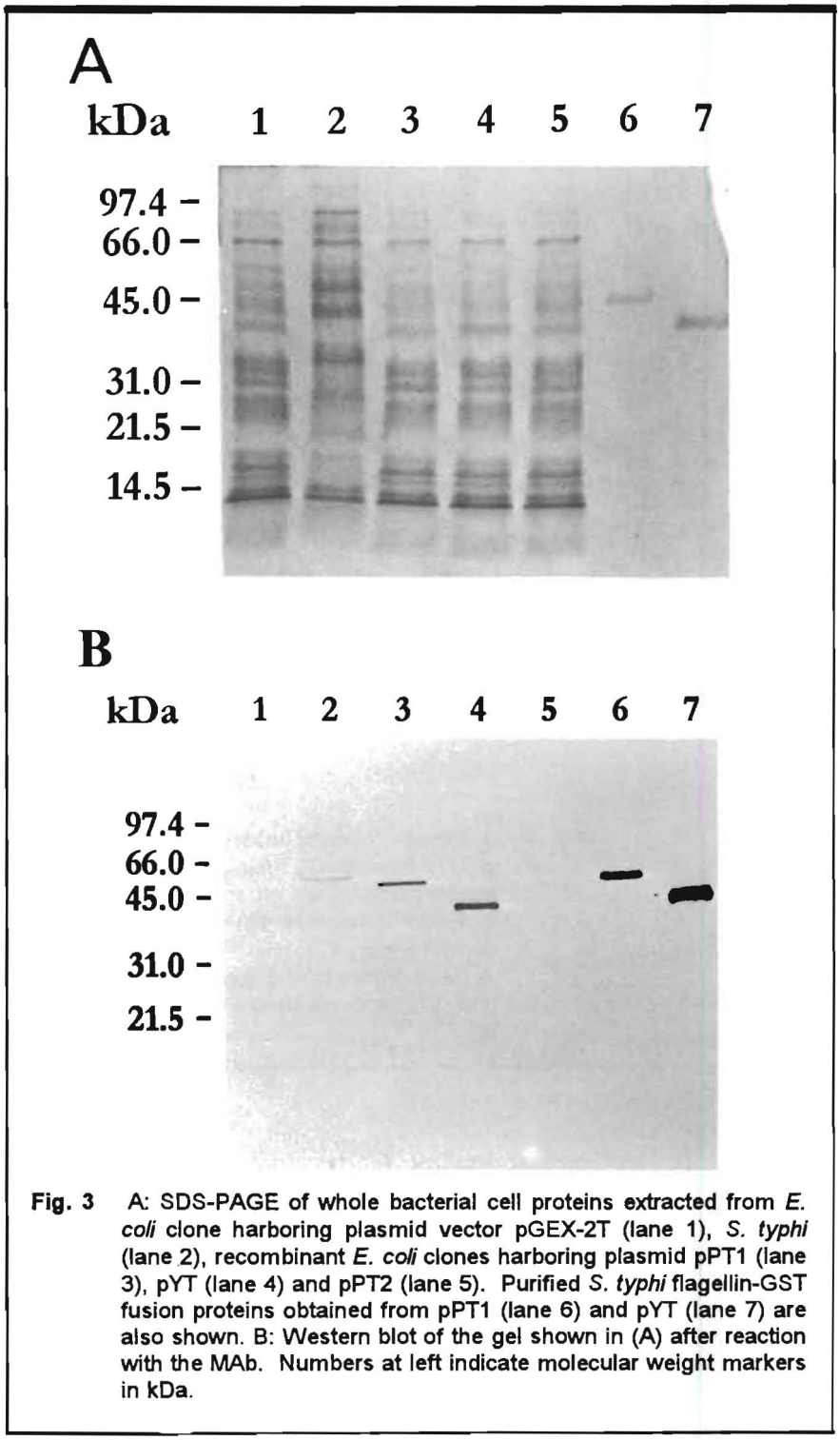

Fig. 3 A: SDS-PAGE of whole bacterial cell proteins extracted from *E. coli* clone harboring plasmid vector pGEX-2T (lane 1), *S. typhi* (lane 2), recombinant *E. coli* clones harboring plasmid pPT1 (lane 3), pYT (lane 4) and pPT2 (lane 5). Purified *S. typhi* flagellin-GST fusion proteins obtained from pPT1 (lane 6) and pYT (lane 7) are also shown. B: Western blot of the gel shown in (A) after reaction with the MAb. Numbers at left indicate molecular weight markers in kDa.

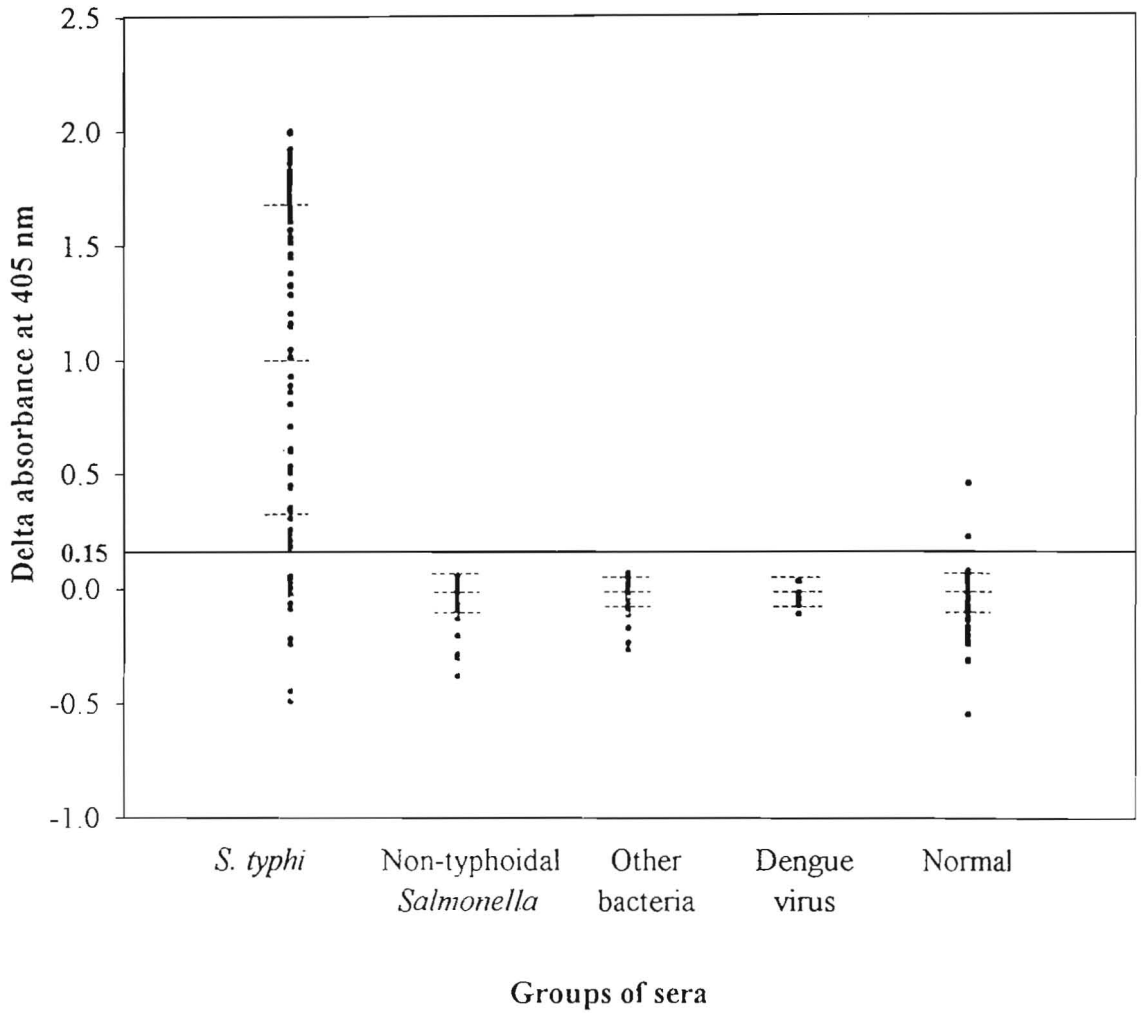

Fig. 4 Distribution of delta absorbance values of IgM antibody against region IV flagellin-GST fusion protein. The delta absorbance values are obtained from 4 groups of patients infected with *S. typhi*, non-typhoidal *Salmonella*, other bacteria and dengue virus, as well as normal control subjects. The mean ± SD of the absorbance values in these 4 groups of patients and normal healthy individuals were 0.98 ± 0.74, 0 ± 0.10, 0 ± 0.06, 0 ± 0.06 and 0 ± 0.09, respectively.

dengue virus and normal healthy individuals were 0 ± 0.10, 0 ± 0.06, 0 ± 0.06 and 0 ± 0.09, respectively.

The sensitivity and specificity of the assay varied according to the cut-off level chosen. By using the delta absorbance 0.1 as the cut-off level for positive results, the sensitivity and specificity of the ELISA were 83.7% and 98.4%. respectively, while these values were found to be 83.7% v.s. 99.0% and 81.5% v.s. 99.0% for the cut-off value of 1.5 and 2.0, respectively. Thus, the cut-off level for the positive results of the developed ELISA was chosen at the delta absorbance 0.15 which gave the highest values of both sensitivity and specificity.

By using the cut-off value chosen, 77/92 samples collected from patients whose hemoculture were positive for *S. typhi*, had detectable levels of IgM against the designed flagellin protein. In con-

Table 1 Findings of clinical samples tested for IgM antibody specific to region IV flagellin of *Salmonella typhi* by indirect ELISA

Group of serum samples	Number of cases	ELISA result*	
		Positive (%)	Negative (%)
Hemoculture positive			
S. typhi	92	77 (83.7)	15 (16.3)
Non-typhoidal *Salmonella*	31	0 (0)	31 (100)
Gram-positive and gram-negative bacteria	34	0 (0)	34 (100)
Dengue virus antibody positive	13	0 (0)	13 (100)
Normal serum	116	2 (1.7)	114 (98.3)

*Samples were considered positive when the delta absorbance value was greater than 0.15.

trast, none of the 78 sera from patients whose hemoculture was positive for non-typhoidal *Salmonella*, other gram-positive and gram-negative bacteria, or had dengue virus infection showed a positive result of specific IgM detection. Among 116 healthy normal subjects, only 2 (1.7%) had positive IgM detection (Table 1). Thus, the sensitivity, specificity, positive and negative predictive values of the developed ELISA were 83.7%, 99.0%, 97.5% and 92.8%, respectively.

DISCUSSION

The diagnosis of typhoid fever can be conducted through culture or by antibody or antigen detection in body fluids. Detection of antibodies against somatic (O) and flagellar (H) antigen in the Widal test was the earliest developed antibody detection method, but is particularly unreliable with a single serum in endemic areas.[2-4] Establishment of many immunoassays to improve diagnosis of typhoid fever has been reported.[6-12] However, none of these tests has gained widespread acceptance in routine microbiological laboratories.

We previously reported the production of a MAb against the 52 kDa flagellin antigen of *S. typhi*.[1,15] The specificity of the MAb was demonstrated by positive reaction with *S. typhi* but not with *S. paratyphi* A (phase 1-a), *S. paratyphi* B (phase 1-b), *S. paratyphi* C (phase 1-c), *S. cholerae-suis* (phase 1-c), *S. enteritidis* (phase 1-g,m), *S. typhimurium* (phase 1-i), *S. krefeld* (phase 1-y), *S. panama* (phase 1-l,v) and three other enterobacteriaceae.[15] Further studies have been undertaken to demonstrate whether the MAb is specific to *S. typhi* or phase 1-d flagellin protein and are reported here.

Data from this study demonstrated that our previously produced MAb recognized not only flagellin from *S. typhi* but also those from other phase 1-d *Salmonella*. Therefore, the epitope recognized by the MAb is phase 1-d specific. In addition, it has been demonstrated that the epitope specific to the MAb is located on hypervariable region IV (nucleotide 514-909 corresponds to amino acids 171-303 from the total of 507 amino acids) of *S. typhi* flagellin protein. Hypervariable region IV is one among 8 regions on *Salmonella* flagellin according to Wei and Joys classification.[16] The data were obtained by expression of different flagellin regions in the central part of the protein before allowing those flagellin fragments to react with the MAb. The demonstration that specific phase 1-d epitope is present on this hypervariable region, together with previously reported evidence that the specific phase 1-i[22] and phase 1-a [our unpublished observation] epitopes are located there, indicates the importance of the hypervariable region IV of *Salmonella* flagellin in determining the specificity of different phase 1 *Salmonella* serotypes.

The hypervariable region IV of *S. typhi* phase 1 flagellin has been shown to be antigenically important for phase 1-d flagellar antigen. Although there are many *Salmonella* that possess phase 1-d flagellar antigen, only *S. typhi* has important frequent associations with severe disease and bacteremia. The other phase 1-d *Salmonella* such as *S. muenchen* and *S. stanley*, are more likely to cause gastroenteritis than septicemia. Therefore, if antibody is detected in patients' sera, this is highly suggestive of *S. typhi* infection.

The hypervariable region IV of *S. typhi* flagellin should be

useful for the serodiagnosis of typhoid fever. Therefore, the flagellin-GST fusion protein of clone pYT was employed to detect IgM antibody in patients' sera. The advantage of IgM detection is that IgM is an indicator of acute infection whereas positive IgG detection can result from previous infections, immunization with typhoid vaccine or repeated *S. typhi* antigen stimulation in an endemic area in addition to acute infection. As a result, comparison of IgG levels between acute and convalescent phase sera is required to confirm the diagnosis of acute *S. typhi* infection. Furthermore, this developed ELISA is aimed to diagnose typhoid fever from a single blood sample rather than paired blood samples which are more difficult to obtain.

In this study, the 40.5 kDa of region IV flagellin-GST fusion protein, instead of 13.0 kDa of region IV flagellin alone, was used as a specific antigen in the test. The advantage of the fusion protein expression system is to allow purification of flagellin from *E. coli* host proteins. After the purification step, GST (MW 27.5 kDa) can be cleaved off from the region IV flagellin by blood clotting factor X or thrombin.[20] However, we found that the yield and quality of purified region IV flagellin antigen obtained was not satisfactory. To overcome the problem that a serum sample might contain antibody against GST, the optical density detected in each serum sample had to be corrected by subtracting the optical density contributed by the GST antigen.

With this system, 77 of 92 patients whose hemoculture was positive for *S. typhi* gave positive results, whereas none of the non-typhoidal *Salmonella* patients showed a positive reaction. The lack of cross-reaction with non-typhoidal *Salmonella* (*S. paratyphi* A, B and C) should be due to the difference in serotype of flagellar antigen. The phase 1 flagellin antigens of *S. paratyphi* A, B and C are phase 1-a, phase 1-b and phase 1-c, respectively. Our established ELISA detects antibody against phase 1-d flagellin. Infection with non-typhoidal *Salmonella* will induce non-phase 1-d antibody in the patients' sera which should not cross-react with the phase 1-d antigen used in the assay. Furthermore, the phase 1-d antigen in this study is only the hypervariable region IV not the whole flagellin molecule. Wei and Joys[16] reported that this region is highly variable. The amino acid sequence homology between phase 1-d, phase 1-a, phase 1-c and phase 1-i is about 30%. These important properties give rise to the high specificity of the test.

The 83.7% sensitivity of ELISA reported in this study is higher than those of the methods previously described by Sukosol *et al.*[23] and Chart *et al.*[8] The possible explanation for this result might be due to the differences in size of flagellin antigens, systems of detection and time of serum sample collection. The latter reason is very important, especially for IgM detection, since IgM is the first immunoglobulin class rising during acute phase of infection then switched to IgG in the late phase. Therefore, an inappropriate time of serum sample collection after onset of fever will affect the sensitivity of the test.

In conclusion, we have developed a specific and sensitive diagnostic test for typhoid fever, using well defined, fully characterized reagents which could be made available in large quantities. Unlike the Widal test, which may require at least a week or two for rise in titre after the onset of fever,[24] it is possible with our assay to diagnose typhoid fever using a single specimen and the result can be obtained within one day.

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