

Fusion Protein of *Salmonella typhi* Flagellin as Antigen for Diagnosis of Typhoid Fever

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Salmonella typhi infection or typhoid fever remains a public health problem in many developing countries, but the rapid and accurate laboratory diagnosis of typhoid fever remains unsatisfactory. The currently available methods to diagnose typhoid fever are the isolation of the causative organism, *Salmonella typhi*, from clinical specimens, mainly by haemoculture; and the demonstration of 4 folds rising antibody titres to the O and H antigens of *S.typhi* from the paired acute and convalescent sera from patients by the Widal test. An ideal diagnostic test for typhoid fever should be sensitive, specific and rapid for early diagnosis. Neither of the above two methods fulfill these requirements. Thus, there is a need to develop a rapid and specific test.

Our group has produced and characterized monoclonal antibodies (MAbs) specific to the *S.typhi* 52 kDa antigen. These antibodies do not cross-react with related protein antigens from 11 bacteria causing enteric fever and enteric fever-like illness (*S.paratyphi* A, *S.paratyphi* B, *S.paratyphi* C, *S.cholerae-suis*, *S.enteritidis*, *S.kr feld*, *S.panama*, *S.*

SUMMARY We previously established the specific 52 kDa antigen of *Salmonella typhi*, detected by our monoclonal antibodies, which was a flagellin protein. Comparison of the nucleotide sequences of phase-1 flagellin of *Salmonella* species available through GenBank database showed high homology at both ends of the genes with lower degree of homology in the middle portion which contained the antigenically variable regions. Thus, proteins from the central regions of flagellin genes should be species specific and could be used as specific antigens for the immunodiagnostic tests. In this report, recombinant protein derived from the central region of *S.typhi* flagellin was produced as a fusion protein with glutathione-S-transferase. This fusion protein was used as specific *S.typhi* antigen for the immunodiagnostic test to detect IgM antibodies in sera using enzyme-linked immunosorbent assay. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of this test were 53.5, 98.0, 91.5, 82.1 and 92.4%, respectively.

typhimurium, *Escherichia coli*, *Pseudomonas pseudomallei* and *Yersinia enterocolitica*).¹ We also demonstrated by SDS-PAGE and Western blot using *S.typhi* whole cell antigen to show the presence of specific IgM antibody to the 52 kDa protein in sera from patients with acute typhoid infection. The result suggested that the 52 kDa protein of *S.typhi* was a strong immunogen and could be used to diagnose the specific antibodies in sera. Because of this evidence, we tried to develop a diagnostic test detecting IgM antibodies against *S.typhi* specific antigen(s) from sera of the patients.

A genomic library of *S.typhi* was constructed² and screened for the recombinant clones expressing specific *S.typhi* antigens by using the specific MAbs. The gene in the selected clone was sequenced³ and

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analysed by comparing the nucleotide sequence with those in the bacterial libraries of GenBank. The result showed a high homology with the phase 1-d flagellin gene of *S. muenchen* (98% similarity) and the 227 bp sequence of part of *S. typhi* flagellin gene (99.56% similarity). Thus, specific 52 kDa antigen of *S. typhi* detected by our MAbs was *S. typhi* flagellin. Comparison of the *S. typhi* 52 kDa antigenic gene and five published phase 1 flagellin sequences from *Salmonella*³⁻⁶ showed high homology at both ends; 400 bp at the 5' ends and 200 bp at the 3' ends were almost identical. The nucleotide sequences showed decreasing homology towards the middle portion which contained the antigenically variable regions.⁴ Therefore, proteins from the central regions of flagellin genes should be species specific and could be used as specific antigens for the immunodiagnostic tests.

The central region of *S. typhi* flagellin DNA was amplified using polymerase chain reaction (PCR) technique and cloned in the pGEX expression system (Pharmacia). The central region of flagellin protein was expressed as a fusion protein with glutathione-S-transferase (GST). This fusion protein was used as the specific *S. typhi* antigen for the immunodiagnostic test to detect specific IgM antibodies in sera using enzyme-linked immunosorbent assay (ELISA). The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of this test were 53.5, 98.0, 91.5, 82.1 and 92.4% respectively.

MATERIALS AND METHODS

Serum samples

Serum specimens used in this study were obtained from 3 groups of patients during admission in the hospital: 43 were collected from patients whose haemocultures were positive for *S. typhi*, 27 were from patients with positive for non-

typhoidal *Salmonella* (*S. paratyphi* A, *Salmonella* gr. B and *Salmonella* gr. C) and 34 were from patients with positive for gram positive cocci (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Proteus mirabilis*) and other gram negative bacilli (*Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Enterobacter aerogenes*) by haemoculture results. The other 189 serum samples were from normal healthy individuals.

Bacteria and cloning vectors

The pGEX expression system (Pharmacia) allowed production of recombinant protein as fusion with glutathione-S-transferase (GST). *Escherichia coli* JM 101 was used for the cloning and production of GST fusion protein.

The 52 kDa flagellin gene and DNA amplification

The pSKM-T7 containing the specific 52 kDa antigen gene of *S. typhi* and its DNA sequencing of the pSKM-T7 have been described previously.³ Because of the homology with other flagellin genes at both ends, the middle region of *S. typhi* flagellin was amplified using the primer sequences designed according to the *S. typhi* 52 kDa antigenic gene and the published phase 1 flagellin sequences from *Salmonella*. The modified primer sequences were 5' GAGGATCCAGGTTGGTGCCA 3' with *Bam* HI site and 5' GTGAATTCGGAGTTGAAACG 3' with *Eco* RI site. DNA amplification was accomplished in the presence of 20 ng of pSKM-T7 template, 5×10^{-3} mM of each oligonucleotide primer and 2 units of *Taq* DNA polymerase (Pharmacia) in a final volume of 100 μ l in the following solution: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, and dNTPs (200 mM final concentration of each nucleotide). Amplification was carried out for 35 cycles: each consisted of 94°C for 1 minute, 50°C for 1 minute and 72°C for 2

minutes. The PCR amplified product was approximately 900 bp in length containing the gene coding nucleotide number 436-1339 of *S. typhi* flagellin sequence. The reaction mixture was subjected to electrophoresis on 0.8% agarose gel to analyze and purify the PCR product.

Cloning of PCR-amplified product

Cloning and DNA techniques were as described by Sambrook *et al.*⁷ Briefly, the PCR product was digested with *Bam* HI and *Eco* RI at 37°C for 3 hours. After complete digestion, the PCR product was purified and ligated into the *Bam* HI and *Eco* RI sites of the pGEX-3X vector (Pharmacia) at 3:1 proportion. Plasmid DNA was introduced into competent *E. coli* JM 101 prepared by DMSO method.⁸ The recombinant *E. coli* containing central region of *S. typhi* flagellin DNA was identified by immunoenzyme assay⁹ using specific MAbs.

Production and purification of glutathione-S-transferase fusion protein¹⁰

Overnight culture of *E. coli* JM 101 containing recombinant pGEX plasmid containing *S. typhi* gene was diluted 1/100 in Luria broth and grown at 37°C with vigorous shaking to an approximate absorbance at 600 nm (A_{600}) of 0.5. Isopropyl- β -D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM to induce the production of fusion protein and the culture was incubated for further 3 hours. Cells were harvested by centrifugation at $3,000 \times g$ and resuspended in 1/50 of the original volume in phosphate-buffer saline (PBS). The cells were then lysed by sonication and discarded debris by centrifugation at $10,000 \times g$. Culture supernatant was applied to glutathione Sepharose 4B column (Pharmacia). The column was washed with PBS prior to the elution of the fusion protein. Fusion protein was eluted by competition with free

glutathione (5mM reduced glutathione (Sigma) in 50 mM Tris-HCl pH 8.0). Protein concentration was calculated from the absorbance at 280 nm.¹¹

Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli¹² and the separated antigens were transferred from the gels to nitrocellulose membrane using a Bio-Rad blotting apparatus according to the manufacturer's instructions and stained by the immunoenzyme staining method⁹ using MAbs specific to the *S.typhi* 52 kDa antigen and alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins.

ELISA for detecting IgM antibodies

ELISA for the detection of IgM antibodies in the serum specimens was carried out by the standardization of various conditions, e.g. antigen concentration, conjugate concentration and serum dilution. Optimal conditions chosen were then used to assay serum specimens. Briefly, fusion protein antigen in 0.05 M carbonate buffer pH 9.8, was used at a concentration of 20 µg/ml for coating each well of a Microelisa Immunol[®] plate (Dynatech Produkta AG, Klonten, Switzerland) at 4°C overnight. Sera were assayed at a dilution of 1:200 in 0.01 M phosphate buffer saline pH 7.1 containing 0.1% Tween 20 (PBST) and 1% bovine serum albumin (BSA). The incubation at this step was at 37°C for 3 hours. The conjugate used was 1:1000 dilution of alkaline phosphatase conjugated anti-human IgM F(ab')₂ fragment (Sigma) and it was incubated at 37°C for 1 hour. The substrate used was *p*-nitrophenyl phosphate at a concentration of 1 mg/ml (Sigma) dissolved in 0.05 M carbonate buffer pH 9.8, containing 0.005 M MgCl₂·6H₂O. The substrate incubation was 37°C for 1

hour. Positive result exhibited yellow color whereas negative result was clear. Weak positive or borderline results were confirmed by reading the absorbances at 405 nm (Titertek Multiskan; Flow Laboratories Ltd., Ayrshire, Scotland) and the samples considered positive when the absorbance was > 0.2.

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, $[d/(b+d)] \times 100$; 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 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.¹³ 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 of subjects were considered false positive results.

RESULTS

Amplification of DNA from variable central region of *S.typhi* flagellin

To produce the specific protein antigen for the immunodiagnosis, the central region of *S.typhi* flagellin DNA was amplified using polymerase chain reaction (PCR) technique. The PCR amplified product yielded approximately 500 ng with 900 bp in length containing the gene coding nucleotide number 436-1339 of *S.typhi* flagellin sequence.

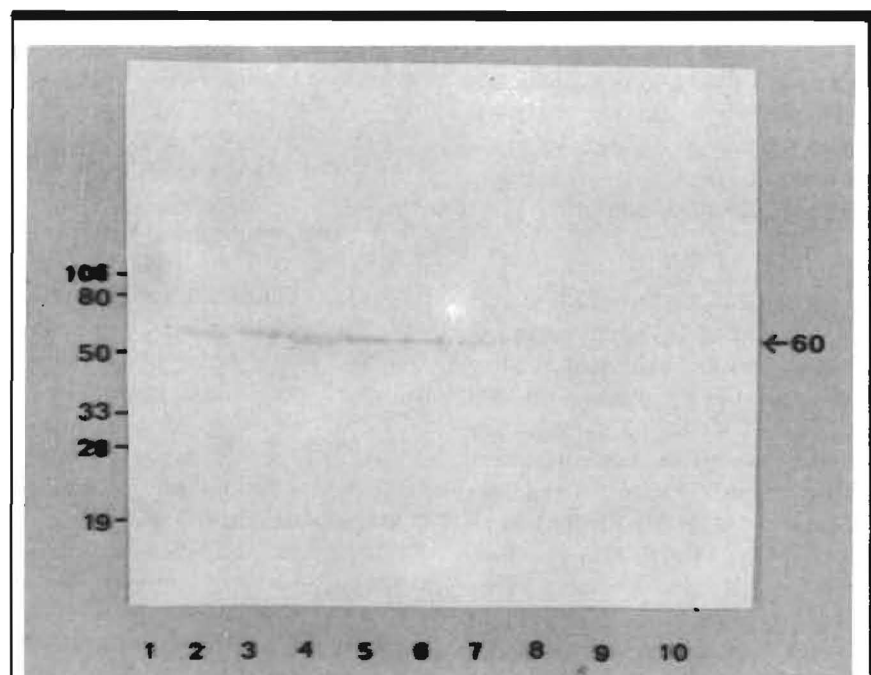


Fig. 1 Production of fusion protein was purified by affinity chromatography and detected by immunoblotting technique using monoclonal antibodies specific to the 52 kDa protein. The result showed positive band at 60 kDa position.

Lane 1 = Standard protein marker

Lane 2-10 = 2nd-10th fusion protein fraction eluted from the column.

Cloning and characterization of central region gene of *S.typhi* flagellin

A recombinant clone (pTST-1) was obtained with 900 bp inserted DNA fragment from the PCR amplified product into the *Bam* HI and *Eco* RI site of the pGEX-3X vector. The recombinant clone was induced with isopropyl- β -D-thiogalactoside (IPTG) to increase the protein production. The cells were lysed and soluble GST fusion protein was purified by affinity chromatography on immobilised glutathione followed by competitive-elution with excess reduced glutathione. The fusion protein fractions eluted from the column were analysed into 2 sets by SDS-PAGE, one set was stained with Coomassie blue and shown only one protein band at 60 kDa (data not shown), another set was transferred to nitrocellulose membrane and stained by immunoenzyme staining assay using MAbs specific to the 52 kDa protein. The result showed position red purple band at 60 kDa positive which contained GST (26 kDa) fused with 900 bp flagellin protein (34 kDa) as expected (Fig. 1). In this synthesis, the fusion protein was purified with yield of 2 μ g/ml of culture.

Analysis of ELISA

The ELISA was used to detect IgM antibodies in serum samples. Positive results exhibited yellow color with the absorbance at 405 nm $>$ 0.2. Of the 43 samples for patients whose haemocultures were positive from *S.typhi*, 23 (53.5%) had detectable levels of IgM antibodies in the sera (Table 1). Two (7.4%) of 27 samples from non-typhoidal *Salmonella*, 1 (2.9%) of 34 samples from other bacteria and 2 (1.1%) of 189 samples from healthy individuals from an endemic area gave positive results. Therefore, the sensitivity, specificity, accuracy, positive predictive value and negative predictive value of this assay were 53.5, 98.0, 91.5, 82.1 and 92.4%, respectively.

Table 1 Enzyme-linked immunosorbent assay for the detection of IgM antibodies in typhoid patients, non-typhoidal *Salmonella* patients and normal controls.

Group	Total	ELISA	
		positive (%)	negative (%)
<i>S.typhi</i>	43	23 (53.5)	20 (46.5)
Non-typhoidal <i>Salmonella</i>	27	2 (7.4)	25 (92.6)
Other bacteria	34	1 (2.9)	33 (97.1)
Normal	189	2 (1.1)	187 (98.9)

DISCUSSION

Because haemoculture and the Widal test, which are currently used as standard methods for the diagnosis of typhoid fever, still possess some disadvantages, many alternative methods to detect antigens or antibodies have been proposed to achieve quick, sensitive and reliable results. In the present study, fusion protein containing the central region of *S.typhi* flagellin protein was produced and used as specific antigen for detecting IgM antibodies for the diagnosis of typhoid fever. The DNA from the central region of *S.typhi* flagellin which determined its antigen character was amplified and cloned in the pGEX expression system. The protein product was expressed as fusion protein with GST. Purification of this fusion protein was achieved by one step affinity chromatography on immobilized glutathione using competitive elution. This provided a readily available purified antigen which was found to be suitable for ELISA. Background reactivity to the GST, the fusion partner, was tested by Thomas *et al*¹⁴ and found that it could be negligible.

Since the d flagellin antigen of *S.typhi* was present as a phase-1 antigen similar to those of some members of *Salmonella* genus¹⁵ and showed 98% similar to *S.muenchen*,

thus the ELISA using central region of *S.typhi* flagellin-GST fusion protein as antigen could give positive result with *S.muenchen*. This cross-reactivity is of relatively little importance since *S.muenchen* infection is infrequent and usually is not a problem for the differential diagnosis of typhoid fever.¹⁶

For the serodiagnosis of typhoid fever, the ELISA for detecting IgM antibodies was performed using the fusion protein derived from only the central region of *S.typhi* flagellin as specific antigen. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of this test were 53.5, 98.0, 91.5, 82.1 and 92.4%, respectively. The result showed high specificity which indicated that the fusion protein antigen used in this test was specific to *Salmonella typhi*. However, the sensitivity was only 53.5% as compared to haemoculture. Several factors could possibly affect the sensitivity of this assay. The limitation of the assay itself, the time of sample collection and the factors that interfered with it might affect its sensitivity as well. It may be able to increase the sensitivity by using other technique such as nitrocellulose membrane slot blot ELISA or IgM capture ELISA. The slot blot ELISA using nitrocellulose membrane would increase the sensi-

tivity since the nitrocellulose membrane had a high binding capacity, approximately 1,000 times more protein per surface area than the microplate.¹⁷ All of samples which were positive *S.typhi* haemocultures but could not detect IgM antibodies by ELISA, specific IgG antibodies to the fusion protein antigen were detected (data not shown). The IgM antibodies might be competitive binding to the specific antigen by these IgG antibodies and gave low sensitive result. Thus, IgM capture ELISA would overcome this problem.

In this paper, we had produced a specific recombinant protein antigen of *Salmonella typhi* and used in an ELISA to determine specific IgM antibodies *S.typhi*. The high specificity of this fusion protein antigen allowed a possible technique for diagnosis of *S.typhi*.

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