DETECTION OF IgM ANTIBODY AGAINST REGION IV FLAGELLIN OF *SALMONELLA PARATYPHI* A

Sunee Korbsrisate[1], Suttipant Sarasombath[1], Nuttaya Praaporn[1], Pattara Iamkamala[2], Moazzem Hossain[3] and Stan Mckay[4]

[1]Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; [2]Department of Medicine, Lardprao General Hospital, Bangkok, Thailand; [3]Sir Salimullah Medical College, Dhaka, Bangladesh; [4]World Vision International-Cambodia, Phnom Penh, Cambodia

Abstract. *Salmonella paratyphi* A is a pathogenic bacterium that causes paratyphoid fever. The current laboratory diagnostic techniques are unsatisfactory. To improve diagnosis, a plasmid (pSK-8E) encoding phase 1 flagellin gene nucleotide position 452-890 from *S. paratyphi* A has been constructed. The recombinant protein expressed from the plasmid has been used to develop an indirect ELISA for IgM antibody detection. Sera from patients with hemoculture positive for *S. paratyphi* A, *S. typhi,* other gram-positive and gram-negative bacteria, and dengue hemorrhagic fever as well as from healthy control subjects were tested. Sensitivity, specificity, positive and negative predictive values of the test were 56.9%, 98.8%, 90.6% and 92.1%, respectively. Since the sensitivity was low, the explanation for this result was investigated. It was found that the sensitivity of the test could be increased to 83.3 % if the sera were obtained 9-12 days after onset of fever. The sera obtained earlier or later gave only 33.3% and 66.6% sensitivity, respectively. This result suggests that the IgM antibody detection assay which we have developed is a valuable tool for diagnosis of *S. paratyphi* A infection when the serum samples are taken at the appropriate time.

INTRODUCTION

Enteric fever due to systemic *Salmonella* infection is rare in developed countries, but remains one of the most prevalent acute infectious diseases in the developing world. The disease can be divided into typhoid fever, caused by *Salmonella typhi,* and paratyphoid fever caused by *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C (Parker, 1991). Currently, the majority of enteric fever is due to *S. typhi* and *S. paratyphi* A infections with rare cases reported as being due to *S. paratyphi* B or *S. paratyphi* C infections. In the past, the prevalence of *S. typhi* infection was higher than *S. paratyphi* A infection in developing countries such as Malaysia and Thailand. However, at present, prevalence of *S. paratyphi* A infection remains the same in spite of quite low prevalence of *S. typhi* infection. One reason might be the development of two effective vaccines, Ty21a and Vi polysaccharide vaccines, against *S. typhi* but not *S. paratyphi* A. Identification of the etiologic agent of *S. paratyphi* A infection is based on laboratory diagnosis, hemoculture and the Widal test. Hemoculture provides a conclusive diagnosis, but it is a time consuming process and sometimes can give false negative results owing to prior antibiotic therapy. The serological diagnosis, Widal test, has been reported to be unreliable because of its cross-reactivity with other bacteria and high prevalence of antibody in normal population. The confirmation result requires both acute and convalescent phase serum samples that are rarely collected.

To improve diagnosis of *S. paratyphi* A infection, a monoclonal antibody (MAb) specific to a 52 kDa protein antigen from this bacteria was produced by our group. The MAb was highly specific to *S. paratyphi* A, and did not react with protein antigen from *S. typhi, S. paratyphi* B, *S. paratyphi* C, *S. typhimurium, S. cholerae-suis,* or other gram-negative bacteria that could produce symptoms similar to *S. paratyphi* A infection (Ekpo *et al,* 1995). Korbsrisate *et al,* (1994) reported that the 52 kDa protein, carrying the target epitope of the MAb, is phase1 flagellin, the monomer of bacterial flagellar filament. The phase 1 flagellin in *S. paratyphi* A has been identified serologically as phase 1-a which is also encountered in more than 90 other bioserotypes of *Salmonella* (Popoff *et al,* 1997). In this study, further characterization of the MAb specific to the 52 kDa protein of *S. paratyphi* A demonstrated that

Correspondence: Dr Sunee Korbsrisate, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
Tel: 66-2-419-7066; Fax: 66-2-418-1636

the immunoreactive epitope is phase1-a specific and has been mapped to variable region IV of the protein. In addition, the flagellin protein fragment carrying phase1-a specific epitope has been used to develop an indirect ELISA to detect IgM antibody in patients' sera for improved diagnosis of *S. paratyphi* A infection.

MATERIALS AND METHODS

Bacterial strains

Salmonella possessing phase1-a flagellar antigen including *S. paratyphi* A (1,2,12:a:[1,5]), *S. assen* (21:a:1,5), *S. hannover* (16:a:1,2), *S. oslo* (6,7:a:e,n,x) and *Salmonella* 40:a:z39 ssp.II were kindly provided by the National Institute of Health, Nonthaburi, Thailand. *Escherichia coli* JM101 was used for the cloning and production of specific flagellin segment fused with glutathione S-transferase (GST) protein (flagellin-GST fusion protein).

Serum samples

Serum specimens used in this study were obtained from 4 groups of patients and 115 healthy normal subjects. The four groups of patients included 51 patients whose hemocultures were positive for *S. paratyphi* A, 90 patients for *S. typhi*, 43 patients for either gram-positive cocci *(Staphylococcus aureus, Streptococcus pneumoniae)* or gram-negative bacilli (*Salmonella* serogroup B and C, *Burkholderia pseudomallei, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *E. coli)*, and 13 patients who had serologically confirmed dengue virus infection.

Cloning of *S. paratyphi* A flagellin gene segments

Our previously constructed pSKA-7 plasmid (Korbsrisate *et al*, 1994), which carries *S. paratyphi* A flagellin gene at nucleotide (nt) 452-1314, was digested with different restriction enzymes. They are *Bam* HI, *Hae* III, *Hinc* II, *Dra* I and *Eco* RI. The flagellin gene fragments obtained were nt 452-890, 890-1182, 1182-1314, 452-611 and 611-890. These DNA fragments were ligated with expression vectors pGEX-3X or pGEX-2T (Smith and Johnson, 1988) in order to get inframe fusion with GST and transformed into *E. coli* JM101. The resulting recombinant DNA molecules were designated pSK-8E, pSK-9E, pSK-10E, pSK-11E and pSK-15E, respectively.

Immunoblotting

Whole bacterial cell protein from phase1-a *Salmonella* or different recombinant *E. coli* constructs were prepared by mixing a loopfull of viable cells of bacteria with 600 µl sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heat for 2.5 minutes in boiling water bath. The whole bacterial cell proteins were separated on 12% SDS-polyacrlamide gel (Laemmli, 1970) before being electroblotted onto nitrocellulose membrane with the buffers described by Towbin *et al* (1979). The blotted nitrocellulose was stained by an immunoenzyme staining method (Korbsrisate *et al*, 1994) using MAb specific to *S. paratyphi* A 52 kDa antigen and alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins.

Preparation of GST and GST-flagellin fusion protein

Overnight culture of *E. coli* carrying plasmid pGEX or pGEX with the flagellin gene insert was diluted 1/100 in Luria-Bertani broth and grown at 37°C with vigorous shaking to an approximate absorbance at 600 nm of 0.5. 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 was incubated for a further 3 hours. Cells were harvested by centrifugation at 3,000g and resuspended in 1/50 of the original volume in phosphate-buffered saline (PBS) pH 7.4. The cells were then lysed by sonication and debris discarded after centrifugation at 10,000g. 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 protein sample was checked by observation of protein pattern after Coomasie brilliant blue staining of electrophoresed protein. The protein concen-

tration 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 at optimal conditions. In brief, microtiter plates (96-well, flat-bottom, Corning Laboratory Sciences Company, New York) were coated overnight at 4°C with 100 µl of 3 µg/ml GST or 20 µg/ml GST-flagellin protein. Plates were washed 3 times 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 of PBS containing 3% skim milk, 0.1% Tween-20 and incubation for 1 hour at 37°C, followed by three washes with PBS-Tween. Each diluted serum sample (1:100 with 1% skim milk in PBS) was added to both GST and GST-flagellin coated wells, then the plate was incubated for 1 hour 37°C. After 3 washes with PBS-Tween, 100 µl of alkaline phosphatase conjugated anti-human IgM F(ab')2 fragment (Sigma) diluted to 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 p-nitrophenyl phosphate (Sigma). The concentration of added substrate was 1 mg/ml dissolved in 0.05 M carbonate buffer pH 9.8, containing 0.005 M MgCl1. $6H_2O$. The color 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 samples were considered positive when the absorbance obtained from wells coated with GST-flagellin minus the absorbance of GST coated wells was greater than 0.15. This cut-off value is derived from the average absorbance value plus 2 SD (mean+2SD) of 115 normal individuals.

Statistical method

The method of Galen (1979) was used for calculating the diagnostic specificity, sensitivity, positive and negative predictive values, using hemoculture as a gold standard. Only positive results obtained from those patients with hemoculture positive for *S. paratyphi* A were considered as true positives.

RESULTS

MAb specific to phase 1-a flagellin protein

We previously reported production of a MAb specific to *S. paratyphi* A 52 kDa flagellin protein (Ekpo *et al,* 1995; Korbsrisate *et al,* 1994). The specificity of the MAb was confirmed by positive staining with *S. paratyphi* A and negative reaction with *S. typhi* (phase1-d), *S. paratyphi* B (phase1-b), *S. paratyphi* C (phase1-c), *S. cholerae-suis* (phase1-c), *S. enteritidis* (phase1-g,m), *S. typhimurium* (phase1-i), *S. krefeld* (phase1-y), *S. panama* (phase1-l,v) and three other entero-bacteriaceae (Ekpo *et al,* 1995). In this report, further studies have been performed to demonstrate whether the specific epitope recognized by the MAb on 52 kDa protein is phase1-a specific or specific to *S. paratyphi* A.

When whole bacterial cell proteins extracted from other phase1-a *Salmonella* including *S. assen, S. hannover, S. oslo* and *Salmonella* 40:a:z39 ssp II were allowed to react with the MAb compared with that from *S. paratyphi* A, the MAb reacted with protein from all phase1-a *Salmonella* tested at the same molecular weight at 52 kDa (Fig 1). This data, together with our previous demonstration that the MAb does not react with the protein antigen from non-phase1-a *Salmonella* and other gram-negative

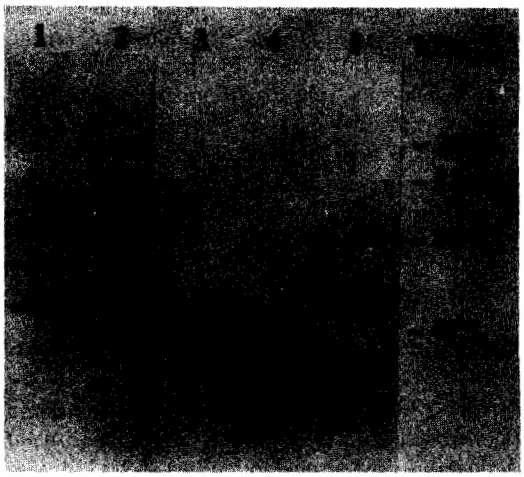

Fig 1–Specificity of the MAb. Western blot of flagellin proteins from phase 1-a *Salmonella* including; *S. paratyphi* A (lane 1), *S. assen* (lane 2), *S. hannover* (lane 3), *S. oslo* (lane 4) and *Salmonella* 40:a:Z39 ssp II (lane 5) were immunostained with the MAb.

bacteria (Ekpo *et al,* 1995), indicates that the epitope recognized by the MAb is not species specific but is specific to phase1-a flagellin.

Specific phase1-a epitope located on hypervariable region IV

To localize this epitope, our previously constructed pSKA-7 plasmid (Korbsrisate *et al,* 1994) which encodes the phase1-a specific epitope was digested with restriction enzymes to generate flagellin DNA fragments at nt 452-890, 890-1182 and 1182-1314. These DNA fragments were cloned into expression vectors and the constructed recombinant plasmids were designated pSK-8E, pSK-9E and pSK-10E, respectively (Fig 2). Western blot analysis of the proteins expressed from these plasmids revealed that all flagellin fragments showed negative staining with phase1-a specific MAb except the flagellin protein expressed from pSK-8E (flagellin DNA nt 611-890, Fig 2). Two plasmid constructs, pSK-11E and pSK-15E, were generated by digestion of flagellin DNA on plasmid pSK-8E. Data from immunoblotting showed that only *E. coli* haboring plasmid pSK-15E can react with the MAb (Fig 3). Among the 8 regions on the *Salmonella* flagellin molecule, the flagellin gene fragment encoding on pSK-15E is comparable to the hypervariable region IV according to the Wei and Joys (1985) study. Since the cloning vector used in this study contained GST gene upstream of the multiple cloning sites, the molecular weight of the fusion protein expressed from pSK-15E is 36.5 kDa (27.5 kDa from GST plus 9.0 kDa from region IV of flagellin protein (Fig 3).

Application of flagellin fragment with phase1-a epitope

The hypervariable region IV from *S. paratyphi* A flagellin is an antigenically important site for phase1-a flagellin. Among *Salmonella* possessing phase1-a flagellar antigen, only *S. paratyphi* A has important frequent associations with severe disease and bacteremia. Therefore, the hypervariable region IV of flagellin might be useful as an antigen for the detection of specific antibodies against *S. paratyphi* A, a causative agent of enteric fever.

An indirect ELISA was developed to detect IgM in patients' sera using flagellin-GST fusion protein expressed from plasmid clone pSK-8E as specific

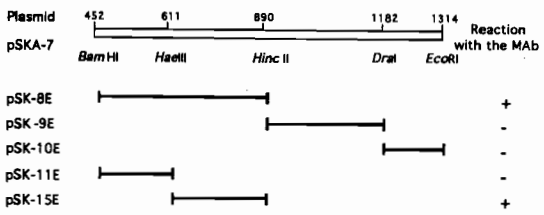

Fig 2–Relative locations of *S. paratyphi* A flagellin gene inserted into either expression vector pGEX-3X or pGEX-2T and their reactivities with the MAb. The upper open bar represents the original clone, pSKA-7.

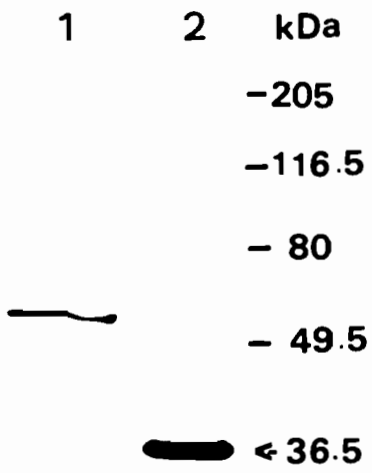

Fig 3–Western blot of whole cell protein extracted from *E. coli* harboring plasmid pSK-15E (lane 2) reacted with the MAb. The 52 kDa flagellin protein isolated from *S. paratyphi* A is included (lane 1).

antigen and GST protein as control antigen to check for false positive results due to the presence of anti-GST or other antibodies that might cross-react with GST in the serum samples. Of the 51 serum samples collected from patients whose hemocultures diagnosed *S. paratyphi* A infection, 29 had detectable levels of IgM against the designed flagellin protein. In contrast, among 90 and 43 patients whose hemocultures were positive for *S. typhi,* and other bacterial pathogens, respectively, only one subject in each group (1.1% and 2.3%) gave a positive IgM reaction against S. *paratyphi* A flagellin. Of 13 dengue virus infected patients, presenting with febrile symptoms similar to *S.*

Table 1

ELISA for the detection of IgM antibody in sera from patients infected with *S. paratyphi* A, *S. typhi*, other gram-positive and gram-negative bacteria, dengue virus and healthy controls.

Group of serum samples	No. of cases	No. of cases with ELISA	
		Positive (%)	Negative (%)
Hemoculture positive			
S. paratyphi A	51	29 (56.9%)	22 (43.1%)
S. typhi	90	1 (1.1%)	89 (98.9%)
Gram-positive and gram-negative bacteria	43	1 (2.3%)	42 (97.7%)
Dengue virus antibody positive	13	0 (0%)	13 (100%)
Healthy controls	115	1 (0.9%)	114 (99.1%)

Table 2

Relationship between specific IgM response against *S. parahyphi* A flagellin protein (clone pSK-8E) and time after the onset of fever.

Time after onset (days)	No. of cases	No. of cases with ELISA	
		Positive (%)	Negative (%)
<3	2	0 (0%)	2 (100%)
3-5	12	4 (33.3%)	8 (66.7%)
6-8	9	4 (44.4%)	5 (55.6%)
9-12	6	5 (83.3%)	1 (16.7%)
13-14	6	4 (66.7%)	2 (33.3%)
>14	1	0 (0%)	1 (100%)

paratyphi A infection, none gave a positive result with the test. Among 115 healthy control subjects, only one (0.9%) showed IgM positivity against *S. paratyphi* A flagellin (Table 1). From the data presented, the calculated sensitivity, specificity, positive and negative predictive values are 56.9, 98.8, 90.6 and 92.1%, respectively. The sensitivity of detection is unsatisfactory. Among 51 *S. paratyphi* A infected patients reported in this study, only 36 patients had clinical information regarding

the time of onset of fever. These patients can be divided into 6 groups. Table 2 shows the result of specific IgM detection from these 6 groups of patients. It demonstrates that sensitivity of detection is variable with time. The maximum sensitivity of IgM detection was 83.3% in serum samples which had been collected 9-12 days after the onset of fever. Serum samples collected earlier or later this period of time showed lower sensitivity of IgM detection against *S. paratyphi* A flagellin.

DISCUSSION

The MAb directed against phase1 flagellin from *S. paratyphi* A previously produced by our group has been shown in this study to be specific to phase 1-a flagellin. The phase 1-a flagellin from *S. paratyphi* A is composed of 499 amino acids. Comparison of the amino acid sequence with 4 other phase1 flagellins has led to the definition of eight regions of different variability (Wei and Joys, 1985). The amino- and carboxy- terminal sequences are conserved whereas the middle part of the molecule is variable. Epitope mapping of our MAb demonstrated that the specific phase1-a epitope is located on the middle part of the flagellin, hypervariable region IV. The remarkable feature of this region is the heterogeneity of amino acid sequences among *Salmonella* flagellins. Wei and Joys (1985) found that the predicted amino acid sequence of region IV showed hypervariation, no greater than 30% homology, for any pairwise comparison between 5 alleles of phase1 flagellin *(a, d, c, i, r)*. This data is in agreement with our result which indicates that the specific epitope of phase1-a flagellin protein is located on this hypervariable region. In addition, the specific phase1-i epitope (Joys and Martin, 1973) and two major epitopes of phase1-d flagellin (Frankel *et al,* 1989; He *et al,* 1994; Newton *et al,* 1991) as previously identified were also located within this region. Thus, the hypervariable region IV of *Salmonella* flagellin is antigenically important for different phases of flagella. In addition to region IV, region V and VI were also found to be responsible for the specific serotype of *Salmonella* flagellin (Asten *et al,* 1995; Joys and Schödel, 1991). From the three-dimensional model of *S. typhimu- rium* flagellin (Namba *et al,* 1989), each of the several thousands of individual flagellins is seen to be folded in a horse-shoe-like structure. The termini, the legs of the horse-shoe, are in the center of the flagellum near the central cavity. There fore, the posibility of these termini to induce specific immune response will be low. The middle part of the flagellin, consisting of regions IV-VII, is exposed at the surface of the flagellar filament. Therefore, hypervariable region IV, with a specific phase1-a epitope, should be a better imunogen when compared with the termini of the modecule.

Enteric fever remains one of the most prevalent acute infectious diseases in the developing world and improved methods of diagnosis are required. Diagnosis of *S. paratyphi* A infection 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. However, the prevalence of H antibodies in adults living in endemic areas is too high for the test to be useful, and false positives may occur because of the crude antigen used in the test. Besides the Widal test, no other serological technique for *S. paratyphi* A diagnosis is available. In this study, the hypervariable region IV of phase1 flagellin of *S. paratyphi* A has been demonstrated to be immunogenic and antigenically important in the structure of flagellar antigen a. Among *Salmonella* possessing phase1-a flagellar antigen, only *S. paratyphi* A has important frequent associations with severe disease and bacteremia. Therefore, this region of flagellin should be useful as an antigen for the detection of specific antibodies against *S. paratyphi* A in serological diagnosis.

Purified flagellin protein was extracted from clone pSK-8E which had been fused with GST. The fusion with GST was constructed to aid purification of flagellin from *E. coli* proteins. GST can be cleaved-off from flagellin by blood coagulation factor X_a or thrombin (Smith and Johnson, 1988). However, we found that the yield and quality of purified flagellin antigen obtained was not satisfactory. Therefore, flagellin-GST fusion protein was used as the specific antigen in this test. To overcome the problem of false positives due to the presence of antibody against GST, IgM antibody against purified GST was also assayed together with that against flagellin-GST fusion protein. From 115 normal serum samples, we found that 5 samples (4.3%) showed the presence of IgM antibody against GST. This strategy of absorbance subtraction not only prevents false positives due to the presence of antibody against GST but also due to the presence of antibody against *E. coli* antigen that might have

been a contaminant in the antigen preparation. With an established system, only 56.9% of sera from patients whose hemocultures were positive for *S. paratyphi* A gave positive results in the test. The characteristic of IgM antibody is that it declines rapidly after infection instead of persisting in the blood circulation like IgG. This low sensitivity of detection might have been a consequence of when the serum samples were collected. Too early or too late a collection of serum samples, when IgM has not been produced or has already declined, would result in a failure to detect the antibody. The time of collection of the serum sample is important as it has been revealed in this study that the maximum sensitivity of IgM detection was obtained when the sera were collected 9-12 days after the onset of fever.

The advantage of our developed IgM detection method over the Widal test (mainly IgG detection method) is that a positive result from a single serum sample can indicate recent infection whereas the IgG detection method needs paired sera to exclude previous infections from recent ones, especially in endemic areas. This is because patients with enteric fever have been reported to have IgG against H or flagellar antigen persisting for years after the end of the illness (Ivanoff, 1994). In addition to the difference in antibody class to be detected, the Widal test and our developed ELISA use different antigens. Our established ELISA system uses flagellar antigen only from the central part of the protein whereas the Widal test uses crude and whole flagellin molecule. This property leads to the high specificity of the ELISA we have developed. However, we found that further reduction of the flagellin protein to contain only hypervariable region IV (pSK-15E) did not improve the specificity. On the other hand, it resulted in decreasing the sensitivity of detection. This might due to the lower number of epitope present on the flagellin antigen that can be recognized by IgM antibody in the serum.

ACKNOWLEDGEMENTS

We would like to thank Mrs Bangtrakulnonth A and Mrs Pornruangwong S (National Institute of Health, Nonthaburi, Thailand) for providing phase1-a *Salmonella*. We also appreciate the suggestions and comments of Dr Cowan during the preparation of the manuscript.

REFERENCES

Asten Van AJAM, Zwaagstra KA, Baay MF, Kusters JG, Huis JHJ, Van Der Zeijst BAM. Identification of the domain which determines the g,m serotype of the flagellin of *Salmonella enteritidis*. *J Bacteriol* 1995; 177: 1610-3.

Ekpo P, Sarasombath S, Banchuin N, Pongsunk S, Korbsrisate S, Sirisinha S. Monoclonal antibodies against protein antigens of Salmonellae causing paratyphoid fever and their diagnostic application. *Asian Pacific J Allergy Immunol* 1995; 13: 63-70.

Frankel G, Newton SMC, Schoolnik GK, Stocker BAD. Intragenic recombination in a flagellin gene: characterization of the H1-j gene of *Salmonella typhi*. *EMBO J* 1989; 8: 3249-52.

Galen RS. The predictive value of laboratory testing. *Orthopedic Clin North Am* 1979; 10: 287-97.

He X, Rivkina M, Stocker BAD, Robinson WS. Hypervariable region IV of *Salmonella* gene *fliC*^{d} encodes a dominant surface epitope and a stabilizing factor for functional flagella. *J Bacteriol* 1994; 176: 2406-14.

Ivanoff B, Levine M, Lambert PH. Vaccination against typhoid fever: present status. *Bull WHO* 1994; 72: 957-71.

Joys TM, Martin JF. Identification of amino acid changes in serological mutants of the i flagellin antigen of *Salmonella typhimurium*. *Microbios* 1973; 7: 71-3.

Joys TM, Schödel F. Epitope mapping of the *d* flagellin antigen of *Salmonella muenchen*. *Infect Immun* 1991; 59: 3330-2.

Korbsrisate S, Sarasombath S, Ekpo P, Pongsunk S. Molecular cloning and expression of *Salmonella paratyphi* A 52 kDa specific protein gene. *Asian Pacific J Allergy Immunol* 1994; 12: 27-37.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

Namba K, Yamashita I, Vondeviszt F. Structure of the core and central channel of bacterial flagella. *Nature* 1989; 342: 648-54.

Newton SMC, Wasley RD, Wilson A, Rosenberg LT, Miller JF, Stocker BAD. Segment-IV of a *Salmonella*-flagellin gene specifies flagellar antigen epitopes. *Mol Microbiol* 1991; 5: 419-25.

Parker MT. Enteric infections: typhoid fever and paratyphoid fever. In: Smith GR, Easmon CFS. Topley and Wilson's princilpes of bacteriology, virology and immunity, vol. 3. Inc., Philadelphia Hamilton: Decker BC, 1991; 423-39.

Popoff MY, Minor LL. Antigenic formulas of the *Salmonella* serovars. WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France. 1997; 151p.

Smith DB, Johnson KS. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 1988; 67: 31-40.

Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc Natl Acad Sci USA* 1979; 76: 4350-4.

Wei LN, Joys TM. Covalent structure of three phase-1 flagellin filament proteins *of Salmonella*. *Mol Biol* 1985; 186: 791-803.