



# Monoclonal Antibodies Against Protein Antigens of Salmonellae Causing Paratyphoid Fever and Their Diagnostic Application

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Enteric fever (typhoid and paratyphoid fever) is caused by four enteric bacteria, namely, *Salmonella typhi*, *S. paratyphi A*, *S. paratyphi B* and *S. paratyphi C*. The disease is still a public health problem in developing countries.<sup>1</sup> The clinical manifestations of the disease are usually nonpathognomonic.<sup>2,3</sup> Thus, the diagnosis is based on clinical suspicion and supported by positive culture and serological finding of paired serum samples.<sup>4</sup> The isolation of *Salmonella* spp from various specimens provided a conclusive diagnosis, but it is a time consuming process and sometimes can give false negative result owing to prior antibiotic therapy. Serological diagnosis of *Salmonella* infection has mainly relied on the detection of antibodies. The tube agglutination (Widal test) traditionally has been used in the diagnosis of enteric fever, but several reports have seriously requested its reliability, especially in endemic area because of its cross-reactivity with other bacteria and high prevalence of antibody in normal population.<sup>5,6</sup> The confirmative result requires both acute and convalescent phase serum samples that are rarely collected.

**SUMMARY** Hybrid clones producing monoclonal antibodies (MAbs) specific for *Salmonella paratyphi A* (72 clones), *S. paratyphi B* (9 clones) and *S. paratyphi C* (8 clones) were produced by using the affinity purified *Salmonella* protein (Bp) as immunogens. MAbs to *S. paratyphi A* and *S. paratyphi B* reacted specifically with the 52 kDa homologous flagellin protein components while those to *S. paratyphi C* reacted with a 61 kDa flagellin protein component. The MAbs against *S. paratyphi A* and *S. paratyphi B* were used to establish a double antibody sandwich ELISA for detection of the 52 kDa flagellin antigens in serum samples from patients with acute paratyphoid A and paratyphoid B fever. With this assay system, 6.25 ng per ml of flagellin antigens of *S. paratyphi A* and *S. paratyphi B* could be detected. However, the assay system could not detect the flagellin antigens in patients' sera. The presence of IgM antibodies to the 52 kDa antigens of *S. paratyphi A* and *S. paratyphi B* in the acute sera from paratyphoid A or paratyphoid B patients suggested that the 52 kDa protein components of both salmonellae are good immunogens for human and might be used as antigens for early diagnosis of paratyphoid A and paratyphoid B fever.

Many investigators try to develop new immunological methods for improving the diagnosis of enteric fever. The methods included antigen detection<sup>7-10</sup> and antibody detection.<sup>11,12</sup> However, there is no single immunodiagnostic test that can be recommended for rapid, sensitive and specific diagnosis of enteric fever. The test should be directed toward detecting the *Salmonella* antigens or detecting of IgM antibodies against the specific antigens in patients' specimens

In previous reports, we established MAbs which reacted specifically

with a 52 kilodalton (kDa) flagellin protein of *S. typhi*.<sup>13,14</sup> The 52 kDa antigen is a good immunogen for human. This was demonstrated by

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the presence of IgM antibody to this antigen in acute typhoid sera. Thus, the MAbs were then used to select specific antigen derived from recombinant DNA technology.<sup>14</sup> This recombinant *S. typhi* protein was found to be very useful for detection of specific IgM antibody in acute typhoid patients' sera in an endemic area.<sup>15</sup>

In an attempt to improve the diagnostic test of paratyphoid fever, specific monoclonal antibodies (MAbs) against *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C were produced in this present study. The MAbs specific to *S. paratyphi* A and *S. paratyphi* B were used to establish double antibody sandwich ELISA for detection of the specific antigens in sera from patients with paratyphoid A and paratyphoid B fever. With the assay system, the MAbs could not detect the specific antigens in the patients' sera. However, when the specific components were studied for IgM antibodies detection in acute sera from patients with paratyphoid fever, we found that the acute sera from patients with paratyphoid A and paratyphoid B fever showed the presence of specific IgM antibodies to the specific components of *S. paratyphi* A and *S. paratyphi* B, respectively. The finding of IgM antibodies to the specific components in acute infection suggested that the specific components of *S. paratyphi* A and *S. paratyphi* B are strong immunogens and would be highly useful for early diagnosis of paratyphoid A and paratyphoid B fever.

## MATERIALS AND METHODS

### Bacterial strains

The bacterial strains used in this study were *S. typhi*, *S. paratyphi* A, *S. choleraesuis*, *S. enteritidis*, *S. krefeld*, *S. panama*, *S. typhimurium*, *Escherichia coli*, *Pseudomonas pseudomallei*, *Yersinia enterocolitica* (all isolated from the patients at Siriraj Hospital), *S. paratyphi* B (obtained from National Salmonella and Shigella Center, Division of Clinical Pathology, Department of Medical Sciences, Ministry of Public

Health, Thailand) and *S. paratyphi* C (kindly supplied by Dr. Bernard Rowe, WHO Collaborating Center for Phage Typing and Resistance of Enterobacteria, London). All of these bacteria were used for preparing the crude Barber protein (Bp) antigens.

Additional bacteria used for the preparation of crude whole cell (WC) homogenate antigens included 18 strains of *S. paratyphi* A, 9 strains of *S. paratyphi* B and one strain each of *S. agona*, *S. blockley*, *S. brunei*, *S. choleraesuis*, *S. chwarzengrund*, *S. derby*, *S. emek*, *S. hardar*, *S. heidelberg*, *S. infantis*, *S. montevideo*, *S. potsdam*, *S. saintpaul*, *S. stanley*, *S. typhimurium* and *S. virchow* (obtained from National Salmonella and Shigella Center).

### Preparation of antigens

Crude Barber protein (Bp) antigens were prepared using the method described in the previous reports.<sup>16, 17</sup> Crude whole cell (WC) homogenate antigen was freshly prepared for each use by mixing a loopfull of viable cells of bacteria grown on trypticase soy agar plate with 600  $\mu$ l of SDS-PAGE sample buffer and heated for 2.5 minutes in boiling water bath. The WC antigen was used in the experiment to rule out any possible proteolytic degradation that might have occurred during the preparation of crude Bp. Flagellin protein antigen of *S. typhi*, *S. paratyphi* A, *S. paratyphi* B or *S. paratyphi* C was purified according to the method of Ibrahim *et al.*<sup>18</sup> Briefly, flagella were detached by exposure of the bacteria to pH 2 with 1 N hydrochloric acid (HCl). After detachment, the flagellin in the supernatant was obtained by centrifugation at 100,000  $\times$  g for 1 hour at 4°C. The supernatant was adjusted to pH 7.2 with 1 N sodium hydroxide, and the flagellin was precipitated with ammonium sulfate at final concentration of 2.67 M for 16 hours at 4°C. Precipitated flagellin was separated by centrifugation at 15,000  $\times$  g for 15 minutes and dialyzed against distilled water for 18 hours at 4°C. The dialyzed flagellin

preparations were then lyophilized and stored at 4°C. Affinity purified Bp antigens of *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C were prepared from affinity columns of Sepharose conjugated with specific IgG against homologous purified Bp antigen. The specific IgG was performed using the method described in the previous report.<sup>13</sup> Briefly, crude Bp antigen was initially purified by successive gel filtration chromatography using a series of Sephadex columns with different molecular weight cutoff. The antigenic reactivity of the eluent was monitored by a method previously described by Savigny and Voller.<sup>19</sup> The fractions with highest activity were pooled, concentrated and cross reactivity components were subsequently removed by successive absorbing with Sepharose conjugated to antibodies against crude Bp of the three heterologous *Salmonella* spp. For example, when *S. paratyphi* A fractions were to be purified, components cross reacting with *S. typhi*, *S. paratyphi* B and *S. paratyphi* C were sequentially removed by passing the *S. paratyphi* A fractions over the anti-*S. typhi* Bp Sepharose column to remove components cross reactive with *S. typhi*. The flow through portion was then passed over the anti-*S. paratyphi* B Sepharose column to eliminate components cross reactivity with *S. paratyphi* B. The flow through portion was then allowed to pass over the final Sepharose column conjugated with anti-*S. paratyphi* C Bp. The final eluent was concentrated by lyophilization and was employed in the production of rabbit antibody. The specific IgG antibody was purified from the rabbit serum by DEAE ion exchange chromatography. The affinity purified Bp antigen was used as immunogen for the production of MAbs.

### Production and Characterization of MAbs

The affinity purified Bp antigens from *S. paratyphi* A, *S. paratyphi* B and



*S. paratyphi C* were used as immunogens for MAbs productions. Six female BALB/cJ mice (6 to 8 weeks of age, kindly provided by the Division of Veterinary Medicine, Armed Forces Research Institute of Medical Science, Bangkok, Thailand) were immunized intraperitoneally with 100  $\mu\text{g}$  of the affinity purified Bp antigen homogenized in complete Freund adjuvant. This priming immunization was followed 2 weeks later with two intravenous injections of 50  $\mu\text{g}$  of the antigen in 0.85% NaCl at 2-week intervals. One day after the last injection, the mouse that gave the highest antibody response as tested by indirect ELISA was sacrificed and spleen cells were taken for fusion with X63-Ag 8.653 myeloma cell line.<sup>20</sup> Hybridomas producing antibodies were screened by indirect ELISA using its corresponding Bp antigen and cloned by limiting dilution (0.3 cells per well). MAbs that produced from each clone were tested for their specificity by indirect ELISA using a panel Bp antigens prepared from *S. typhi*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. choleraesuis*, *S. enteritidis*, *S. krefeld*, *S. panama*, *S. typhimurium*, *E. coli*, *Ps. pseudomallei* and *Y. enterocolitica*. The hybridomas producing MAbs that reacted specifically to only *S. paratyphi A*, *S. paratyphi B* or *S. paratyphi C* were cloned again for three times and were then expanded in 250 ml tissue culture flasks for bulk production. MAbs were partially purified from the culture supernatants by precipitation with 50% ammonium sulfate. MAb isotypes were also determined by indirect ELISA using rabbit antibodies to mouse IgG1, IgG2a, IgG2b, IgG3, IgM,  $\kappa$  light chain and  $\lambda$  light chain conjugated to alkaline phosphatase (Sigma).

The MAbs were further tested for their specificity by SDS-PAGE and immunoblotting method using the panel of crude Bp antigens, WC antigens and purified flagellin protein antigens.

#### Indirect ELISA for detection of MAbs to Bp antigens

Flat-bottom 96-well plate (Dynatech Immulon I) was coated with 20  $\mu\text{g}$  per ml Bp antigen diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 37°C for 5 hours. After washing the wells with 0.15 M phosphate buffer saline (PBS) pH 7.2-0.05% Tween 20 (0.05% PBST), mouse serum or culture supernatant was added to each well and the plate was incubated overnight at 4°C and washed again. Alkaline phosphatase conjugated rabbit anti-mouse immunoglobulins (Dakopatts), diluted 1:250 in 1% bovine serum albumin (BSA) in 0.05% PBST was added and the plate was incubated at 37°C for 3 hours. After washing with 0.05% PBST, the enzymatic reaction was revealed by adding a substrate solution, 1 mg per ml p-nitrophenylphosphate (Sigma). The OD was read at 405 nm. A criterion for a positive result was set arbitrarily at 0.2 OD unit above the background that was determined from the mean OD value performed without test supernatant (the mean OD was 0.05).

#### SDS-PAGE and immunoblotting

The antigen with amounts among 50-100  $\mu\text{g}$  per well was subjected to SDS-PAGE in 10% acrylamide gel and was run at 40 mA constant current per gel slab at 25°C for 3 hours. After electrophoresis, the protein bands were electroblotted onto nitrocellulose membrane in methanol-tris glycine buffer at 6 volts per cm for 16 hours. The proteins blotted onto the nitrocellulose membrane were identified by immunoblotting, protein and glycoprotein staining.

The nitrocellulose membrane was rinsed in PBS-1% Tween 20 (1% PBST) and then immersed in 3% BSA dissolved in 1% PBST (3% BSA-1% PBST) at room temperature for 3 hours. After washing with 1% PBST, the membrane was incubated with the MAb or patient serum at dilution of 1:200 (in 1% BSA-0.05% PBST) at room temperature for 2 hours, washed again and incubated

with 1:1,000 dilution of rabbit antimouse immunoglobulin-alkaline phosphatase conjugate (Dakopatt) or goat anti-human IgM-alkaline phosphatase conjugate (Sigma) in 3% BSA-1% PBST at room temperature for 1 hour. The substrate o-dianizidine tetrazotised (6 mg per ml) and beta-naphthyl acid phosphate (1 mg per ml) (Sigma) was added and the reaction was stopped by washing with distilled water.

#### Staining of protein components

The nitrocellulose membrane was soaked for 8 minutes in 0.2% amido Schwartz 10 B (Merck). Excess staining was removed by placing it in destaining solution (45% methanol, 10% glacial acetic acid in distilled water).

#### Staining of glycoprotein components

The nitrocellulose membrane was immersed in 1% PBST at 37°C for 30 minutes, transferred to 3% BSA-1% PBST and incubated at room temperature for 2 hours. After incubation, the membrane was washed with 10 mM tris-HCl, pH 8.0 and 200  $\mu\text{g}$  per ml concanavalin A (con A) (Sigma) in 10 mM tris-HCl was added and incubated at 4°C overnight. Then the membrane was washed with tris buffer before allowing it to react with 100  $\mu\text{g}$  per ml horseradish peroxidase (Sigma) in 3% BSA-1% PBST for 2 hours at room temperature. The membrane was washed again before a substrate solution (0.3 mg per ml of 3,3'-diaminobenzidine in 50 mM tris-HCl, pH 7.4 with 0.05%  $\text{H}_2\text{O}_2$ ) was added and the reaction was stopped by washing with distilled water.

#### Clinical specimens

Serum specimens from patients with hemoculture positive for *S. typhi* (41), *S. paratyphi A* (27), *S. paratyphi B* (5), *Salmonella* group B (23), *Salmonella* group D other than *S. typhi* (1), gram negative bacteria (31) such as *Acinetobacter lwoffii*, *Citrobacter diversus*, *E. coli*, *Enterobacter* spp, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas* spp, gram posi-



tive bacteria (18) such as *Bacillus* spp, *Clostridium perfringens*, *Staphylococcus* spp, *Streptococcus* spp and 35 normal sera were included in this study for antigen detection by double antibody sandwich ELISA with our established MAbs.

The sera from patients with paratyphoid A and paratyphoid B fever were also studied for IgM antibodies specific to the components of *S. paratyphi* A and *S. paratyphi* B.

#### Double antibody sandwich ELISA for antigen detection

Rabbit IgG anti-flagellin protein was purified from the rabbit serum anti-flagellin protein (Biotechnical, Bangkok, Thailand) by DEAE ion exchange chromatography. Double antibody sandwich ELISA for antigen detection was performed by coating the microelisa plate with 20 µg per ml of the rabbit IgG in carbonate buffer at 4°C overnight. After incubation, the plate was washed with 0.05% PBST, blocked with 1% BSA-0.05% PBST at 37°C for 1 hour and washed again. Various concentrations of flagellin protein antigen or patient serum at dilution of 1:100 (in 1% BSA-0.05% PBST) was added to the plate and incubated at 37°C for 1 hour. The plate was washed again and 10 µg per ml of pooled representative MAbs specific to each *Salmonella* spp was added into the wells and the plate was incubated at 37°C for 1 hour, then washed. The 1:250 dilution of rabbit anti-mouse immunoglobulin-alkaline phosphatase conjugate (Dakopatts) in 1% BSA-0.05% PBST was added. The plate was incubated at 37°C for 1 hour and washed. Then the substrate (p-nitrophenyl phosphate) was added and the absorbance value was read at 405 nm. Each sample was performed in duplicate.

## RESULTS AND DISCUSSION

### Production and characterization of MAbs

#### MAbs against specific component

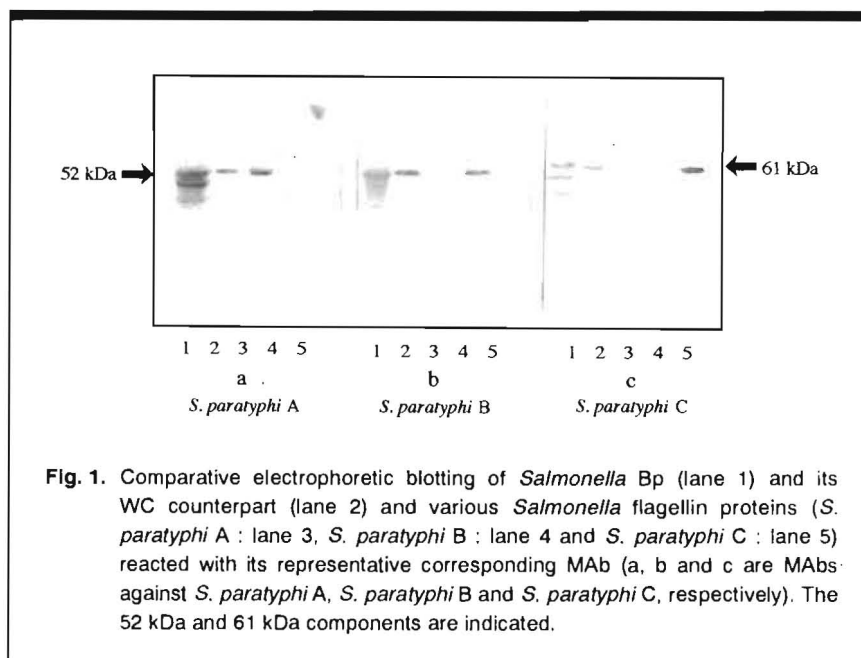
in Bp of *S. paratyphi* A, *S. paratyphi* B or *S. paratyphi* C were produced by immunization of BALB/cJ mice with the homologous affinity purified Bp antigen. The mouse producing the highest titre of antibodies was sacrificed and spleen cells were taken for fusion with X63-Ag 8.653 myeloma cell line. The hybridomas that secreted MAbs specific to only *S. paratyphi* A, *S. paratyphi* B or *S. paratyphi* C were cloned for 3 times. After clonings, there were 72, 9 and 8 stable clones secreting high levels of MAbs specific to *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C, respectively.

It was found that the 72 and 9 MAbs secreted from the clones were highly specific to *S. paratyphi* A and *S. paratyphi* B, respectively as determined by indirect ELISA against a panel of crude Bp antigens prepared from *S. typhi*, *S. paratyphi* A, *S. paratyphi* B, *S. paratyphi* C, *S. choleraesuis*, *S. enteritidis*, *S. krefeld*, *S. panama*, *S. typhimurium*, *E. coli*, *Ps. pseudomallei* and *Y. enterocolitica*. For MAbs against *S. paratyphi* C, however, the 8 MAbs secreted from the 8 stable clones were equally reactive with *S. paratyphi* C Bp and *S. choleraesuis* Bp but failed to react with other crude Bp antigens, as determined by indirect

ELISA. Isotyping of these MAbs showed all to be IgG with κ light chains.

All 72 *S. paratyphi* A MAbs exhibited identical immunoblotting pattern when tested against *S. paratyphi* A Bp. The pattern exhibited smearing reaction from the 30-52 kDa positions (Fig. 1a, lane 1). The 9 *S. paratyphi* B MAbs also exhibited identically and smearing immunoblotting pattern (30-52 kDa positions) when tested against *S. paratyphi* B Bp (Fig. 1b, lane 1). However, all 8 *S. paratyphi* C MAbs exhibited specific discrete bands at the 45, 47, 52 and 61 kDa positions (Fig. 1c, lane 1). These MAbs also reacted with *S. choleraesuis* Bp at the same positions. In contrast to these results, the crude Bp antigens prepared from all other bacteria did not show any specific reactive band with these MAbs. The characteristics of the 72 *S. paratyphi* A, 9 *S. paratyphi* B and 8 *S. paratyphi* C MAbs are summarized in Table 1 and Table 2.

These MAbs were arbitrarily classified into groups according to the original wells, isotypes and immunoblotting patterns. From these criteria, we divided the MAbs specific to *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C into 4, 1 and 2 group (s),



**Fig. 1.** Comparative electrophoretic blotting of *Salmonella* Bp (lane 1) and its WC counterpart (lane 2) and various *Salmonella* flagellin proteins (*S. paratyphi* A: lane 3, *S. paratyphi* B: lane 4 and *S. paratyphi* C: lane 5) reacted with its representative corresponding MAb (a, b and c are MAbs against *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C, respectively). The 52 kDa and 61 kDa components are indicated.

respectively. One representative clone from each of these groups, its reactivity in an indirect ELISA against a panel of crude Bp antigens and its characteristics were shown in Table 3. These representative MABs were used for detailed analysis.

It was rather unexpected to find that MAB reacted with more than one component in the crude Bp (Fig. 1a, b and c, lane 1). To determine whether these various components were degradative products produced during the preparation of crude Bp or were distinct entities possessing common epitopes, a similar immunoblotting experiment was repeated using freshly prepared *Salmonella* WC as antigens and reacted with each of the 4 and 1 homologous representative MAB to *S. paratyphi* A and *S. paratyphi* B. It was found that the MABs each did give a single staining band at a 52 kDa position (Fig. 1a and b, lane 2). However, with the 2 representative MABs to *S. paratyphi* C the positive reactions were noted at 61 kDa instead of at the 52 kDa position (Fig. 1c, lane 2). This evidence indicated that the epitope reactive with the MABs specific to *S. paratyphi* A or *S. paratyphi* B or *S. paratyphi* C was represented on a single component. But these MABs appeared to react with several components when crude Bp antigens were used. The evidence obtained with crude Bp preparation suggested that the specific components were degraded during the preparation.

To further explore the specificity of these MABs, the WC antigens from homologous species but different strains and WC species from other species within the same serogroup (the same somatic antigen) were used to react with these MABs by immunoblotting technique. The 4 representative MABs to *S. paratyphi* A were reacted with 18 strains of *S. paratyphi* A WC antigens (somatic antigens: 1, 2, 12 and phase 1-a flagellin) and they reacted with all 18 strains at the 52 kDa component.

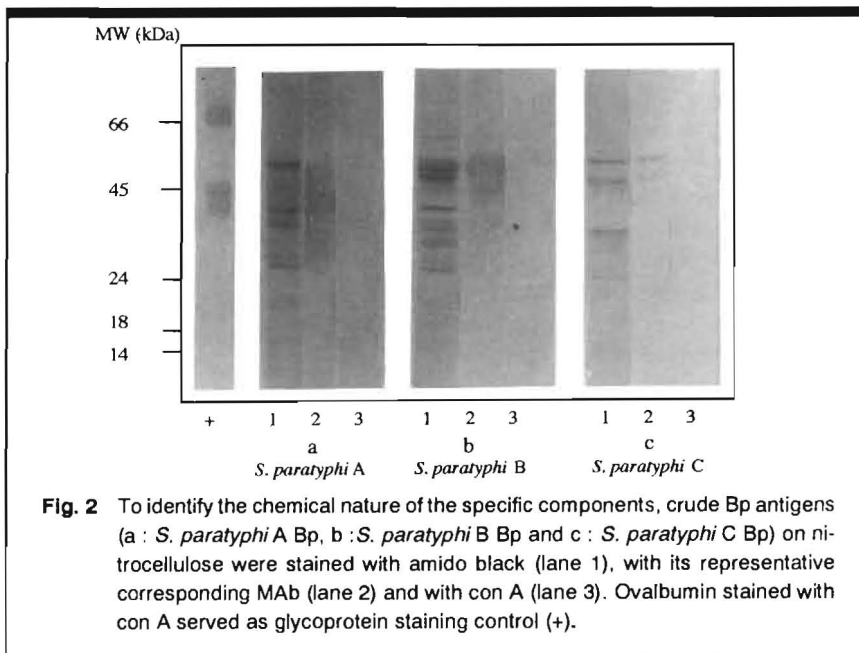
The representative MAB to *S. paratyphi* B was reacted with WC anti-

gens from 9 strains of *S. paratyphi* B (somatic antigens: 1, 4, 5, 12 and phase 1-b flagellin) and 7 other serogroup B with different phase 1 flagellin protein from *S. paratyphi* B (*S. agona*, *S. derby*, *S. heidelberg*, *S. saintpaul*, *S. chwarzengrund*, *S. stanley* and *S. typhimurium*). The MAB reacted only with homologous species at the 52 kDa position and not with the other members in the same serogroup.

The 2 representative MABs to *S. paratyphi* C were also tested for their specificity with WC antigen from 8 other *Salmonella* serogroup C (but no phase 1-c flagellin) (*S. blockley*, *S. brunei*, *S. emek*, *S. hardar*, *S. infantis*, *S. montevideo*, *S. potsdam* and *S. virchow*) and with *S. choleraesuis* which is the same serogroup C (Somatic antigens: 6, 7) and phase 1-c flagellin protein. The positive reactions were found with *S. paratyphi* C and *S. choleraesuis* at 61 kDa.

To identify the chemical nature of the specific components of *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C, the crude Bp antigens on nitrocellulose membrane were stained with amido black that stained the protein components and conA that stained the glycoprotein components. The specific components of the 3 *Salmonella* spp

gave positive staining with amido black but negative with con A (Fig. 2). From the results that MABs could not react with salmonellae within the same serogroup and the negative con A staining suggested that the specific components of these 3 salmonellae were not somatic polysaccharide (O antigen) or a glycoprotein antigen but they were protein in nature. Among *Salmonella* protein antigens, the flagellin antigen is a promising antigen that should contain the specific component. Since there are more than 60 different specific flagellin antigens in *Salmonella* spp,<sup>21</sup> the specificity of the flagellin has been used for classification of *Salmonella* as described by Kauffman and White.<sup>22</sup> In this study, however, only *S. paratyphi* C and *S. choleraesuis* which contained the same phase 1-c flagellin antigen were reacted with MABs to *S. paratyphi* C. To confirm that the specific protein components are flagellin proteins, we purified phase 1 flagellin proteins from *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C and allowed them to react with the heterologous and homologous MABs (Fig. 1a, b and c, lanes 3, 4 and 5). The result showed that the MABs were highly specific to its corresponding flagellin protein and they failed to react with the heterologous flagellin



**Fig. 2** To identify the chemical nature of the specific components, crude Bp antigens (a : *S. paratyphi* A Bp, b : *S. paratyphi* B Bp and c : *S. paratyphi* C Bp) on nitrocellulose were stained with amido black (lane 1), with its representative corresponding MAB (lane 2) and with con A (lane 3). Ovalbumin stained with con A served as glycoprotein staining control (+).



proteins. In addition, the DNA that coded for the specific components of *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C were cloned, sequenced and analyzed.<sup>23-25</sup> It was as expected that the DNA sequences were almost homologous to the phase 1-a, phase 1-b and phase 1-c flagellin genes of *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C, respectively. From these evidences, we concluded that the 52 kDa specific protein component of *S. paratyphi* A or *S. paratyphi* B or the 61 kDa specific protein component of *S. paratyphi* C is the phase 1-a or phase 1-b or phase 1-c flagellin of *S. paratyphi* A or *S. paratyphi* B or *S. paratyphi* C, respectively.

#### Clinical application

The 4 pooled representative MABs against *S. paratyphi* A and the representative MAB against *S. paratyphi* B were used to establish a double antibody sandwich ELISA for detection of the 52 kDa flagellin antigens in serum samples from patients with acute paratyphoid A or paratyphoid B fever. With this assay system, as little as 6.25 ng per ml of *S. paratyphi* A or *S. paratyphi* B flagellin antigen in normal serum could be readily detected (Fig. 3). However, when the clinical specimens from patients with hemoculture positive for *S. typhi* (41), *S. paratyphi* A (27), *S. paratyphi* B (5), *Salmonella* group B (23), *Salmonella* group C (9), *Salmonella* group D other than *S. typhi* (1), gram negative bacteria (31), gram positive bacteria (18) and 35 normal sera were tested, no positive result could be demonstrated. It is possible that the 52 kDa flagellin epitopes are blocked in the form of immune complexes or changed after being metabolized and our MABs could not recognize. This result is similar to our previous reports that the MABs specific to *S. typhi* phase 1-d flagellin protein could not detect the specific antigen in patients' specimens.<sup>13,14</sup> However, the fusion protein of *S. typhi* flagellin was very useful for detection of specific IgM in acute typhoid patients'

sera.<sup>15</sup>

The significance of the 52 kDa flagellin proteins of *S. paratyphi* A and *S. paratyphi* B could be demonstrated in our preliminary study by using sera from patients with an acute paratyphoid A and paratyphoid B infec-

tion. In these experiments, *S. paratyphi* A WC and *S. paratyphi* B WC antigens were prepared from one local strain, separated by SDS-PAGE, immunoblotted and then probed with patients' sera (diluted 1:200). The results from 2 and 4 acute sera from paratyphoid A

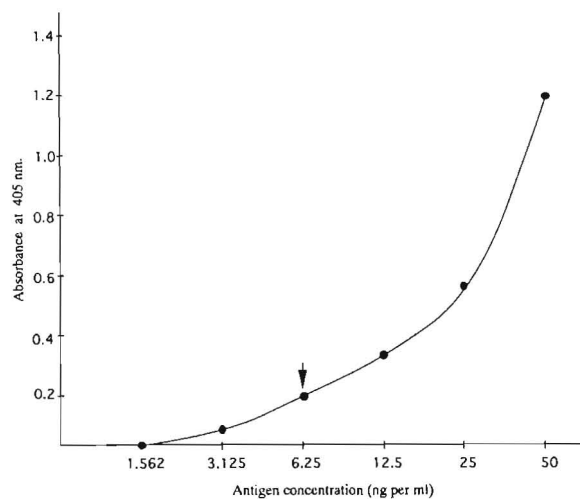


Fig. 3. Dose response curve obtained from the assay of flagellin protein antigen of *S. paratyphi* A at various concentrations with double antibody sandwich ELISA. This assay could detect approximately 6.25 ng per ml of the antigen (arrow).

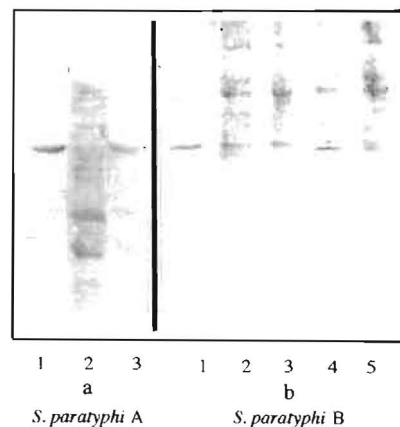


Fig. 4 To determine the significance of the 52 kDa flagellin protein components of *S. paratyphi* A and *S. paratyphi* B for IgM antibodies detection in acute phase sera from patients with paratyphoid fever, the immunoblotting using WC antigen reacted with the patients' sera and human IgM antibodies were detected by alkaline phosphatase conjugated goat anti-human IgM.

a : WC antigen from *S. paratyphi* A reacted with acute phase sera from patients with paratyphoid A fever (lanes 2, 3) and with its representative MAB (lane 1).

b : WC antigen from *S. paratyphi* B reacted with acute phase sera from patients with paratyphoid B fever (lanes 2-5) and with its representative MAB (lane 1).



tients infected with this bacterium either in Thailand or other countries for more than twenty years. Thus, the specific antigen detection and the immunoblotting for detecting the specific IgM to this bacterium were not established.

For further studies, the flagellin fusion proteins of *S. paratyphi* A and *S. paratyphi* B will be produced and they will be used for detection of the specific IgM in acute paratyphoid A and acute paratyphoid B patients' sera. Furthermore, the DNA coding for these specific epitopes will be analyzed and used as DNA probes or DNA primers for diagnosis of the paratyphoid fever.

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