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*Full Length Research Paper*

# Identification and evaluation of immunogenic *Salmonella* Typhi proteins in sera of acute typhoid fever patients using two protein separation methods

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**Pooled sera from typhoid fever patients and normal subjects were subjected to protein fractionation using 2 different methods: 1) affinity chromatography by removing albumin and immunoglobulin, or 2) ultrafiltration membrane by removing proteins above 100 kDa; in order to unmask low abundance and low molecular weight proteins. Protein preparations from both separation methods were subjected to Western blot analysis. The results showed that different separation methods gave different antigen-antibody binding profiles inherent of the technology used. A total of five immunoprecipitin bands were found to be specific for typhoid fever. Mass spectrometry and bioinformatic analyses indicated the presence of *Salmonella* Typhi (*S.* Typhi) proteins in bands 12, 15 and 50 kDa; and bands 18, 25 and 50 kDa contained human proteins. *S.* Typhi proteins, namely: blue copper oxidase (CueO), outer membrane protein C (OmpC), and flagellar hook-associated protein 1 (HAP1), were selected and cloned using *Escherichia coli* expression system. Using indirect ELISA, the individual recombinant proteins gave diagnostic sensitivities of <50%, whereas combining all 3 proteins in a single test gave a higher sensitivity of 70% and specificity of 93%. This study has shown that serum fractionation can uncover immunogenic proteins unique to the pathogen, which can be used as biomarkers for specific diagnosis of the disease.**

**Key words:** Typhoid fever, affinity chromatography, ultrafiltration membrane, blue copper oxidase (CueO), outer membrane protein C (OmpC), flagellar hook-associated protein 1 (HAP1), *in vivo* immunogenic proteins.

## INTRODUCTION

Typhoid fever is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). It is a facultative anaerobic gram-negative bacterium which belongs to the family of *Enterobacteriaceae*. About 2,500 serotypes in the *S. enterica* species exist, but only one serotype (*S.* Typhi) causes typhoid fever in humans (Edsall et al., 1960). In the year 2010, the worldwide incidence of typhoid fever was estimated to be 26.9 million with more than 269,000 deaths annually (Buckle et al., 2012). Blood culture is the gold standard for diagnosis of typhoid fever. However, this technique is time-consuming, laborious, expensive, requires multiple equipment, and has low sensitivity for bacterial detection (Akoh, 1991).

Since typhoid fever is a systemic disease, serum could serve as the diagnostic fluid for biomarker identification. In theory, serum should contain protein signatures from host and pathogen cells within the body (Musselman and Speicher, 2005). Biomarkers of interest might be from tissue leakage products during cell death or damage, aberrant secretions by diseased tissues of the host, as well as from foreign proteins of the invading microbe (Anderson and Anderson, 2002). It was reported that

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some of the proteins produced by the microbes in response to the host's immune system would only be found in the host during host-microbe interactions. However, the major problem is in the identification of these microbial biomarkers present in the patients' sera against a background of innumerable host proteins (Nuti et al., 2011). Human serum consists of a complex mixture of proteins and exhibit broad and dynamic range of concentrations up to 12 orders of magnitude during an acute phase reaction. Two major proteins in serum, that is, albumin and immunoglobulin G (IgG), comprise 90% of the total serum proteins (Tirumalai et al., 2003). These high abundance proteins may mask detection of low abundance proteins, which are thought to be present at nanogram levels and mostly of low molecular weights. Candidate protein biomarkers that are relevant for the diagnosis of a disease have been reported to exist in low concentrations, and to comprise less than one percent of the total serum proteins, making them difficult to be identified in whole serum (VanMeter et al., 2012). To address this challenge, several approaches for simplifying serum-protein profiling have been described including biophysical fractionation, enrichment of target sub-proteomes, and removal of the most abundant interfering proteins by affinity chromatography (Whiteaker et al., 2007).

It is hypothesized here that in acute typhoid fever, the excessive breakdown of the causative microbe and the host tissues could lead to release of specific proteins into circulation. In addition, the blood circulation will also carry antibody molecules raised by the body against these proteins. Therefore, it is conceivable that sera from acute typhoid fever patients could serve as a source of both antibodies and antigens for discovery of biomarkers of the disease. Immunogenic *S.* Typhi proteins, such as flagellin, outer membrane proteins (OMPs), porin and hemolysin, were reported to be small molecules with molecular weights less than 100 kDa (Ismail et al., 1991; Kumar et al., 2009; Nambiar et al., 2009; Ong et al., 2013). Thus, the focus of this study was to discover immunogenic proteins with molecular weights less than 100 kDa in sera of typhoid fever patients. Typhidot™ is a rapid diagnostic test for typhoid fever based on the detection of antibodies against a 50 kDa antigen reported to be specific for *S.* Typhi. This test has reported sensitivities of 26.7 to 100% and specificities of 37.5 to 90% as compared to the culture method (Jesudason et al., 2002; Keddy et al., 2011; Mehmood et al., 2015; Narayanappa et al., 2010; Olsen et al., 2004).

This study aimed to use 2 different separation methods to concentrate the low molecular weight immunogenic proteins in sera, namely: 1) affinity chromatography-based technique to remove albumin and IgG, and 2) membrane filtration-based technique to remove serum proteins of molecular weights above 100 kDa. The low molecular weight proteins were then subjected to Western blot and Mass spectrometry (MS) analyses to identify immunogenic proteins specific for typhoid fever.

## MATERIALS AND METHODS

### Collection of sera

Human sera used were obtained from the Serum Bank of the Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Malaysia. These sera comprised samples from normal controls, patients diagnosed with acute typhoid fever, and patients with non-typhoid fever. All samples were collected in accordance with the guidelines approved by the Human Ethical Committee of Universiti Sains Malaysia.

### Clinical and laboratory history of sampled patients

Typhoid fever patients (n=30) were individuals with fever of more than 5 days duration and positive for *S.* Typhi blood culture and/or Typhidot™ test (antibody test). Non-typhoidal fever patients (n=30) were individuals who had fever but were negative for both *S.* Typhi blood culture and Typhidot™ test. This group consisted of patients diagnosed with dengue, malaria or leptospirosis. Normal controls (n=30) were individuals who have not had clinically-diagnosed typhoid fever or typhoid vaccination in their life time, and had no symptoms of any other infection at the time of blood collection. All individuals were free from any immunosuppressive diseases such as cancer, and were not under any immunosuppressive medication.

### Protein separation methods

Ten individual sera, each from *S.* Typhi blood culture positive patients and normal control subjects, were mixed to obtain pooled typhoid fever sera (PTFS), and pooled normal human sera (PNHS), and were each subjected to two protein separation methods as follows:

#### *Method 1: Separation using affinity chromatography column (Ahmed et al., 2003)*

The two most abundant proteins in human serum, that is, albumin and IgG, were removed using Aurum™ Serum Protein Mini Kit (Bio-Rad, USA) according to the manufacturer's protocol. Sixty microliters of PTFS were diluted with 180 µL Serum Protein Binding buffer and pipetted into the washed Aurum™ column, followed by incubation at room temperature for 15 min. The eluate containing PTFS depleted of albumin and IgG was stored at -20°C until it was ready for use. The same procedure was repeated using PNHS.

#### *Method 2: Separation using ultrafiltration column (Harper et al., 2004)*

Prior to separation by ultrafiltration column, pre-treatment

of the sera was required. Two hundred microliters of PTFS was diluted with 120 µL of 25 mM ammonium bicarbonate containing 20% (v/v) of acetonitrile (64 µl). The mixture was placed in a 40°C water bath for 15 min and then centrifuged at 14,000×g for 10 min to remove serum precipitate (Harper et al., 2004). The supernatant was transferred to a pre-wetted ultrafiltration column containing a 100 kDa cut-off membrane (Amicon Ultra-2 ml Centrifugal Filters, Millipore, USA) and centrifuged at 4,000×g for 30 min. The retentate was flushed with 500 µl of 25 mM ammonium bicarbonate and centrifuged again at 4,000×g for 30 min. This step was repeated twice. The filtrates from the 100 kDa cut-off membrane were pooled and transferred to another pre-wetted ultrafiltration column containing a 3 kDa cut-off membrane, and centrifuged for 1 h at room temperature to concentrate the filtrate. This time, the retentate containing proteins of molecular weights 3 to 100 kDa was recovered by inverting the column and centrifuging at 4,000×g for 1 min. The protein preparation containing low molecular weight proteins was stored at -20°C until it was ready for use. The same procedure was repeated using PNHS.

**Western blot analysis of fractionated PTFS and PNHS proteins with individual typhoid fever, non-typhoid fever and normal human sera**

The fractionated PTFS protein at a concentration of 200 µg was loaded onto a preparative 12% SDS-PAGE gel, and electrophoretically separated at a constant current of 25 mA for 40 min, in order to obtain its protein profile. The proteins in the gel were then transferred onto a pre-activated 0.2 µm PVDF membrane using a Trans-Blot™ SD Semi-Dry Transfer Cell (Bio-Rad, USA) at 10 V for 45 min. The unbound sites on the PVDF membrane were blocked with 5% (w/v) skimmed milk for 1 h at room temperature. The blocked membrane was then washed with PBS, and cut into 4 mm strips using a scalpel. To detect the presence of immunogenic antigens in the fractionated PTFS preparation, the strips were placed in troughs and incubated with individual sera as the source of antibodies, either from acute typhoid fever, non-typhoid fever or normal patients at 1:100 dilution in PBS. Antibodies from the sera were allowed to bind with the separated PTFS proteins overnight at 4°C. Following incubation, the sera were aspirated and the strips washed 3 times in PBS. In order to visualize the antigen-antibody complexes and to ascertain the immunoglobulin class of the bound antibodies, the washed strips were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG or IgM secondary antibodies (Dako, Denmark) at 1:200 dilution in PBS for 2 h at room temperature. The antigen-antibody complexes formed on the membrane were visualized by adding the substrate 4-chloronapthol, which gave purplish colored bands on the strips. Bands which appeared with typhoid fever sera but not with non-typhoid fever or normal sera, were considered immunogenic proteins associated with typhoid fever. The above procedure was repeated using fractionated PNHS preparation as the source of control antigens in order to compare the antigen profiles between PTFS and PNHS.

**Identification of immunogenic proteins using mass spectrometry analysis**

The bands seen in Western blot were excised from the SDS-PAGE gel containing the fractionated PTFS proteins, and analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) by First Base, Malaysia. The mass spectrometry data were compared with the primary sequence databases from MASCOT (Perkins et al., 1999) to characterize and identify the proteins. Parameters were set as follows: type of search = MS/MS ion search; enzyme = trypsin; variable modification = oxidation (M), carbamidomethyl (C), or deaminated (NQ); peptide mass tolerance = ±4.0 Da; database = Swiss-Prot; and taxonomy = *Homo sapiens* and *Salmonella*.

**Cloning, expression and antigenicity testing of *S.* Typhi recombinant proteins using indirect ELISA**

The antigenicity of each *S*. Typhi protein identified by LC-MS/MS was analyzed using Scratch Protein Prediction software (Magnan et al., 2010), and to avoid cross-reactivity with its closely related genus, *Escherichia coli* in the family of *Enterobacteriaceae*, the protein amino acid sequence similarity was compared using NCBI BLAST software (Tatusova et al., 2014). Based on the high antigenicity prediction scores and the low sequence similarity, three *S.* Typhi proteins, namely, blue copper oxidase (CueO), outer membrane protein C (OmpC), and flagellar hook-associated protein 1 (HAP1) were selected for further studies. The target proteins were cloned and the antigenicity of the recombinant proteins was tested using indirect ELISA.

The genome of *S.* Typhi strain ATCC CT18 was used as the reference for all bioinformatics sequences alignment. Primers were designed to anneal with target DNA sequences (*S.* Typhi strain ATCC 7251) for PCR amplification using BioEdit software. The amplified PCR fragments with gene sizes of 1,611 bp (CueO), 1,137 bp (OmpC) and 1,662 bp (HAP1) were ligated into vector pET28a (Novagen-Merck, Darmstadt, Germany) and transformed into *E. coli* dH5α cells (NEB, UK). The resulting plasmids were sequenced (First Base, Malaysia) to ensure that the sequence of the DNA gene was correct. Following successful transformation of the sequenced-plasmids into *E. coli* Lemo21 (DE3) (NEB, UK) competent cells, the recombinant proteins were expressed by induction with 0.1 mmol/L isopropyl β-D-1-thiogalactopyranoside and shook overnight at 200 rpm at

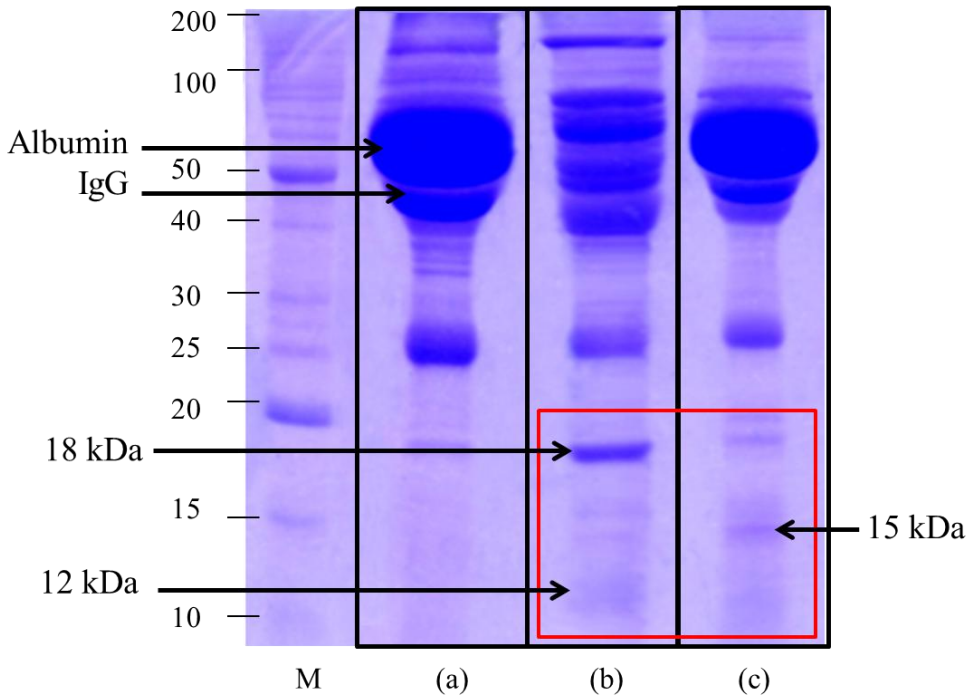


**Figure 1.** SDS-PAGE protein staining profile of the final protein preparations of PTFS using two separation methods. New bands at molecular weight below 20 kDa could be observed following serum fractionation (red box).
M = Molecular weight marker;
(a) = Whole untreated PTFS;
(b) = Method 1 - affinity chromatography separation;
(c) = Method 2 - ultrafiltration membrane separation.

37°C. The His-tagged recombinant proteins, rCueO (58,264 Da), rOmpC (41,239 Da) and rHAP1 (59,074 Da) were purified using Ni-NTA affinity spin column according to the manufacturer's protocol (QIAGEN, Germany), and stored at -20°C until it was ready for use.

The antigenicity of the eluted recombinant proteins was tested using indirect ELISA with a panel of 30 typhoid fever, 30 non-typhoid fever, and 30 normal subjects' sera. The recombinant proteins were diluted using 50 mM carbonate-bicarbonate buffer (pH 9.6), pipetted into a 96-well polypropylene microtiter plate (Greiner Bio-One, Germany) and incubated overnight at 4°C at a concentration of 5 µg/ml for single protein and 7.5 µg/ml for mixture of 3 proteins (2.5 µg/ml for each recombinant protein) in a single test. The unbound sites were blocked with 5% (w/v) skimmed milk in PBS for 2 h, followed by incubation with test human sera (primary antibody) at 1:100 dilution at 37°C for 2 h. The microtitre plate was washed six times with PBS-Tween 20 and HRP-conjugated rabbit anti-human IgG antibody at 1:4000 dilution or rabbit anti-human IgM antibody at 1:1000 dilution was added and incubated at 37°C for 1 h. Finally, the plate was washed and 100 µl of the substrate tetramethylbenzidine (TMB) (Invitrogen, Singapore) was added. After 20 min, the reaction was stopped using 100 µl 2N sulfuric acid and the optical density (OD) of the colored solution read at λ450 nm.

Using SPSS version 20.0, One-way ANOVA was used to compare the significance of the difference between the OD readings of the three groups, that is, typhoid fever, non-typhoid fever and normal human sera. Based on the blank-subtracted OD readings of the sera from the normal group individuals against the recombinant proteins in ELISA, the sample was set either as single protein or as combination of 3 proteins. The cut-off values for IgG and IgM were set as the mean ± 2SD of the OD readings of 2 replicates. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the recombinant proteins were calculated based on Estimation of Post-test Probability (Parikh et al., 2008).

## RESULTS

As evident in Figure 1, using method 1 (affinity chromatography method), albumin and IgG (heavy chain) were removed at the expected molecular weights of about 68 kDa and 50 kDa, respectively. This helped to unmask proteins at molecular weights between 40 to 90 kDa (Figure 1a and b). Method 2 (ultrafiltration method) helped to remove proteins above 100 kDa (Figure 1a and c). However, both separation methods also helped to

concentrate proteins below 20 kDa, allowing them to be visualized following fractionation (Figure 1a, b and c).

Western blot analyses were done to elucidate the presence of immunogenic proteins specific for typhoid fever infection. Using fractionated PTFS proteins as source of antigens, the IgG class of antibodies from individual sera of typhoid fever patients formed an immunoprecipitin band at 12 kDa but not with individual sera of non-typhoid fever or control normal human sera. This 12 kDa band was found in both separation methods (Figure 2a and b). Also, an additional band reactive with typhoid fever IgG class of antibodies was seen at 15 kDa in method 2 (Figure 2b). Western blot analysis with anti-human IgM showed the presence of bands at 25 kDa and 50 kDa, which were reactive with individual sera of typhoid fever patients in both separation methods (Figure 2c and d). Additional bands were seen at 18 kDa (Figure 2c) in method 1 which was also reactive to typhoid fever IgM class of antibodies. Thus, the two methods showed five immunoprecipitin bands from PTFS proteins (12, 15, 18, 25 and 50 kDa) that were reactive with antibodies from typhoid fever sera. However, the sera from typhoid fever patients also contained antibodies reactive with proteins from PNHS at bands 18, 25 and 50 kDa (Figure 2c, d, c and d), indicating that auto-antibodies were also produced to self-antigens during typhoid fever infection.

Mass spectrometry analysis of the fractionated PTFS proteins which bind to antibodies found in typhoid fever sera identified many *Salmonella* and human proteins (Tables 1 and 2). Three *S.* Typhi proteins, namely: blue copper oxidase (CueO), outer membrane protein C (OmpC), and flagellar hook-associated protein 1 (HAP1) identified from bands 12, 15 and 50 kDa, respectively, were selected for further studies based on their high antigenicity prediction scores and low similarity with *E. coli* (Table 2). The most significant human proteins identified in bands 18, 25 and 50 kDa were haptoglobin, apolipoprotein A-1 and alpha-1-antitrypsin, respectively (Table 1).

One-Way ANOVA statistical analysis of the indirect ELISA results showed that there was a significant difference in the mean OD reading of the typhoid fever group compared to either the non-typhoid fever group or the normal control group for IgG class of antibodies ($p<0.05$) for individual recombinant proteins (rCueO, rOmpC and rHAP1) and in combination (Figure 4). When the 3 proteins were used in a single test, the sensitivity of the indirect ELISA increased from 50% to 70%, and the specificity was 93.3%. The PPV of the assay was 84.0% and the NPV was 86.2% (Table 3).

## DISCUSSION

The fractionation process carried out in this study helped to concentrate or enrich the low molecular weight proteins from PTFS by removing high molecular weight and high abundance proteins, which enabled them to be visualized in SDS-PAGE (Figure 1). This study also showed that different fractionation methods have different abilities to unmask the low abundance proteins. Four prominent immunoprecipitin bands at molecular weights 12, 18, 25 and 50 kDa were found in PTFS using method 1, whereas four were found at molecular weights 12, 15, 25 and 50 kDa using method 2. Both methods showed bands at 12 kDa (Figure 2a and b), 25 kDa and 50 kDa (Figure 2c and d). The visibility of the 15 kDa band in SDS-PAGE gel was poor in method 1 (Figure 1b) compared to method 2 (Figure 1c). The emergence of the 15 kDa band in method 2 might be due to the effect of acetonitrile. Acetonitrile is an organic solvent that disrupts protein-protein interaction by breaking hydrophobic and hydrogen bondings so that low molecular weight proteins that may be bound to larger molecules are released (Tirumalai et al., 2003). Also, this could probably be due to the effect of non-specific binding of the small proteins to Affi-Gel Blue and Affi-Gel Protein A in method 1 which might result in their removal. Non-specific binding of small proteins to the planar ring structure of Cibacon Blue dye (Affi-Gel Blue) and to albumin molecules can unwittingly remove these small protein molecules from the sample and thus result in their lower visibility in Western blot analysis (Ahmed et al., 2003; Tirumalai et al., 2003). As evident from Figure 1b, method 1 helped to concentrate the 18 kDa proteins more, which enabled this band to be visualized more in Western blot than method 2 (Figure 2c).

Western blot analysis of the low abundance proteins of PTFS showed 12 kDa antigens reactive with antibodies from typhoid fever sera but not with non-typhoid fever or normal human sera (Figure 2a). Although all 10 typhoid fever sera used to evaluate the immunogenicity of the PTFS proteins were *S.* Typhi culture positive, their antibody binding profiles showed differences. Figure 2a showed that typhoid fever sera 1, 2, 9 and 10 did not contain antibodies reactive with the 12 kDa protein unlike sera 3, 4, 5, 6, 7 and 8. This showed that a single biomarker is not sufficiently indicative for the disease as individual biomarkers gave sensitivities as low as 50%. This may be due to the temporal expression of bacterial proteins in which different antigens are expressed at different stages of infection in the host and thus the ensuing antibody production. Also, the nature of the humoral immune response of individuals is dependent on genetic and environmental factors that can lead to different antibody profiles. Hence, the antibodies expressed vary among the typhoid fever patients. This could probably be the reason for the lack of 100% analytical sensitivity of currently available diagnostic test kits that use only a single antigen (Fadeel et al., 2011).

Mass spectrometry analysis of the immunogenic proteins from SDS-PAGE gels showed discrepancies in the molecular weights of the *Salmonella* proteins (Table 2). The molecular weights of the proposed (predicted) protein monomers were not identical to the molecular

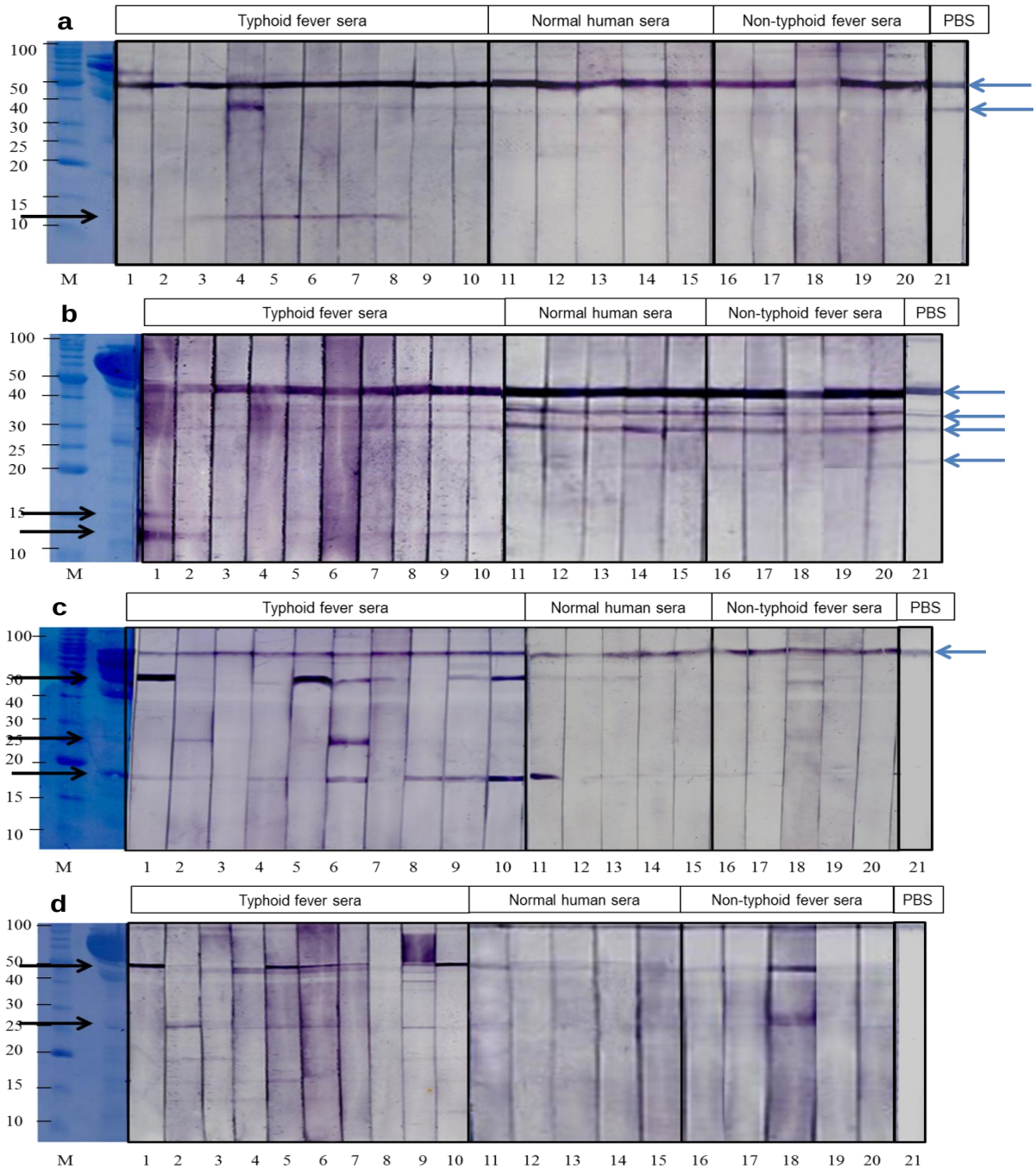


**Figure 2. (a)** Antibody profile of three groups of sera against antigens derived from PTFS using method 1, and visualized using HRP-conjugated rabbit anti-human IgG as secondary antibody. **(b)** Antibody profile of three groups of sera against antigens derived from PTFS using method 2, and visualized using HRP-conjugated rabbit anti-human IgG as secondary antibody. **(c)** Antibody profile of three groups of sera against antigens derived from PTFS using method 1, and visualized using HRP-conjugated rabbit anti-human IgM as secondary antibody. **(d)** Antibody profile of three groups of sera against antigens derived from PTFS using method 2, and visualized using HRP-conjugated rabbit anti-human IgM as secondary antibody.
M = Molecular weight marker;
Lanes 1-10 = Individual typhoid fever sera as the primary antibody;
Lanes 11-15 = Individual normal human sera as the primary antibody;
Lanes 16-20 = Individual non-typhoid fever sera as the primary antibody; and
Lane 21 = PBS control;
Black arrows = Significant positive bands for typhoid fever sera;
Blue arrows = Heavy chains of IgG or IgM antibodies.

**Table 1.** *Homo sapiens* proteins identified using LC-MS/MS analysis.

| Molecular weight | *Homo sapien* proteins | Molecular weight | Score | No. of peptides | Coverage (%) |
|---|---|---|---|---|---|
| 12 | **Hemoglobin β** | **15988** | **321** | **9** | **51** |
| | Albumin | 69321 | 270 | 12 | 16 |
| | Hemoglobin α | 15248 | 129 | 7 | 36 |
| | Ig gamma-1 chain C region | 36083 | 126 | 5 | 13 |
| | Ig kappa C region | 11602 | 100 | 9 | 30 |
| | Protein S100-A9 | 13234 | 65 | 2 | 24 |
| | Transthyretin | 15877 | 58 | 2 | 9 |
| | Fibrinogen gamma chain | 51479 | 55 | 1 | 5 |
| | Fibrinogen alpha chain | 94914 | 44 | 2 | 2 |
| | Haptoglobin | 45177 | 44 | 2 | 3 |
| | Ig mu heavy chain | 43030 | 42 | 1 | 3 |
| | Ig gamma-2 chain C region | 35878 | 39 | 1 | 5 |
| 15 | **Haptoglobin** | **45177** | **90** | **2** | **6** |
| | Fibrinogen alpha chain | 94919 | 63 | 5 | 6 |
| 18 | **Haptoglobin** | **45177** | **152** | **14** | **13** |
| | Ceruloplasmin | 122128 | 39 | 4 | 4 |
| | Clustered mitochondria homolog | 146577 | 35 | 1 | 1 |
| 25 | **Apolipoprotein A-1** | **30759** | **413** | **16** | **55** |
| | Ig kappa chain C region | 11602 | 272 | 19 | 80 |
| | Ig kappa chain V-I region | 11654 | 163 | 3 | 16 |
| | Ig kappa chain V-III region | 11628 | 136 | 2 | 16 |
| | Complement C3 | 187030 | 95 | 7 | 5 |
| | Serum albumin | 69321 | 89 | 5 | 8 |
| | Immunoglobulin J chain | 18087 | 65 | 2 | 12 |
| | Ig kappa chain V-III region | 12567 | 45 | 1 | 7 |
| 50 | **Alpha-1-antitrypsin** | **46707** | **386** | **30** | **67** |
| | Vitamin D-binding protein | 52929 | 147 | 11 | 23 |
| | Alpha-2-HS-glycoprotein | 39300 | 142 | 3 | 8 |
| | Ig gamma chain C region | 35878 | 141 | 10 | 20 |
| | Serum albumin | 69321 | 126 | 5 | 9 |
| | Fibrinogen beta chain | 55892 | 110 | 9 | 22 |
| | Ig alpha-1 chain C region | 37631 | 101 | 3 | 10 |
| | Inter-alpha-trypsin inhibitor | 103293 | 81 | 6 | 6 |
| | Ig kappa chain C region | 11602 | 47 | 2 | 35 |
| | Isocitrate dehydrogenase | 39566 | 46 | 1 | 2 |

Bold texts represent *S.* Typhi proteins selected for further analysis.

weights of the observed bands in Western blot. This might be due to damage of the proteins by proteolytic activities occurring during infection, triggered either by host cell proteases or bacterial cell proteolytic enzymes (Cain et al., 2014; Ribet and Cossart, 2010). Blue copper oxidase (CueO), also known as multi-copper oxidase, is important for bacterial survival by expressing genes associated with bacterial cell autoaggregation to resist various host immune responses (Tree et al., 2007). Flagellar hook-associated protein 1 (HAP1) are essential for filament formation (Homma et al., 1984), and plays an important role in bacterial motility (Ikeda et al., 1987) to invade the host and for pathogenesis. OmpC is the major outer membrane protein which is expressed throughout the infection period (Muthukkaruppan et al., 1992). It is a permeable channel for nutrients, toxins and antibiotics,

**Table 2.** *Salmonella* Typhi proteins identified using LC-MS/MS analysis.

| Molecular weight (kDa) | *Salmonella* Typhi proteins | Molecular weight (Da) | Score | No. of peptides (% coverage) | Antigenicity prediction score | Similarity with *E. coli* (%) |
|---|---|---|---|---|---|---|
| **12** | **Blue copper oxidase (CueO)** | **58586** | **38** | **2 (5%)** | **0.561215** | **88** |
| **15** | **Outer membrane protein C (OmpC)** | **41214** | **29** | **2 (5%)** | **0.911465** | **81** |
| | Flagellar motor switch protein FliG | 36828 | 24 | 2 (2%) | 0.223074 | 97 |
| | 2-methylcitrate dehydratase | 53735 | 14 | 6 (2%) | 0.253528 | 94 |
| **50** | Glycine-tRNA ligase beta subunit | 76598 | 31 | 3 (10%) | 0.331186 | 95 |
| | Osmolarity sensor protein EnvZ | 50388 | 21 | 3 (6%) | 0.323013 | 96 |
| | HTH-type transcriptional activator AllS | 34237 | 20 | 3 (15%) | 0.596103 | 86 |
| | **Flagellar hook-associated protein 1** | **59074** | **16** | **4 (11%)** | **0.938566** | **80** |

Bold texts represent *S.* Typhi proteins selected for further analysis.

**Table 3.** Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of the recombinant proteins.

| Recombinant proteins | Typhoid fever sera (n=30) | Non-typhoid fever and normal sera (n=60) | PPV (%) | NPV (%) |
|---|---|---|---|---|
| | Sensitivity (%) | Specificity (%) | | |
| rCueO | 50.0 (15/30) | 96.7 (58/60) | 88.2 | 79.5 |
| rOmpC | 50.0 (15/30) | 95.0 (57/60) | 83.3 | 79.2 |
| rHAP1 | 50.0 (15/30) | 93.3 (56/60) | 78.9 | 78.9 |
| Combination | 70.0 (21/30) | 93.3 (56/60) | 84.0 | 86.2 |

and plays an important role in bacterial virulence by conferring resistance to antibiotic and serum bactericidal effectors (Liu et al., 2012). Despite their physiological importance, no study has reported the immunogenicity of these proteins except for OmpC, which was extracted from *S.* Typhi under *in vitro* laboratory culture conditions (Nambiar et al., 2009; Verdugo-Rodriguez et al., 1993). As a single biomarker, OmpC gave a sensitivity of only 40% in uncomplicated typhoid fever patients as compared to 85% in complicated typhoid fever cases (Nambiar et al., 2009), suggesting that this could be an antigen released during severe infection. Thus, combining these OMPs (OmpC, OmpF and OmpA) gave a detection sensitivity approaching 100% and a specificity of 94% (Verdugo-Rodriguez et al., 1993).

Differences in types and profiles of antibodies expressed in patients are reported in the literature substantiating our hypothesis of differences in antigen expression based on infection status and host genetic makeup. Antibodies to H-antigen have been found to be elevated earlier in comparison to O-antigen antibody titer (Chew et al., 1992); and higher urinary Vi-antigen is detected at the first week of infection (Fadeel et al., 2004). Also, reports have shown that the effect of antimicrobial treatment will reduce the antibody response against certain antigens (Bowden et al., 1999; Pang and Puthucheary, 1983). As the time of collection of sera following infection and patients' management data were not available in this study, it was not possible to ascertain the correlation between duration of infection with seropositivity rates, and the effect of antibiotic treatment on the antibody profile. In this study, the diagnostic sensitivities of the individual recombinant proteins as single biomarkers were 50% (Table 3), indicating the variation of the response expected of a heterogenous population. When all three recombinant proteins were mixed in a single test, the sensitivity of the test increased to 70%, with specificity of 93%. This shows the potential usefulness of these biomarkers in combination for diagnosis of typhoid fever to overcome variations in individual immune responses.

Antibodies to self-proteins were also detected in the sera of typhoid fever patients used to evaluate reactivities against PNHS proteins (Figure 3c and d). Such reactions could be due to five possible mechanisms: 1) the immunological homunculus where naturally occurring autoantibodies are present in the body and play a vital role in the regulation of the host immune system; 2) the excessive breakdown of cells during infection which releases antigens that share similar antigenic determinants as the host proteins, thus resulting in cross-reactivity with autologous antigens; 3)

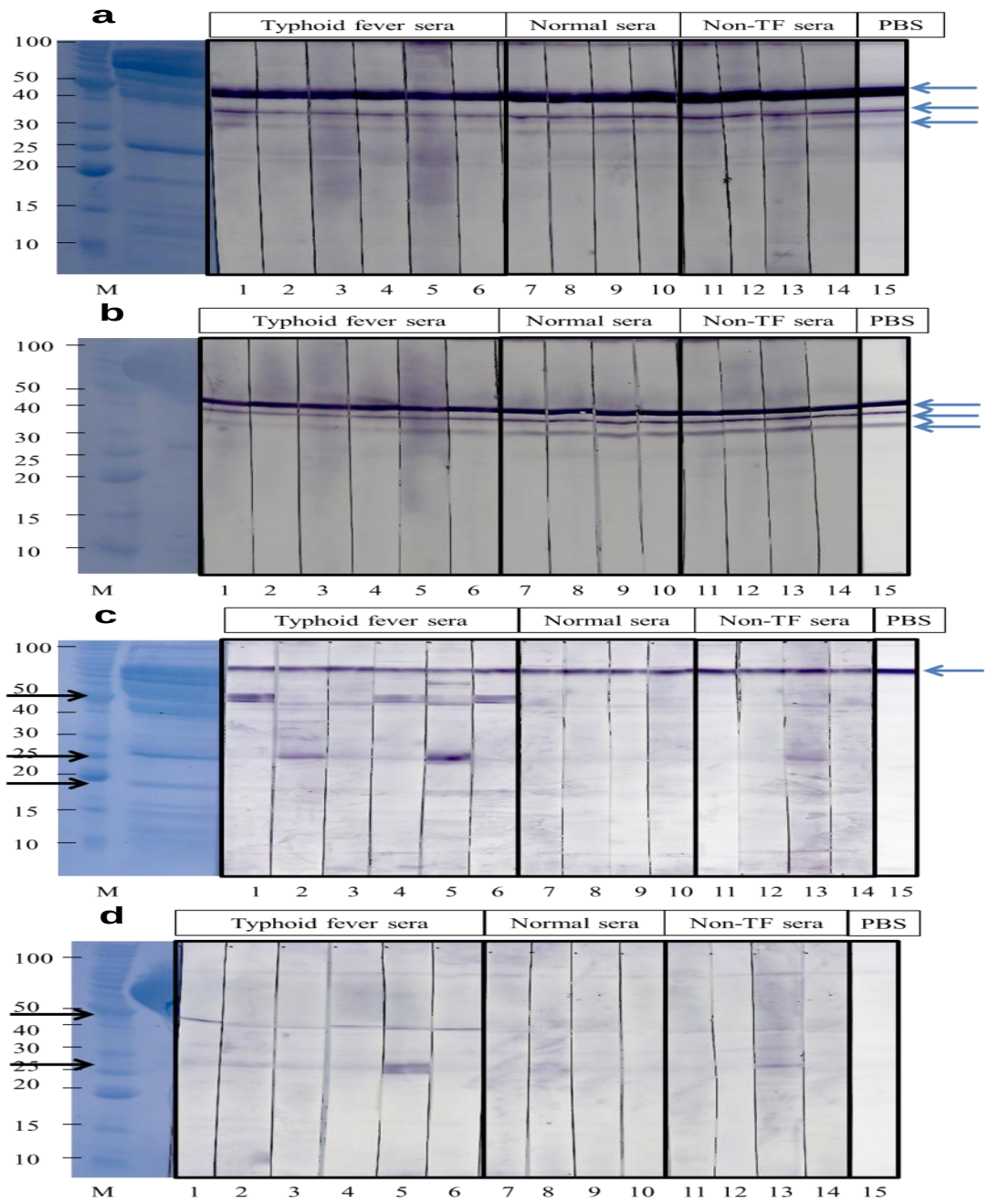


**Figure 3. (a)** Antibody profile of three groups of sera against antigens derived from PNHS using method 1, and visualized using HRP-conjugated rabbit anti-human IgG as secondary antibody. **(b)** Antibody profile of three groups of sera against antigens derived from PNHS using method 2, and visualized using HRP-conjugated rabbit anti-human IgG as secondary antibody. **(c)** Antibody profile of three groups of sera against antigens derived from PNHS using method 1, and visualized using HRP-conjugated rabbit anti-human IgM as secondary antibody. **(d)** Antibody profile of three groups of sera against antigens derived from PNHS using method 2, and visualized using HRP-conjugated rabbit anti-human IgM as secondary antibody.

M = Molecular weight marker;
Lanes 1-6 = Individual typhoid fever sera as the primary antibody;
Lane 7-10 = Individual normal human sera as the primary antibody;
Lane 11-14 = Individual non-typhoid fever sera as the primary antibody;
Lane 15 = PBS control;
Black arrows = Significant positive bands for typhoid fever sera;
Blue arrows = Heavy chains of IgG or IgM antibodies.

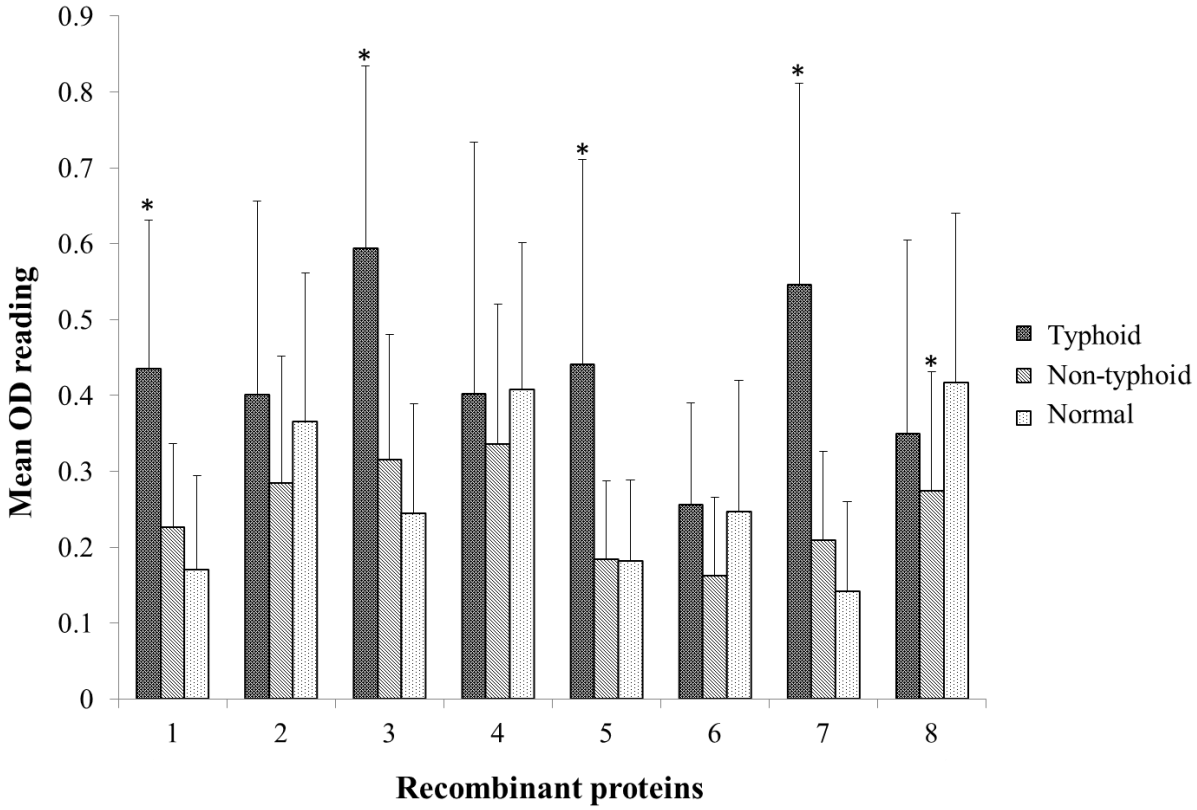


**Figure 4.** Mean OD ± 1 SD (λ450 nm) for typhoid fever patients (n=30), non-typhoid fever patients (n=30) and normal subjects (n=30) for each recombinant proteins as follows: 1 = rCueO IgG; 2 = rCueO IgM; 3 = rOmpC IgG; 4 = rOmpC IgM; 5 = rHAP1 IgG; 6 = rHAP1 IgM; 7 = rCueO, rOmpC and rHAP1 IgG; 8 = rCueO, rOmpC and rHAP1 IgM.
* represent statistically significant difference ($p$<0.05) when compared with normal control group using One-Way ANOVA.

molecular mimicry of host proteins by the pathogen in order to confuse and tolerize the host; 4) non-specific immune clearance of human proteins that are present in excess amounts during an infection; and 5) disruption of the immune tolerance mechanism resulting in non-specific activation of forbidden B-cells.

As per the theory of the immune homunculus, homology of biologically active determinants between bacterial antigens and human proteins are considered essential for maintenance of a healthy immune system and associated with protection against infection (Cohen, 2007; Perschinka et al., 2003). Studies have shown increased production of alpha-1-antitrypsin (Bostian et al., 1976) and haptoglobin (Bostian et al., 1976; Kumar et al., 2014) during typhoid fever infection, and autoantibodies against these acute phase proteins have been noted in many acute infections (Lakota et al., 2011). An earlier study reported that certain host proteins, namely, hemoglobin α, apolipoprotein A-1 and fetuin, interact specifically with the Vi antigen of *S.* Typhi to provoke an inflammatory response (Garg and Qadri, 2010). The observation in this study of autoantibodies directed against these acute phase proteins were restricted to the IgM class of antibodies, that is, the first antibody to appear in response to an initial exposure to an antigen. This reinforces the hypothesis that these autoantibodies are immune clearance agents. However, due to the non-specific nature of these antibodies to typhoid fever, they are not suitable as biomarkers for this disease.

## Conclusion

Antigens isolated from laboratory-grown bacteria (*in vitro*) are usually used as a source for biomarker discovery. However, since an infection is a dynamic phenomenon, antigens produced by the bacteria *in vivo* may vary with the stage of the disease, and thereby the quantity and quality (specificity) of the antibody response. This study has demonstrated the presence of immunogenic *in vivo S.* Typhi antigens in the sera of acute typhoid fever patients that have potential for diagnosis of the disease. Fractionation of serum to remove high abundance and high molecular weight proteins helped to unmask these

antigenic proteins. Western blot and mass spectrometry analysis showed that the proteins belonged to both *Salmonella* and the human host. Three *S.* Typhi proteins showing the highest predicted antigenicity were cloned and the diagnostic sensitivity of the recombinant proteins in combination showed higher diagnostic sensitivity compared to individual proteins. This study has shown the usefulness of typhoid fever sera as a source of *in vivo* antigens, and highlights the importance of the method used for antigen preparation in biomarker discoveries. The use of multiple biomarkers in designing diagnostic tests should be considered in order to improve the sensitivity and specificity of the assay.

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