Journal of Infectious Diseases and Immunity Vol. 2(2), pp. 30-40, 2010
Available online at http://www.academicjournals.org/JIDI
ISSN 2141-2375 ©2010 Academic Journals

*Full Length Research Paper*

# Immunogenic evaluation of a recombinant 49-kilodalton outer membrane protein of *Salmonella typhi* as a candidate for a subunit vaccine against typhoid

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Accepted 14 October, 2010

**Typhoid poses a global public health concern. Presently available vaccines against typhoid have certain drawbacks that make them unsuitable for mass immunization, especially to children and elderly people. The outer membrane proteins (OMPs) of Salmonella have strong potential for the development of a candidate subunit vaccine against typhoid. We previously reported that a 49 kDa OMP confers protection against experimental salmonellosis. In the present study we report the cloning, expression of a recombinant protein to evaluate its potential to elicit an immune response against a challenge with Salmonella. The gene encoding the 49 kDa *Salmonella typhi* protein was cloned in a bacterial expression plasmid pQE-60 under the control of an IPTG inducible *lac* promoter and high-level expression of the 447 amino acid long protein with 6xHis tag was achieved in *Escherichia coli* strain SG 13009. The recombinant protein was purified to homogeneity by a single step Ni-NTA affinity. The yields of expressed protein were ~26 mg/L. The immunogenic evaluation of the recombinant protein vis-a-vis its native counterpart in *S. typhi* and *Salmonella typhimurium* was carried out using protection studies, bacterial clearance from erythropoitic system, cell-mediated immunity and antibody response. The immungenecity of the r-protein is comparable to 49 kDa proteins from *S. typhi* and *S. typhi*mirium. These results may provide keen insights into the development of a conjugate-subunit vaccine against typhoid.**

**Key words:** Outer membrane protein, expression, salmonella, typhoid, vaccine.

## INTRODUCTION

Salmonellosis is an important public health concern all over the world (Shahane et al., 2007). Typhoid and paratyphoid fevers result from systemic infection with the human-adapted *Salmonella enterica* serovars Typhi and Paratyphi A. In contrast, infection with the broad-host-range-adapted *S. enterica* serovar typhimurium usually causes gastroenteritis in humans, but produces a systemic infection similar to typhoid fever in susceptible mice. Typhoid fever remains a public health problem with an estimated 22 million cases and 200,000 related deaths occurring worldwide each year (Crump et al., 2004). Implementation of adequate food handling practices and establishment of safe water supplies are the cornerstone for the development of an effective prevention program.

The emergence of resistance to antimicrobial chemotherapeutic agents and the possible reversal of the resistance in *Salmonella* has become a significant issue leading to difficulties in the management of disease for both developed countries where typhoid is largely a disease of returning travelers as well as for developing countries where typhoid fever is still endemic. However, vaccination against typhoid fever remains an essential tool for the effective management of this disease. The development of effective vaccines for protection against

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diseases caused by *Salmonella* species has been extensively studied. Different antigens such as O, H, Vi polysaccharide, heat shock proteins and whole cell parental vaccines have been exploited for their protective role against typhoid. Presently a number of vaccines against typhoid such as Ty 21a are available; however, there is a need for better and improved new generation vaccines against *Salmonella*. Effective typhoid vaccines available cannot be used for children under two years of age and also have some limitations in older age groups (Podda et al., 2010). The new-generation Vi polysaccharide single-dose injectable typhoid vaccine developed in the 1980s is widely used in countries where it is locally produced, such as China, and in travelers from industrialized countries going to typhoid-endemic settings. However, Vi vaccine use is limited to private practice at a small scale by residents of high-burden countries such as India, Pakistan and Bangladesh (Khan et al., 2010).

The collaborations between *Salmonella* researchers and clinicians worldwide have made significant contributions in understanding the interaction between virulence determinants and immunity required to check the spread of this pathogen (Erin et al., 2007). The integration of research on pathogenesis of bacterial infections with studies on the effects of vaccines and antibiotics should provide insight to counteract bacterial infections (Mastroeni and Sheppard, 2004). The development of effective vaccines for protection against diseases caused by *Salmonella* species continues to be an extensively studied field (Nasser et al., 2002). Various approaches of immunological manipulations, either alone or in combination with chemotherapy and vaccination have been explored. Use of well-defined recombinant protein in combination with appropriate adjuvant is more likely to overcome many limitations and provide species or strain specificity.

The outer membrane is a continuous structure on the surface of Gram-negative bacteria and has particular significance as one of the potential targets for protective immunity. Recent studies have focused attention on the outer membrane proteins (OMPs) suggesting the existence of protective immunogenic components in *Salmonella*. The outer membrane proteins of *Salmonella typhimurium* have a role in the virulence of the organism and are potential candidates for vaccine development. A protein from outer membrane of *S. typhimurium* has been identified as a potential candidate for the development of a subunit vaccine against typhoid. This protein is highly immunogenic, evoking both humoral and cell mediated immune responses. The protein has been purified to homogeneity and the gene coding for its related protein in *Salmonella typhi* has been identified, amplified and sequenced (Hamid and Jain, 2008). The goal of the present study was to clone and express the gene; to further characterize the OMP as a protective immunogen against *Salmonella* pathogenesis and to evaluate its immunological potential both in native and expressed forms.

## MATERIALS AND METHODS

### Construction of recombinant *Escherichia coli* expression cassette

The 1.3 kb gene for 49 kDa OMP was cloned into the bacterial expression vector pQE-60 (3.4 kb, having NcoI and BamHI sites at the MCS region) under the control of the IPTG-inducible promoter PT5/lacO (Khanam et al., 2006). The gene was engineered to have a NcoI site at the 5\` end and BamHI site at the 3\` end to facilitate its directional cloning. The resulting construct, pNJ-4.7, had the inserted gene fused in frame with the initiator ATG codon and 6xHis tag provided by the vector at its 3\` end. The construct was used to transform *E. coli* DH5α cells. The resultant transformants were selected for ampicillin resistance and screened for the presence of the cloned gene using insert specific primers by colony Polymerase Chain Reaction (PCR).

### Isolation of plasmid DNA from *E. coli* DH5α

Plasmid DNA was isolated by an alkali lysis method (Bimboim and Doly, 1979). For screening of recombinant clones mini preps of individual colonies were prepared and analyzed on agarose gels to check the mobility shift due to insertion of a foreign gene.

### Expression screening of clones harboring 49 kDa protein

The plasmid DNA from positive clones was used to transform the *E. coli* expression host strain SG13009 containing the PREP4 plasmid. The PREP4 plasmid encodes the lacI repressor (required for regulated recombinant gene expression) and the kanamycin resistance marker. Double recombinants harboring both pNJ-4.7 and PREP4 plasmids were selected in the presence of ampicillin and kanamycin. Four randomly selected clones were inoculated into 3 ml test tube cultures and allowed to grow at 37°C in a shaker at 200 rpm. The cultures (in logarithmic growth phase, corresponding to an OD of 0.5 - 0.6 at 600 nm) were induced with 1 mM IPTG for 4 h. After induction, an equal number of cells from the different cultures (normalized on the basis of $A_{600}$ values) were lysed in sample buffer and analyzed by SDS-PAGE. Uninduced cultures were also analyzed in parallel. One of the clones that expressed maximum levels of the recombinant protein was chosen for further studies.

### Purification of recombinant protein by Ni-NTA affinity chromatography

Purification of the induced protein using Ni-NTA affinity chromatography was done essentially as described by Khanam et al. (2006) with minor modifications. The induced cultures were harvested by centrifugation at 6000 rpm for 15 min at 4°C and the cell pellet (approximately 1.5 g wet weight) was lysed by re-suspending in 50 ml ice-cold lysis buffers (100 mM sodium phosphate, pH 7.5, 8 M urea, 0.5 M NaCl, 10 mM imidazole and 1 mM freshly added PMSF) and sonication (W-385 sonicator; Heat systems-Ultrasonics) for 10 min. The lysate was stirred for 2 h at 4°C and clarified by centrifugation using a Sorvall SS34 rotor (17000 rpm for 1 h at 4°C). The resulting supernatant was mixed with a 4 ml Ni-NTA super flow resin that had been pre-equilibrated with the lysis buffer. This suspension was gently rocked overnight at 4°C and

then packed into a column. The 6xHis tagged protein remained bound while other proteins passed through the matrix. After collecting the flow through, the column was washed extensively with buffers I (50 mM sodium phosphate, pH 6.3, 8 M urea and 0.5 M NaCl) and II (50 mM sodium phosphate, pH 5.9, 8 M urea) and bound protein was eluted with buffer III (50 mM sodium phosphate, pH 4.5, 8 M urea). Fractions of 3 ml were collected for analysis. All fractions obtained during purification were analyzed by SDS-PAGE. Relevant fractions were pooled and dialyzed against 1x PBS containing 50mM each of arginine and glutamic acid. The dialyzed protein was mixed with gentamicin to a final concentration of 50 µg/ml, flash-frozen in liquid nitrogen and stored at -80℃ until use.

### High-performance liquid chromatography (HPLC) of Ni-NTA purified protein

The purity of the recombinant protein was further assessed by reverse phase-HPLC system equipped with photodiode array detector (Waters, USA) using C8 column (Waters Spherisorb $^{R}$) of 4.6 x 250 mm and particle size 5 µ (Stone et al., 1991). The column was washed with HPLC grade methanol and pre-run with acetonitrile:water (30:70). The protein was eluted with acetonitrile:water (30:70) containing 0.05% trifluoroaceticacid (TFA) at 2000 psi pressure and a flow rate of 0.8 ml/min flow rare. The eluent was analyzed by recording its absorbance at 220 nm.

### Expression kinetics

The induction time for maximum expression of 49 kDa recombinant protein was optimized by inducing 100 ml cultures of transformed *E. coli* SG13009 harboring the recombinant plasmid pNJ-4.7 for different time intervals viz 1, 2, 4, 8h, 16, 24, 48 and 72 h with 1mM IPTG and r-protein was isolated by affinity chromatography on Ni-NTA super flow columns. The quantity of the 49 kDa protein was estimated and also analyzed by SDS-PAGE.

### Immunological studies

The immunological potential of the recombinant 49 kDa protein was evaluated vis-a-vis` natural 49 KDa protein isolated from *S. typhi* and *S. typhimurium* outer membranes.

### Immunizations

All the experiments were carried out on six to eight week old Swiss-albino male mice (20 – 24 g), bred and maintained at the Central Animal Housing Facility of Jamia Hamdard. The animals were housed at 25℃ in polypropylene cages and fed *ad libidum* with pellet diet (Hindustan Lever Ltd) and water. The studies were conducted according to ethical guidelines of the Committee for the purpose of Control and supervision of Experiments on Animals (CPCSEA) on the use of animals for scientific research, immunized with an emulsion of purified 49 kDa proteins in Freund`s adjuvant (recombinant 49 kDa, native 49 kDa from *S. typhi* outer membrane and native 49 kDa from *S. typhimurium* outer membrane, one group for each of the proteins). The animals received four subcutaneous injections on days 1, 7, 14 and 21 and a booster on day 31. Freund's complete adjuvant was used for the first immunization and Freund`s incomplete adjuvant for subsequent immunizations. The antigen (25 µg) in 200 µl 1X PBS was mixed with an equal volume of the adjuvant to form an emulsion that was injected subcutaneously. The animals were bled on day 40 and sera were stored at -20℃ till further use. The control group received saline: adjuvant emulsion at the same immunization schedule.

### Protection studies

For each dose four groups comprising of six animals each with one group in each set serving as control, were used for these studies. Three groups of animals were immunized subcutaneously with 25 µg of protein each (recombinant 49 kDa, native 49 kDa OMP from *S. typhi* and native 49 kDa OMP from *S. typhimurium* outer membrane, one group for each of the protein). The fourth group received vehicle only and served as control. After four weeks of immunization, the animals were challenged with three different doses (1 x $LD_{50}$, 2 x $LD_{50}$ and 50 x $LD_{50}$) of *S. typhimurium* (wild-type), injected intraperitoneally. The animals were observed for 14 days of post-bacterial challenge and their condition as well as mortality was recorded. The $LD_{50}$ of the bacteria was determined by the method of Reid and Muench (Reid and Muench, 1938).

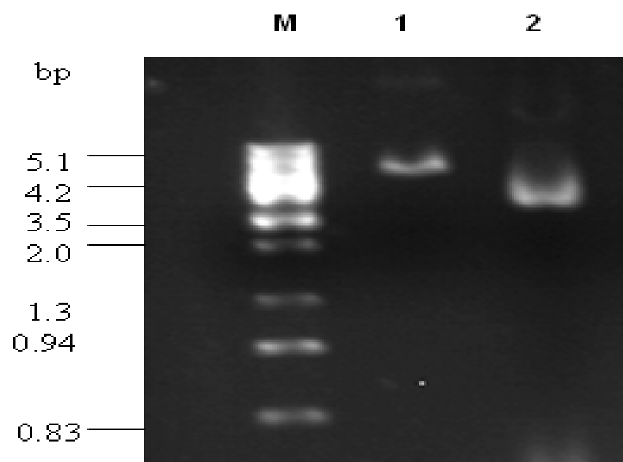
### Bacterial clearance studies

As the mice are exposed to *S. typhimurium*, the bacterium gains entrance to the reticulo-endothelial system, especially in the liver and spleen. The number of bacteria (as seen by CFUs) increases slowly up to 7 days and then attains a constant value. To evaluate the efficacy of selected antigens in accelerating the clearance of bacteria from the recticulo-endothelial system, mice were sacrificed on day 7, post challenge, livers were removed under aseptic conditions and homogenized separately in PBS. An aliquot from each homogenate was cultured on nutrient agar plates. *S. typhimurium* colonies obtained after overnight incubation at 37℃ which were identified by standard dabbing methodology on TSI agar plates and counted. (*Salmonella* imparts a blackish hue to pink colored plates). The results were expressed as number of viable bacteria ($log_{10}$CFU/gm tissue).

### Peripheral blood mononuclear cell (PBMC) proliferation studies

For separating mononuclear cells, 10 ml blood was withdrawn and pooled from each group comprised of five mice (immunized by test antigens in experimental group and unimmunized in control group) by retro-orbital sinus by heparinised capillary tubes. PBMC were obtained from the blood by density gradient centrifugation on Histopaque-1077. To summarize, 10 ml blood was diluted with an equal volume of PBS and carefully layered on 6 ml of Histopaque-1077 solution in a sterile conical centrifuge tube and centrifuged at 600 x g for 30 min at room temperature. The mononuclear cells that separated as white ring at the interphase were carefully collected with a sterile pasteur pipette. These cells were pelleted at 400 x g for 10 min and the cell pellet was suspended in 1 ml growth medium. The cellular viability was checked by the trypan blue dye exclusion method and the cells were plated in a 96-well culture plate at cell density of $1x10^{6}$ cells/ml. Several dilutions of the different test antigens, positive control (Concanavalin A, 50 µg), negative control (vehicle only) were added and culture plates were incubated at 37℃ in $CO_2$ (5%) incubator (Forma Scientific Inc., USA), with water-saturated air for 6 days. After 6 days the cultures were pulsed with 1 µCi of [$^{3}$H] methyl thymidine (specific activity 82Ci/mM) for 18 h and incorporated radioactivity was estimated as follows.

The media was removed and the cells were incubated with lysis solution for 10 min at room temperature. 10 µl of cell lysate was poured onto glass-fiber filters, the filters were dried and washed twice with 5% TCA followed by washing with ethanol. After washing the filters were allowed to air dry and counted in a liquid scintillation



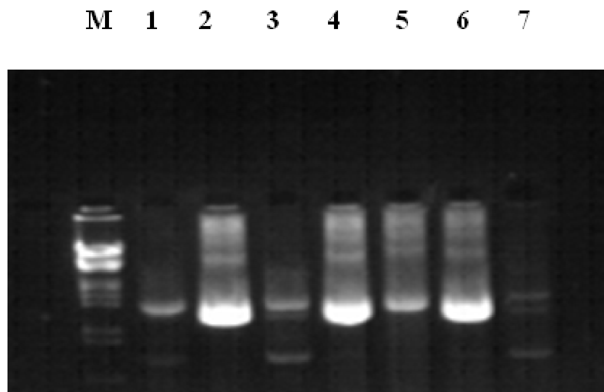




**Figure 1.** Panel A: Ligation of PCR amplified 1.3kb gene for 49 kDa OMP to pQE-60 at NcoI/BamHI sites. Lane 1 has ligated construct, lane 2 has unligated vector DNA and lane M has EcoR1 and HindIII digested λDNA marker. Panel B: Map of expression construct pNJ 4.7 having 1.3kb gene cloned into pQE-60 vector. The size of the construct is 4.7 kb. Panel C: Colony PCR plasmid from transformants using insert specific F1 and R1 primers. Lanes 2, 4 and 6 show strong amplification of 1.3kb gene indicating the insertion of the gene in plasmid vector. Lane M EcoR1 and HindIII digested λDNA marker.

counter ($LS^{6500}$, Beckman Instruments, Inc., USA) to determine the amount of thymidine incorporated into the DNA.

**Delayed-type hypersensitivity (DTH) studies**

DTH studies were carried out by using a standard footpad swelling method (Collins and Mackaness, 1968). The animals were divided into four groups of five animals each with one group serving as control. The test animals received an injection of 50 µg of 49 kDa protein (dissolved in100 µl of 1X PBS) into their right hind footpad, while the left hind footpad received an injection of equal volume of saline and served as control. The control animals were injected with an equal volume of PBS (100 µl) in their right hind footpad and an injection of saline in their left hind footpad. The footpad swelling was measured at different time intervals (3, 6, 24, 48 and 72 h post injection). The values obtained for the swelling induced by saline in the left footpad were subtracted from the values obtained for the swelling induced by the test antigen (r-protein, n-49 kDa OMP from *S. typhi* and n-49 OMP from *S. typhimurium*) or the PBS alone. Finally the mean value of swelling induced by PBS alone was subtracted from all the values of swelling induced by the proteins to eliminate the effect of PBS.

**Humoral response**

Blood was taken from retro orbital sinus using non-heparinised capillary tubes collected in dried centrifuge tubes and allowed to clot. Serum was separated by centrifugation for five minutes at 800 x g at room temperature. After centrifugation, serum was carefully collected using a pasteur pipette and kept at 4°C till use.

**ELISA**

ELISA was performed by the method of Engvall and Perlman (Engvall and Perlman, 1971). Sera from animals of three groups immunized subcutaneously with 25 µg of purified outer membrane proteins each (r-49 kDa, n-49 kDa OMP from *S. typhi* and n-49 kDa OMP from *S. typhimurium* one group for each of the protein) and control animals (mock immunized with saline at the same schedule). The recombinant protein was used as the antigen while the different groups of sera were used as primary antibodies. HRPO-conjugated anti-IgG was used as the secondary antibody.

## RESULTS

### Molecular cloning of gene for 49 kDa protein of *S. typhi* in pQE-60 vector

A fragment of 1.3 kb was PCR amplified which was eluted and purified for further use. The pQE-60 vector was used for cloning of the fragment. This system is designed for high-level expression of His-tagged proteins. This vector contains the initiation codon ATG upstream and 6xHis tag down stream of multiple cloning site. NcoI/ BamHI digested vector and PCR amplified 1.3kb fragment were ligated using T4 DNA ligase and used to transform *E. coli* DH-5α cells. The presence of the insert in transformants was confirmed by restriction enzyme digestion analysis and also by PCR amplification screening using inserts specific primers. The details of these analyses are shown in Figure 1a and 1b. One of the positive clones (pNJ-4.7) was selected for further work. It had the inserted gene fused in frame with ATG codon (at 5'end) and 6x His tag coding sequence (at 3'end) present in the vector pQE-60 (confirmed by sequence analysis- data not shown).

### Expression of 49 kDa protein in *E. coli* SG13009

The expression construct was used to transform *E. coli* strain SG13009. The host contains a PREP4 plasmid

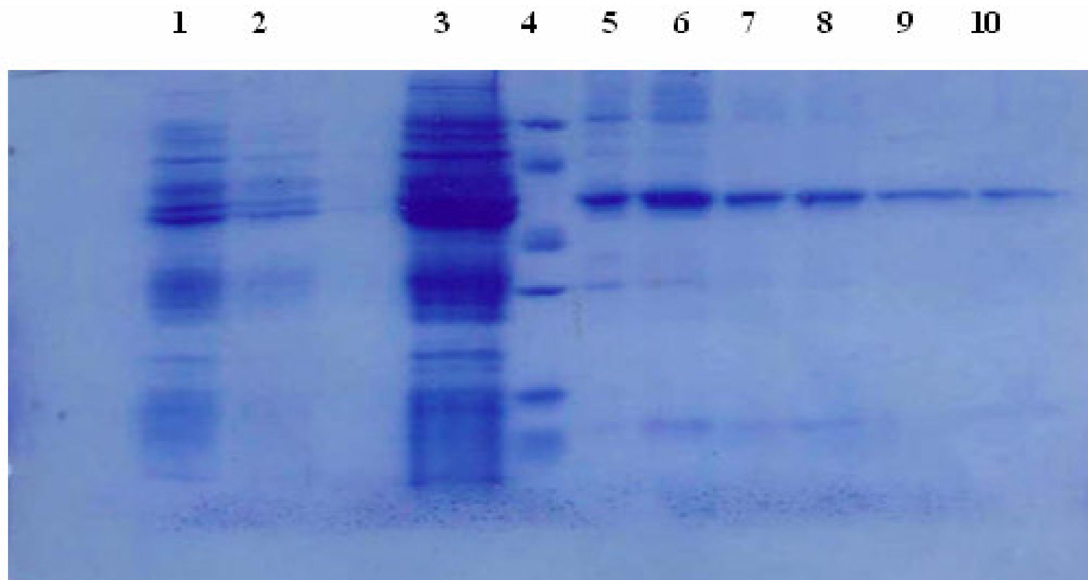



**Figure 2.** Single step purification of recombinant 6x-His tag containing 49 kDa OMP by Ni-NTA affinity chromatography. Lanes 1 and 2 flow through, lane 3 total cell lysate, lane 4 MW protein marker lanes 5 - 10 are eluted fractions in buffer III (See methods for details).

with a kanamycin resistance gene. Double recombinants harboring plasmid pNJ-4.7 with ampicillin resistance gene and PREP4 plasmid with kanamycin resistance gene were selected on ampicillin (50 µg/µl) and kanamycin (25 µg/µl) plates and grown under selection pressure. Minipreps were induced with IPTG as described in methods. Uninduced cultures were kept to serve as controls. Total lysates (prepared by directly boiling the cell pellets in SDS-PAGE sample buffer) were analyzed by SDS-PAGE. The polypeptide profiles obtained in the absence and presence of IPTG indicated that induction of the 1.3kb gene results in the appearance of 49 kDa protein, which is consistent with the predicted size of the cloned gene product.

### Purification of induced protein using Ni-NTA affinity chromatography

Ni-NTA affinity chromatography was used for purification of expressed protein. The fractions collected during different steps of purification were analyzed by SDS-PAGE as shown in Figure 2. Approximately 80 - 90% of the induced protein was bound to the column. The purified recombinant protein was eluted from the column when pH 4.5 buffer was applied. The protein profiles in Figure 3 suggest that almost 95% purity of recombinant proteins has been obtained. The purity of induced protein obtained by Ni-NTA affinity chromatography was further confirmed by HPLC using $C_8$ column. A single peak was obtained that revealed the presence of single protein and absence of the contaminating protein fractions in the sample (Figure 2).

### Kinetics of induction of 49 kDa protein

In order to determine the time for optimal induction of expressed protein, cultures of clone pNJ-4.7 were grown at 37°C until O.D 600 reached 0.6 and induced with 1.0 mM IPTG. The aliquots were taken every hour, purified on a Ni-NTA column and the yield of induced protein was estimated in each fraction. Maximum induction was obtained at 4 h after the addition IPTG to the medium.

### Yield of r-protein

A good yield of 26 mg of purified protein was obtained from one litre of induced culture indicating a high expression of recombinant protein.

### Immunological evaluations

To evaluate the immunological potential of the recombinant 49 kDa protein vis-a-vis` natural proteins of *S. typhi* and *S. typhimurium* the following parameters were studied.

### Protection studies

To assess the protection conferred against bacterial challenge, three preparations of the 49 kDa protein; (recombinant, native isolated from *S. typhi* and native from *S. typhimurium*) were used to immunize the animals (see methods for details). The control group received

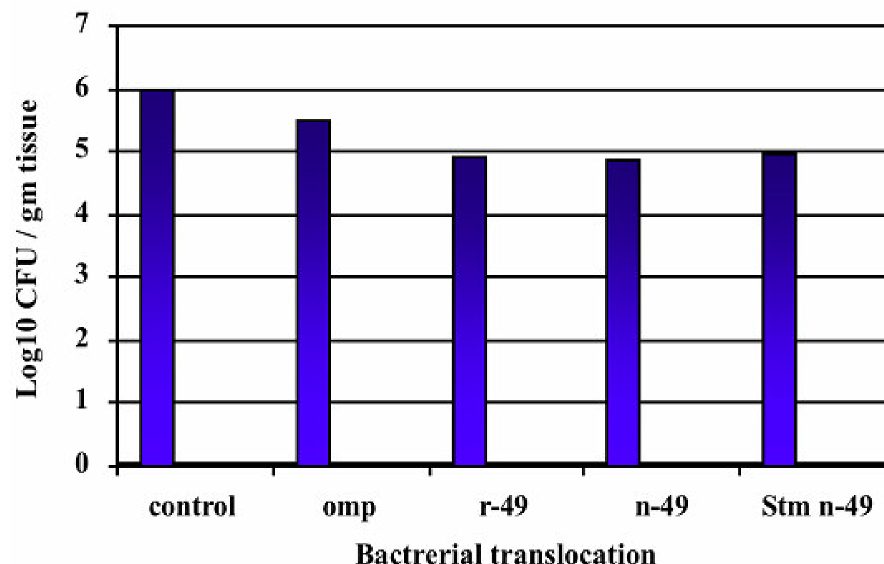



**Figure 3.** The liver homogenates of the animals (immunized with three different 49 kDa proteins used in our studies) exposed to a sublethal dose of *S. typhimurium* were cultured on nutrient agar and the colonies obtained were screened for *S. typhimurium* by culturing them on TSI slants.

**Table 1.** Protection studies of recombinant expressed protein vis-a-vis native protein from *S. typhi* and *S. typhimurium.*

| Group | Treatment | No. of animals | No. of animals survived | | |
|---|---|---|---|---|---|
| | | | Bacterial dose | | |
| | | | 1 x $LD_{50}$ | 2 x $LD_{50}$ | 50 x $LD_{50}$ |
| Control (unimmunized) | Saline | 4 | 2 | 0 | 0 |
| Immunized | r-49 | 4 | 4 | 4 | 4 |
| Immunized | n-49 | 4 | 4 | 4 | 4 |
| Immunized | Stm n-49 | 4 | 4 | 4 | 4 |

saline. After 4 weeks of immunization, the animals were challenged with *S. typhimurium*. The results of the protection studies are shown in Table 1.

All animals in the control groups started to show the signs of salmonellosis such as ruffled hair, lethargic movements, decreased response to external stimuli and loss of apetite at the end of the day 1 post infection. Unprotected animals challenged with 2 x $LD_{50}$, 50 x $LD_{50}$ and 100 x $LD_{50}$ doses of bacteria died after 3 - 4 days. However, half of the animals survived when the dose of infection was low (1 x $LD_{50}$). At 1 x $LD_{50}$ and 2 x $LD_{50}$ all the animals immunized with 49 kDa OMP from *S. typhimurium* were protected. However, no protection was seen in animals immunized by this protein when 100 x $LD_{50}$ dose of bacterium was used. The native 49 kDa protein from *S. typhi* on the other hand, provided 100% protection at 1 x $LD_{50}$, 2 x $LD_{50}$ and 50 x $LD_{50}$. The immunization with recombinant 49 kDa protein from *S. typhi* was equally effective, conferring the same degree of protection against the bacterial challenge (100% against 1x, 2x and 50 x $LD_{50}$ doses).

## Bacterial clearance studies

The clearance of bacteria from the reticulo-endothelial system of immunized mice following *S. typhimurium* challenge was studied by estimating the viable bacteria (cfu) in liver and spleen. The liver homogenates of immunized and control animals were prepared under non-denaturing conditions and cultured seven days after challenge with a sublethal dose (0.1 x $LD_{50}$ i.p) of wild *S. typhimurium*. The number of bacterial colonies was counted. The results of these studies are shown in Figure 3. As can be seen, the livers of animals in the unimmunized control group were highly infected with *S. typhimurium* indicating that in the absence of any resistance from the host, the bacteria reach the liver and

rapidly colonize the hepatocytes before migrating elsewhere. The immunization of the animals with 49 kDa protein had marked effects not only in inhibiting the translocation of *S. typhimurium* from the peritoneal cavity to liver, but also on decreasing the number of viable bacteria once they invade the liver. These mice showed very little viable (approximately 8% as compared to the control liver) bacteria in the liver when the homogenates were cultured overnight on TSI plates. Marked reduction in the number of viable bacteria was seen in homogenates of livers of all the animals that were immunized with recombinant and native 49 kDa protein. Similar results were obtained with spleen homogenate (data not shown).

## Cell-mediated immune responses

### Peripheral blood mononuclear cell (PBMC) proliferation studies

To analyze the proliferative responses of PBMCs induced by 49 kDa proteins blood was withdrawn from each group. The proliferative response of the PBMC was expressed by stimulation index (SI values). Three different dilutions (undiluted, 1:10 and 1:20 diluted) of antigens were used in the studies. Concanavalin A (50 µg) was used as a positive control (also used at three dilutions, undiluted, 1:10 diluted and 1:20 diluted). The results of these studies are shown in Figure 4. The uninduced PBMC cells served as the negative control. The potential of three selected proteins (recombinant 49 kDa protein of *S. typhi*, native 49 kDa of *S. typhi* and *S. typhimurium* outer membranes) used as antigens in inducing the proliferation of the PBMC was assessed by calculating their SI values. To calculate the stimulation index (ratio of mean counts per minute [cpm] of test antigen and mean cpm of control uninduced PBMC cells) of proteins, the mean cpm of three dilutions of negative control were subtracted from the mean cpm obtained from cultures stimulated with three dilutions of r-49, n-49 and Stm n-49, respectively.

The cultures stimulated with all the three 49 kDa proteins showed a significant proliferation at all the three dilutions tested. When undiluted r-49 kDa, n-49 kDa and Stm n-49 kDa were used as eliciting antigen SI values of 2.99, 3.07 and 3.05 were obtained respectively, while SI values of 2.58, 2.72 and 2.74 were recorded with cultures stimulated with 1:10 dilution of r-49 kDa, n-49 kDa and Stm-49 kDa respectively. The SI values of 3.34, 2.66 and 2.58 were obtained from PBMC stimulated with 1:20 dilution of the r-49 kDa, n-49 kDa and Stm-49 kDa respectively. These values of SI obtained were significant ($P < 0.05$ by Mann-Whitney U-test) as compared to the control. The SI values of 3 - 7 are considered as a moderate response and a value of more than 7 is considered as a strong response. All the three proteins were effective at 1:10 and 1:20 dilutions. The response was higher with recombinant 49 kDa protein relatively at 1:20 dilution then the native proteins.

### Delayed type hypersensitivity

These proteins were also screened for their ability to induce DTH response in mice sensitized earlier with a sublethal dose (0.5 x $LD_{50}$) of *S. typhimurium*. The animals immunized with the 49 kDa proteins were also able to mount a significant DTH response as indicated by the increase in footpad edema. The results are depicted in Figure 5. Immunization of mice r-49 kDa, n-49 kDa and Stm 49 kDa with induced significant footpad swelling after 3h of administration. The induced swelling further increased at 6 h. There was a gradual increase in the footpad thickness reaching a maximum at 24 h thereafter decreasing gradually at 48 h. At 72 h the footpad swelling is reduced almost too normal in all the immunized groups. The DTH profile of the control group varies from that of the immunized animals considerably. In control animals, the footpad swelling reached a maximum at 6 h and thereafter decreases abruptly. The results of DTH studies confirm the efficacy of 49 kDa protein as a potent immunogen in eliciting strong immune response.

### Humoral immune response

The effect of immunization with the 49 kDa protein to induce an antibody response was studied by ELISA. Sera were collected from immunized and control animals. The plate was coated with antigens (2 µg per well). The negative control (lane A) was coated with coating buffer alone, while lanes B, C and D had coating buffer containing r-protein *S. typhi* immunized sera, n-protein *S. typhi* immunized sera and n-protein *S. typhimurium* immunized sera respectively. Lane E had coating buffer containing non-immunized sera. 1-12 wells of each row had serially diluted serum prepared in PBS in 1:50 (well 1), 1:100 (well 2), 1:200 (well 3), 1:400 (well 4) and so on. From the results obtained, we can interpret that all the protein preparations (recombinant 49 kDa of *S. typhi*, native 49 kDa of *S. typhi* and native 49 kDa of *S. typhimurium*) elicited significant levels of antigen-antibody reaction and are therefore equally immunogenic.

## DISCUSSION

Vaccination with an attenuated live strain as in patients with natural typhoid fever elicits an immune response to OMPs (Blanco et al., 1993). Increasing antibiotlc resistance to different strains typhoid complicates therapy for travel-related enteric fever. New vaccines, offering protection against both *S. typhi* and *S. typhimurium* are

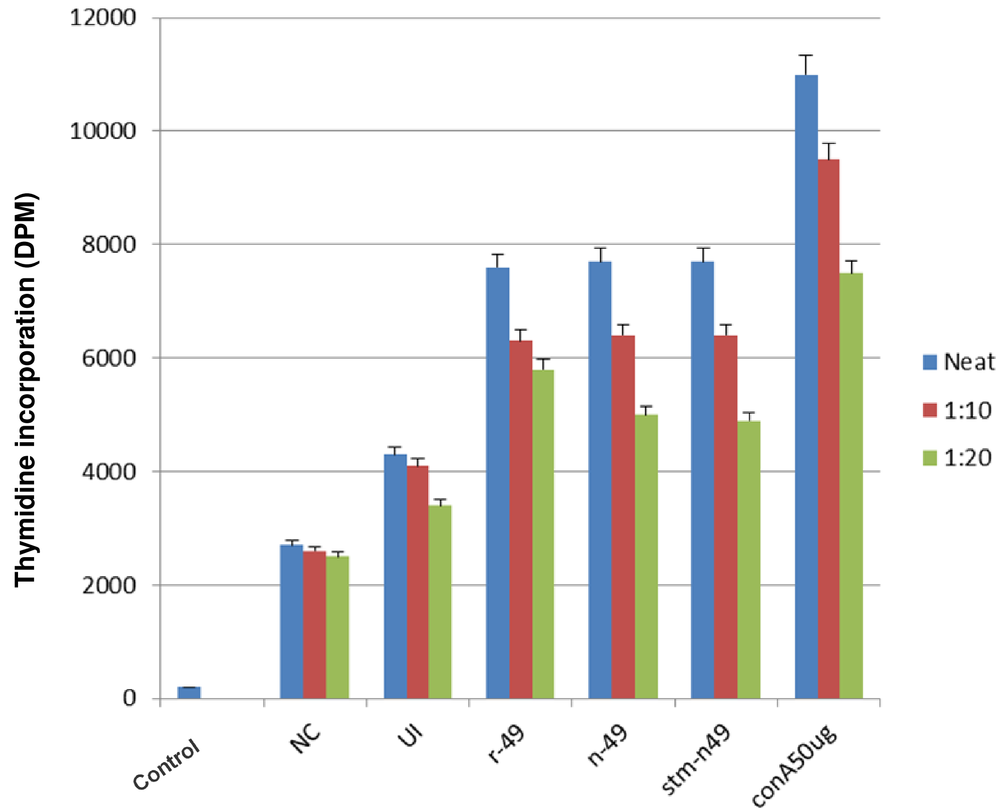



**Figure 4.** Proliferative responses of PBMC to different dilutions (undiluted, 1:10 and 1:20 diluted) of various 49 kDa proteins preparations. RPM medium devoid of any PBMC cells was used as a negative control while 50 µg of concanavalin A was used as a positive control. Unstimulated PBMC cells served as an additional control. The extent of thymidine incorporation was used as the measure of cell proliferation. The results obtained indicate the efficacy of these proteins to enhance proliferation. Mitogenic activity of the 49 kDa protein in native or recombinant forms is significant.

urgently needed; however, none of the current vaccine candidates are likely to be available for several years (Meltzer and Schwartz, 2010). The precise role of OMPs in pathogenicity of *Salmonella* is not well understood. Earlier studies from our laboratories have shown that a protein of apparent molecular weight of 49 kDa is immunogenic and confers protection against *Salmonella* challenge and therefore might be a good vaccine candidate. The gene encoding the 49 kDa protein of *S. typhi* was sequenced, cloned and expressed in *E. coli*.

Using the gene sequence data, oligonucleotide primers were designed and used to amplify a DNA segment from *S. typhi* genomic DNA by PCR. The PCR amplification generated a product of 1.3 kb, cloned in pQE-60 between Nco I and Bam HI sites in MCS region (Figure 1a and b). The recombinant plasmids of 4.7kb molecular weight were selected for expression studies. PQE-60 vector combines a powerful phage $T_5$ promoter (recognized by *E. coli* RNA polymerase) with a double *lac* operator repression module to provide tightly regulated, high-level expression of recombinant proteins in *E. coli*. In the absence of an inducer (IPTG) the gene expression is effectively blocked due to presence of high levels of *lac* repressor. The pQE-60 vector places a 6xHis tag at the C-terminus of the recombinant protein. The expression construct, pNJ-4.7 was expressed in *E. coli* SG13009 cells. Addition of 1 mM IPTG was found to be optimal for induction of r-protein expression. An increase in the IPTG concentration from 1 mM did not show any significant improvement in the expression of 49 kDa protein. The 49 kDa r-protein was purified from *E. coli* by nickel-NTA chromatography.

The protein was present as inclusion bodies. In our study buffers of different pH and urea as denaturant were used for solubilization. Dialysis was performed for removal of excess denaturing agent. After solubilization, refolding was accomplished by controlled removal of the excess of denaturants, thus creating an appropriate environment where protein can fold spontaneously (Singh and Panda, 2005). The addition of small molecules such as arginine (0.4 -1 M) that inhibit intermolecular interactions can be used to avoid unwanted aggregation (Tsumato et al., 2003).

The purified fractions were analyzed by SDS-PAGE

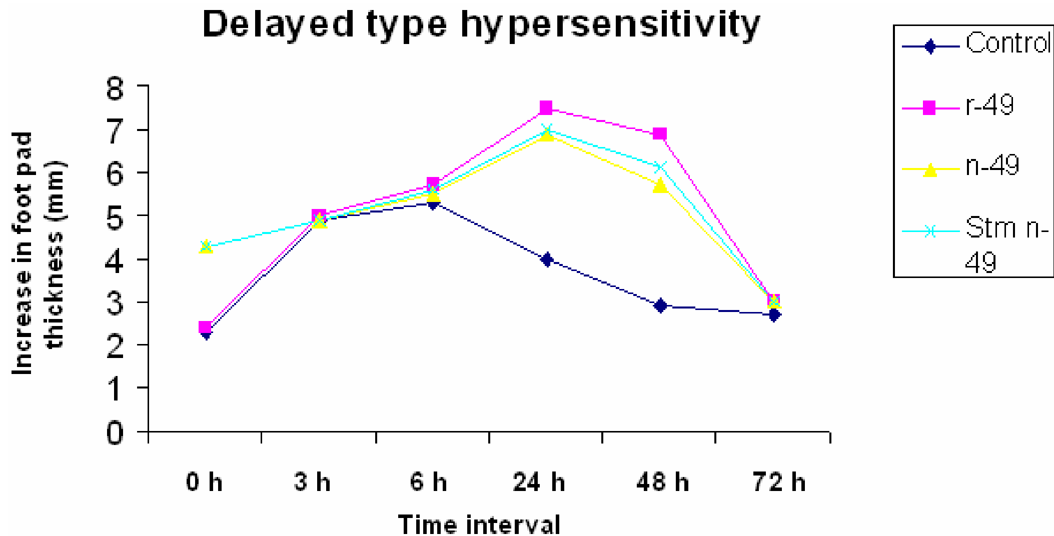



**Figure 5.** DTH response induced upon injection of three 49 kDa proteins (recombinant 49 kDa of *S. typhi*, native 49 kDa from *S. typhi* and native 49 kDa from *S. typhimurium* outer membranes). The values plotted are obtained after subtracting the values of footpad swelling induced in animals that received an injection of saline and serving as control. These results demonstrate the role of 49 kDa proteins to act as modulators of protective cell-mediated immune responses.

(Figure 2). As evidenced by the comparison of the protein profiles of the eluted material and the crude lysate, it is clearly evident that > 95% purity has been obtained, which was further confirmed by HPLC analysis. A single peak of the purified fraction indicated the purity of the protein collected. A maximum expression level of the 49 kDa protein was attained at 4 h post induction after which, the expression level of the protein was reduced.

In order to confirm the authenticity of expressed protein, we tested whether it would display antigenic reactivity that are characteristic of the native 49 kDa protein from *S. typhi* and *S. typhimurium* outer membranes or would possess an entirely new antigenic phenotype. Recombinant and native 49 kDa proteins were used as eliciting antigens in the next set of experiments. The protein-adjuvant (PA) complexes of all the test antigens were prepared and administered to animals.

The recombinant and native 49 kDa proteins were used as immunizing agents in mice that were later exposed to four doses of *S. typhimurium* wild-type (1 x $LD_{50}$, 2 x $LD_{50}$, 50 x $LD_{50}$ and 100 x $LD_{50}$). The results of protection studies indicated that these proteins conferred a similar degree of protection (Table 1).

The immunization with recombinant 49 kDa protein from *S. typhi* was equally effective, conferring the same degree of protection against the bacterial challenge (100% against 1x, 2x and 50x$LD_{50}$ doses and 50% protection against a 100 x $LD_{50}$ dose of bacteria). This protection can be attributed to many factors including elucidation of both specific and non-specific immune responses. To be effective against *Salmonella* infections the immunogen has to be able to induce both humoral and cell mediated immune responses (Mittrucker and Kufmann, 2000).

The immunized animals had significantly lower bacterial translocation to their livers as compared to the control animals. Further, the mean bacterial burdens were considerably lower (more than 92% reduction in cfu) in the liver homogenates of immunized animals (Figure 3). Earlier studies have also indicated a correlation between protective efficacy and prevention of bacterial translocation (Perez et al., 1996).

*S. typhimurium* encounters a diversity of environments throughout the course of systemic infection. In small intestine, *S. typhi* moves across the intestinal epithelial cells and reaches the M-cells overlaying Peyer`s patches where the bacteria are rapidly internalized and get partially phagocytized and neutralized. Some cells escape the mucosal immunity and the T and B lymphocyte activation is provoked. *S. typhimurium* is an intracellular facultative bacterium; therefore cell-mediated immune responses are very important for removal of invading organism. Studies in both humans and mouse model of typhoid fever suggest the importance of these responses.

However, little is known about the cellular immune responses in humans with typhoid and most of the studies have been carried out using crude antigenic extracts or whole cell extracts and the principal antigens involved in stimulation of cellular immunity remain largely undefined. Although, T-cell activation occurs during the early stages of infection, it is possible that the lymphocyte response is blocked by NO induced transient suppression. Eventually the immune system overcomes

suppression and achieves bacterial eradication (Miller et al., 1989).

The capability of recombinant as well as native 49 kDa protein to stimulate the growth of the PBMCs was evaluated. The PBMC cultures stimulated with all the preparations showed significant proliferation of macrophages as evident from the enhanced incorporation of 3H-thymidine (Figure 4). The SI values showed increase in proliferation when compared to the SI values of the non-induced group. These results demonstrate that the 49 kDa protein elicits T-cell responses, which may contribute to the protective immunity.

These proteins were also screened for their ability to induce DTH reactions in mice sensitized earlier with a sublethal dose (0.5 x $LD_{50}$) of *S. typhimurium*. The animals immunized with the 49 kDa proteins were also able to mount a significant DTH response (Figure 5). After the first description of a delayed-type hypersensitivity reaction induced after injection of *Salmonella* antigens into the foot-pads of *S. typhimurium* infected mice (Collins and Mackaness, 1968), significant information on the role of T-cells in *Salmonella* infection has been accumulated. The T-cells participate in protective immunity (Hess et al., 1996; Sinha et al., 1999).

Earlier it was thought that humoral immunity has very little role to play in conferring protection against salmonellosis. Recent reports have suggested that infection of mice with *S. typhimurium* results in a profound antibody response against the non-protein as well as protein antigens (Meltzer and Schwartz, 2010). Our studies showed that the 49 kDa protein induced an antibody response. ELISA studies were carried out to determine the antibody titers to separate antigens. Sera were collected from animals immunized by test antigens in the experimental and unimmunized in control group. The results indicated that the recombinant 49 kDa of *S. typhi*, native 49 kDa of *S. typhi* and native 49 kDa of *S. typhimurium* could elicit significant levels of antigen-antibody reaction and are therefore equally immune reactive.

Despite a large number of published reports, the role of antibodies during different stages of *Salmonella* infection and particularly in protection against typhoid has not been fully evaluated (Brown and Hormaeche, 1989). Though recent reports suggest that antibodies participate and have a role in protection against *S. typhimurium* infection (Miller et al., 1989). The results of our studies showing reduced bacterial loads in the liver and spleen may be attributed to antibody production along with the process of phagocytosis.

Taking these observations together, it becomes clear that 49 kDa OMP evoked both cellular and humoral immune responses. The effect of this protein was pronounced in both its recombinant and native form. Based on the bacterial clearance, protection studies, production of protective antibodies and elicitation of significant CMI response, the 49 KDa protein has a real potential as a candidate vaccine against typhoid.

## ACKNOWLEDGEMENTS

The authors wish to thank Ms. Jasmine Kaur for her help in the preparation of the manuscript. These studies were supported by the Department of Science and Technology, Government of India in the form of a research project to SKJ and NH as University Grants Commisiion Senior Research Fellowship.

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