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High Level Expression of 49kDa Outer Membrane Protein of *Salmonella enterica* serovar Typhi

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ABSTRACT

Salmonella enterica serovar Typhi (S. Typhi) is a facultative intracellular pathogen that causes typhoid fever in humans. The emergence of multidrug-resistant strains of S. Typhi and certain drawbacks in presently available vaccines against typhoid, has added a sense of urgency to develop more effective typhoid vaccines. Outer membrane protein (OMPs) has been reported to be immunogens for eliciting active/protective immunity against Salmonella and thus, have great potential for use in vaccination. Earlier, in a study done in our laboratory, a protein from an outer membrane protein (OMP) of S. Typhi with apparent molecular weight of 49kDa was identified in S. Typhi. The 1.3 kb gene for this was cloned in pQE60 plasmid, expressed in E. coli SG13009 cells and purification of r49 kDa OMP was done by Ni-NTA affinity chromatography under different pH conditions. The yield of expressed protein of ~26 mg/L was obtained. We now report the expression of this protein in E. coli BL21 cells using the expression vector pET28a. The r-49kDa OMP was purified by Ni-NTA metal chelate affinity chromatography using 150mM imidazole under denaturing conditions and a yield of ~39 mg/L was achieved.

Key words: *S.* Typhi, Outer membrane protein, high level expression, purification, typhoid.

INTRODUCTION

Salmonella enterica serotype Typhi (*S.* Typhi) is a human-restricted pathogen that is the primary cause of enteric fever [1, 2]. The organisms are noncapsulated, nonsporulating, Gram-negative anaerobic bacilli, which have characteristic flagellar, somatic, and outer coat antigens [3] (Shahane et al. 2007). Typhoid remains a major health problem, especially in developing world where there is substandard water supply and lack of sanitation [4, 5]. It is a multi-organ parasite that inhabits the lymphatic tissues of small intestine, liver, spleen and bloodstream of infected individuals [6]. It is estimated each year that *S. Typhi* infects over 20 million individuals and kills approximately 600,000 people globally [4, 7]. The risk of acquiring typhoid fever is increased in individuals living under unhygienic conditions particularly in areas with open sewage and unclean drinking water, among clinical microbiologists and travellers to regions where the disease is endemic [8].

With the rapid emergence of widespread of *S. Typhi* serotypes, multi drug-resistant strains of *Salmonella*, and changing modes of bacterial presentation, typhoid fever is becoming increasingly difficult to diagnose and treat [9-11], which has added a sense of urgency to develop more effective typhoid vaccines [8]. None of the currently available typhoid vaccines is ideal. Currently two licensed vaccines against Salmonella are in use globally, Vi polysaccharide vaccine (Typhim-Vi®) and Ty21a live attenuated vaccine (Vivotif Berna®) [12, 13]. Though these two vaccines are well tolerable, they provide only moderate protection against typhoid [11]. Many studies are undergoing to develop Vi conjugated vaccines [14-16] and a number of new genetically defined attenuated strains of *S. Typhi* have been constructed as candidate live oral vaccines [17-20]. However, the emergence of MDR strains of

S. Typhi and less than desired efficacy of currently available vaccines have stirred an urgent need to evaluate the efficacy of other immunogens for developing better and efficient typhoid vaccines.

Many studies have documented, the ability of OMPs to induce a protective immunity in infection caused by diverse Gram-negative bacteria, such as *Haemophilus influenza* type b [21], *Pseudomonas aeruginosa* [22], *Neisseria meningitides* group B [23], *S. typhimurium* [24] and *Bordetella bronchiseptica* [25]. Crude OMP preparations have been used as immunogens for eliciting active immunity against *Salmonella* in animal models [26-28]. Vaccination with an attenuated live strain, as well as the patients with typhoid fever elicits immune response to OMPs [29-30] and the detection of specific antibodies against OMPs in sera of patients has been useful for diagnosis of typhoid [31]. Singh et al, [32] reported the monoclonal antibodies (MAbs) against *Salmonella* OmpA and showed that a single, highly conserved, sequential epitope on the C-terminal domain of OmpA was immunodominant in the mouse response to infection by serovar Typhimurium. The outer membrane proteins (OMPs) of Salmonella seem to have strong immunogenic potential and have been implicated as possible candidates for conferring protection against typhoid [33]. However, only limited success has been achieved using porins as the immunizing agent [26]. Studies have shown that some non-porin OMPs may evoke strong immune response in animals and some of these proteins have very strong potential for the development of a subunit vaccine against typhoid [34].

In previous a study done in our laboratory, a protein from outer membrane of *S.* Typhimurium was identified that elicited protective immunity against *S.* Typhimurium infection. Later, an identical 49 kDa OMP was identified in *S. Typhi* that is immunogenic and evoked both cell mediated and humoral immune responses [35]. The 1.3 kb gene for this was cloned in pQE60 plasmid, expressed in *E. coli* SG13009 cells and purification of r49 kDa OMP was done by Ni-NTA affinity chromatography under different pH conditions. The yield of expressed protein of ~26 mg/L was obtained [36]. Therefore, present study was undertaken to clone 1.3 kb gene in different expression vector pET28a, its high level expression in *E. coli* BL21 and purification by Ni-NTA affinity chromatography using imidazole under denaturing conditions to obtain a higher yield of protein.

MATERIALS AND METHODS

Bacterial strains, enzymes, vectors and reagents

Salmonella enterica serovar Typhi (*S.* Typhi) strain (MTCC No-733) (wildobtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India, was used for isolation of genomic DNA. Plasmid pET28a, *E. coli* BL-21 and E. coli DH5α were obtained from International Center for Genetic Engineering and Biotechnology, New Delhi, India. PCR amplification kit, Taq DNA polymerase, T4 DNA Ligase, isopropylthiogalactoside (IPTG) were procured from Bangalore Genei, India. Restriction enzymes were obtained from MBI Fermentas. PVDF membranes, Anti rabbit polyclonal anti His probe peroxidase-conjugated secondary antibody were obtained from Santacruz Biotechnology, USA. Primers for amplification of 49kDa gene, DAB (3,3,9-diaminobenzidine tetrahydrochloride) were obtained from Sigma, USA.

Bacterial cultivation and DNA purification

The bacterial strains were grown in Luria Bertani (LB) medium at 37 °C. The recombinants were grown in LB broth/ LB agar supplemented with ampicillin (100µg/ml) and kanamycin (50µg/ml). Genomic DNA was isolated by method of Sambrook *et al*. [37] and plasmid DNA was isolated by alkali lysis method as described by Birnboim and Doly [38].

The cloning of 1.3 kb gene for 49kDa OMP of *S. Typhi*

The full-length coding region i.e 1.3 kb gene of 49 kDa OMP of *S. Typhi* was amplified by polymerase chain reaction (PCR) using the following sets of primers - Forward primer: 5` CATG**CCATGG**GAGACGCATCGATAA 3` and Reverse primer: 5`CGC**GGATCC**TT GAGAGGAATTCATT 3`**.** The PCR reaction was performed in a 25µl reaction volume with 100ng genomic DNA, 2.5mM dNTPs, 10 micromols primer and 1.5U Taq DNA polymerase using the following conditions: 94 °C for 5 min (initial denaturation), 94 °C for 45 sec (denaturation), 58 °C for 45 sec (annealing), 72 °C for 1 min 30 s (extension) and 72˚C for 7 min (final extension). The reaction was carried out for a total of 30 cycles. The primers were designed to generate amplification products containing NcoI and BamH1 restriction sites at the 5` end of the forward and reverse primers respectively. The sequence of eluted and purified single fragment of 1.3kb size was confirmed by DNA sequencing at TCGA (The Centre for Genomic Application) from both the ends using forward and reverse primers.

The 1.3 kb PCR product was inserted into expression vector pET 28a in frame with Histidine 6 tag sequence at C-terminal end (5.3 kb, having NcoI and BamHI sites at the MCS region) under the control of the IPTG-inducible

PT7/lacI promoter. The derivative named pJJ-6.6 was introduced into E. coli DH5α by $CaCl_2$ transformation and plasmid isolation was done. The presence of right insert was confirmed by double digestion, plasmid PCR.

Expression of 49kDa OMP of *S. Typhi*

The plasmid DNA from positive clones was used to transform the *E. coli* expression host strain BL21. To express the recombinant protein, transformed *E. coli* BL21 cells were grown in LB medium (500 ml) containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) 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 0.8 mM isopropylthiogalactoside (IPTG). The expressed 49 kDa OMP of *S. Typhi* was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of Recombinant 49kDa OMP of *S. Typhi*

The insoluble pellet fraction (of 500 ml induced culture) was resuspended in 30 ml lysis buffer containing 50 mM Tris–Cl (pH 8), 300 mM NaCl, 8 M urea, 10 mM imidazole and 1mM PMSF and lysed by sonication of 15 cycles of 30seconds (pulse-1 sec on/ 1 sec off) at 65% Amp. After sonication, the lysate was stirred for 3-4 h at room temperature, the contents were clarified by centrifugation at 8000 rpm for 30 min and the recombinant protein in the supernatant was purified by Ni-NTA chromatography under denaturing conditions.

The resulting ~15ml supernatant was mixed with a 1-2 ml Ni- NTA super flow resin that had been pre-equilibrated with the equilibration/binding buffer (20 mM phosphate buffer, 10 mM imidazole and 0.5 M NaCl). This suspension was gently rocked for 2-3 hrs at room temperature 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 wash buffer I (20 mM phosphate buffer, 20 mM imidazole and 0.5 M NaCl, 6 M urea) and wash buffer II (20 mM phosphate buffer, 50 mM imidazole and 0.5 M NaCl, 3 M urea) and bound protein was eluted with elution buffer III (20 mM phosphate buffer, 150 mM imidazole and 0.5 M NaCl). Individual fractions were collected from the affinity column and subjected to electrophoresis on a 12% SDS-PAGE along with protein molecular mass marker. Proteins were visualized by silver staining. The purified protein was dialyzed and then refolded using 50mM each of arginine and glutamic acid, and concentrated using amicon filtration columns (Millipore) and used for further use.

Western blot analysis

For western blotting, eluted protein fraction was collected from the affinity column and subjected to electrophoresis on a 12% SDS-PAGE along with protein molecular mass marker. Protein was transferred onto immunoblot-PVDF membranes (Santacruz Biotechnology, USA), by placing the entire set up Trans Blot apparatus such that the gel was towards the negative terminal and membrane was towards the positive terminal. Transblot was carried out for 50 minutes at 50 mAmp. Western blots were blocked in 3% Bovine Serum Albumin (BSA). Blots were incubated with primary Antibody (1: 1000; Anti rabbit polyclonal anti His probe) for 2 hr at room temperature, followed by washing in PBS containing 0.5% Tween-20. Thereafter the blot was incubated in Goat anti rabbit IgG- peroxidase-conjugated secondary antibody of 1:5000 dilution (Santacruz Biotechnology, USA) followed by washing in PBS containing 0.5% Tween-20 and detected by using DAB (3,39-diaminobenzidine tetrahydrochloride, Bangalore Genei; India) substrate or by chemiluminescence detection (Santacruz Biotechnology, USA).DAB is 3-3' diaminobenzidine tetrahydrochloride, a substrate for horseradish peroxidase, which yields a colored deposit, which is insoluble in aqueous medium, alcohols, xylenes. It should be immediately used after dissolving and should be stored at 4°C.

RESULTS

Molecular cloning of 1.3 kb gene for 49kDa protein of *S. Typhi* in pET- 28a vector

The full-length coding region i.e 1.3 kb gene of r49 kDa OMP of *S. Typhi* was amplified (Figure 1) from genomic DNA. The amplified fragment was eluted, purified and visualized on 0.8% agarose gel (Figure 2). The sequence of eluted and purified single fragment of 1.3kb size was confirmed by DNA sequencing at TCGA from both the ends using forward and reverse primers. Sequence analysis of the purified PCR product revealed a 1300bp DNA sequence that matched exactly the hypothetical 49kDa gene reported in *S. Typhi* and the homology analysis revealed 100% homology of the PCR amplified gene with the reported gene sequence in *S.* Typhi.

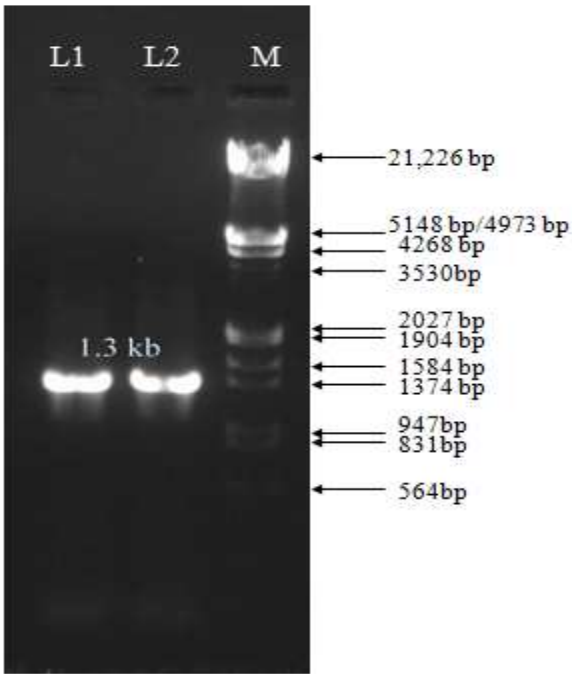

Figure 1: PCR amplification of the 1.3-kb gene for the 49kDa OMP of *S.* Typhi. Lane 1 and 2 shows amplification of 1.3kb band in *S. Typhi* showing the presence of 49 kDa encoding gene. Lane M has EcoR1 and Hind III double digested λ DNA marker.

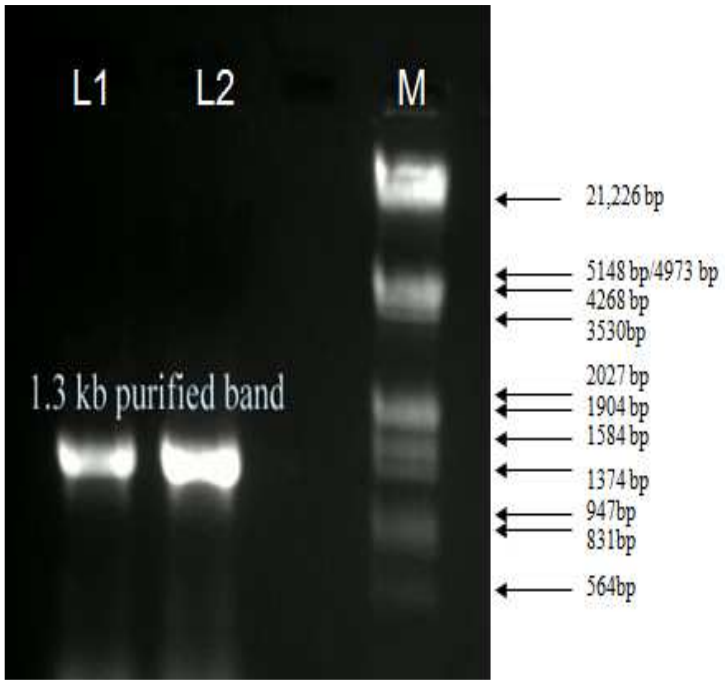

Figure 2: PCR amplified product of 49kDa OMP of *S. Typhi* purified by gel elution method and run on 0.8% agarose gel. Lane 1and 2 shows the 1.3kb purified fragment. Lane M has EcoR1 and Hind III double digested λ DNA marker.

The eluted and purified single fragment of 1.3kb size of *S. Typhi* was cloned in pET 28a expression vector (5.3kb) under the control of IPTG inducible PT7/lac promoter. This system is designed for high-level expression of His-tagged proteins. This vector contains the initiation codon ATG upstream and 6xHis tag downstream of MCS (multiple cloning sites). The NcoI/ BamHI digested vector (Figure 3) and PCR amplified 1.3kb fragment were ligated using T4 DNA ligase and used to transform *E. coli* DH-5α cells.

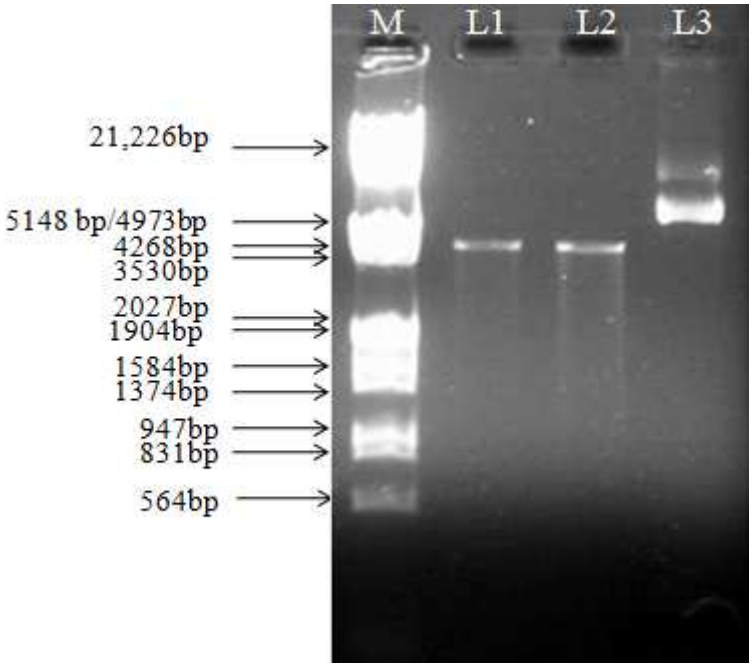

Figure 3: Restriction of pET-28a double digested with BamH1 and Nco1. Lane 1 and 2 has double digested pET-28a and Lane 3 has undigested pET-28a. Lane M has EcoR1 and Hind III double digested λ DNA marker.

The presence of the insert in transformants was confirmed by restriction enzyme digestion analysis (Figure 4) and also by PCR amplification screening using inserts specific primers (Figure 5).

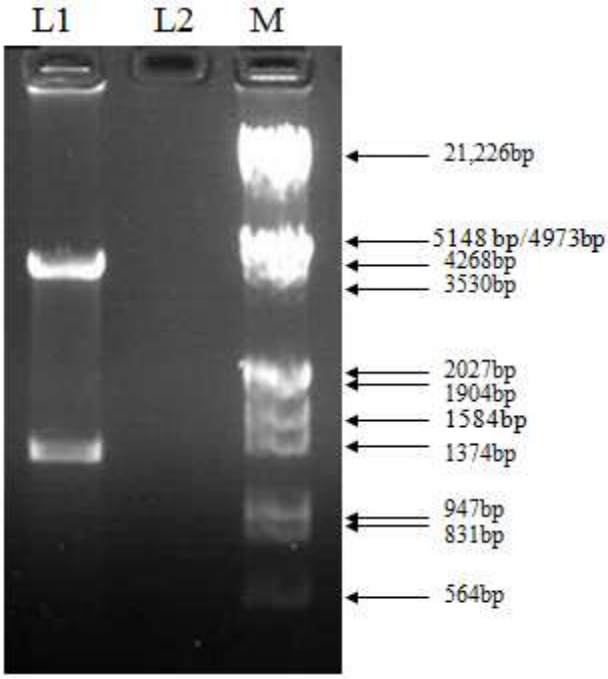

Figure 4: Restriction digestion analysis of the plasmids of the colonies grown on kanamycin positive LA plates using Nco1 & Hind III. Lanes 1 shows restricted band of 1.3kb and 5.3kb. Lane M has EcoR1 and Hind III double digested λ DNA marker.

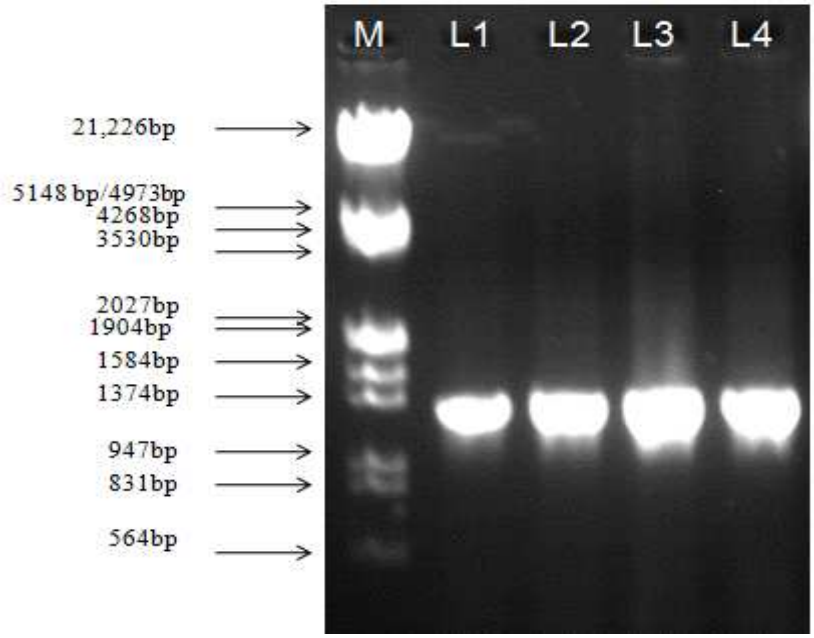

Figure 5: Colony PCR of the plasmids from transformants using insert specific F1 and R1 primers. Lanes 1-4 shows strong amplification of 1.3kb gene indicating the insertion of the gene in plasmid vector. Lane M has EcoR1 and Hind III double digested λ DNA marker.

The expression construct was used to transform *E. coli* expression host strain BL21. Recombinants were selected on ampicillin (100µg/ml) and kanamycin (50µg/ml) plates, grown under selection pressure and were confirmed by PCR amplification screening using inserts specific primers (Figure 6).

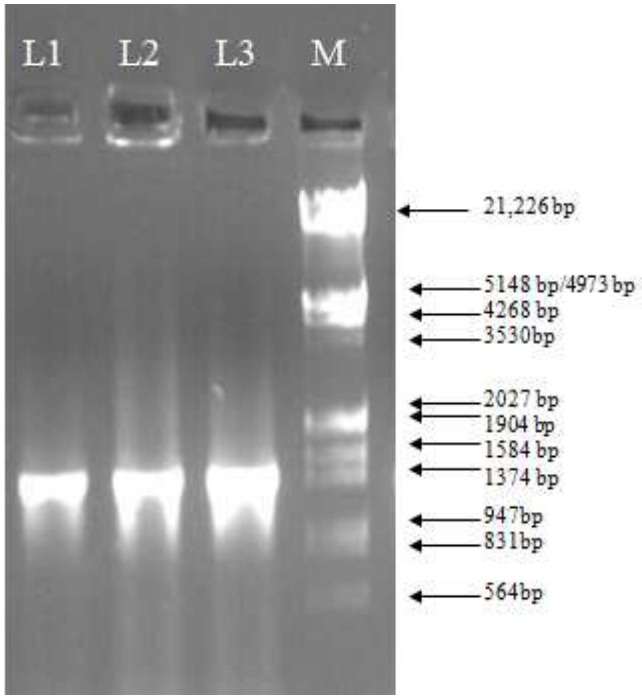

Figure 6: Colony PCR of the expression plasmids of the colonies grown on ampicillin and kanamycin positive LA plates using insert specific F1 and R1 primers. Lanes 1, 2 and 3 shows strong amplification of 1.3kb gene indicating the insertion of the gene in Expression vector. Lane M has EcoR1 and Hind III double digested λ DNA marker.

Purification of induced 49kDa protein using Ni-NTA affinity chromatography

Transformed E. coli BL21 cells were induced with 0.8mM IPTG. 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 12% SDS-PAGE gels (Figure 7).

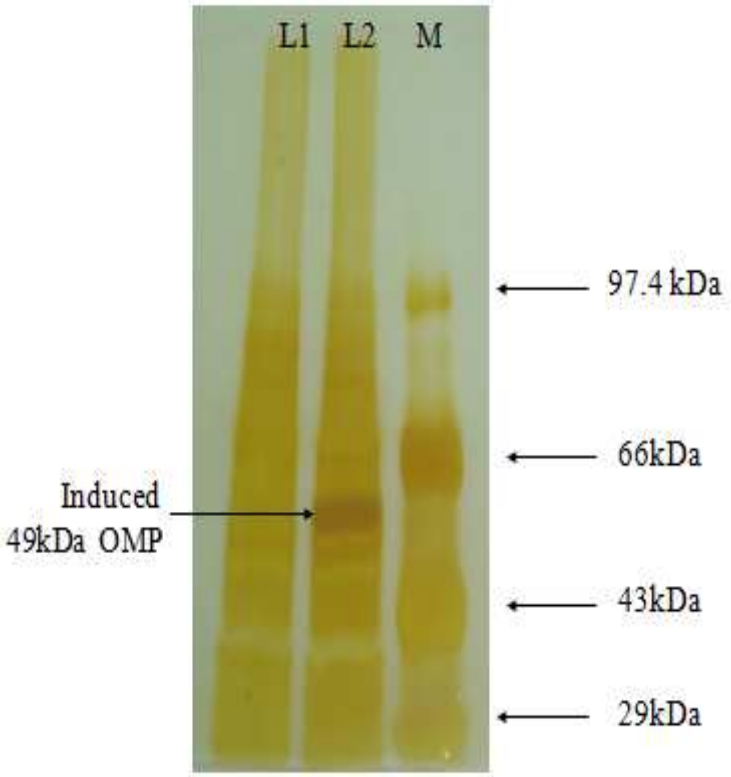

Figure 7: SDS-PAGE profile depicting Expression of the r49 kDa OMP of *S. Typhi* in *E. coli* BL21 cells. The cells were grown up to log phase and induced with 0.8mM IPTG for 4 h at 37 °C Induction was obtained by 0.8 mM IPTG. Lane 1 shows uninduced culture while lane 2 shows induced cultures. Lane M has Protein mol. Wt Marker (Broad range). The figure clearly shows increased expression of r49kDa OMP in induced culture of BL21 cells.

The protein was predominantly expressed in insoluble fraction and was purified by Ni-NTA metal chelate affinity chromatography under denaturing conditions. The fractions collected during different steps of purification were analyzed by 12 % SDS-PAGE gels as shown in (Figure 8). The purified recombinant protein was eluted from the column with 150 mM imidazole. SDS-PAGE analysis of the affinity purified protein revealed a single band of approximately 49 kDa.

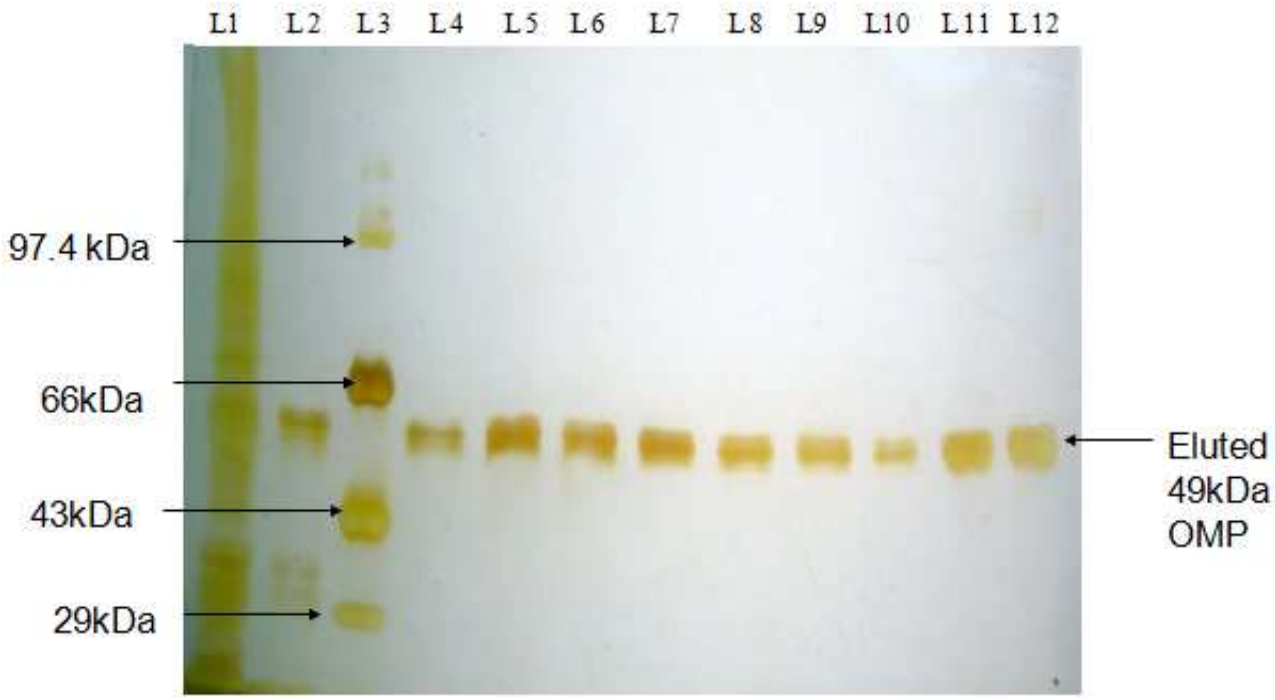

Figure 8: SDS-PAGE profile depicting Single Step Purification of recombinant 6x His tag expressed r49 kDa OMP of *S. Typhi* using Ni-NTA affinity chromatography. Lane 1 has protein flow through, lane 2- wash fraction, lane 3 has Protein mol. Wt Marker (Broad range), lane 4 - lane 12 has purified protein with 150 mM imidazole.

Immunoblot analysis of the His-tagged recombinant protein (49kDa) of *S. Typhi* with anti His-tag antibody

To confirm that the over expressed band seen in SDS-PAGE is recombinant protein with C-terminal His-tag, Western blotting was performed with anti rabbit polyclonal anti His antibody (GenScript, USA) and HRPO conjugated anti-mice IgG antibody (Sigma) as secondary antibody, thereby giving a single band of approximately 49 kDa (Figure 9).

The purified protein was dialyzed and then refolded using 50mM each of arginine and glutamic acid and used for further use.

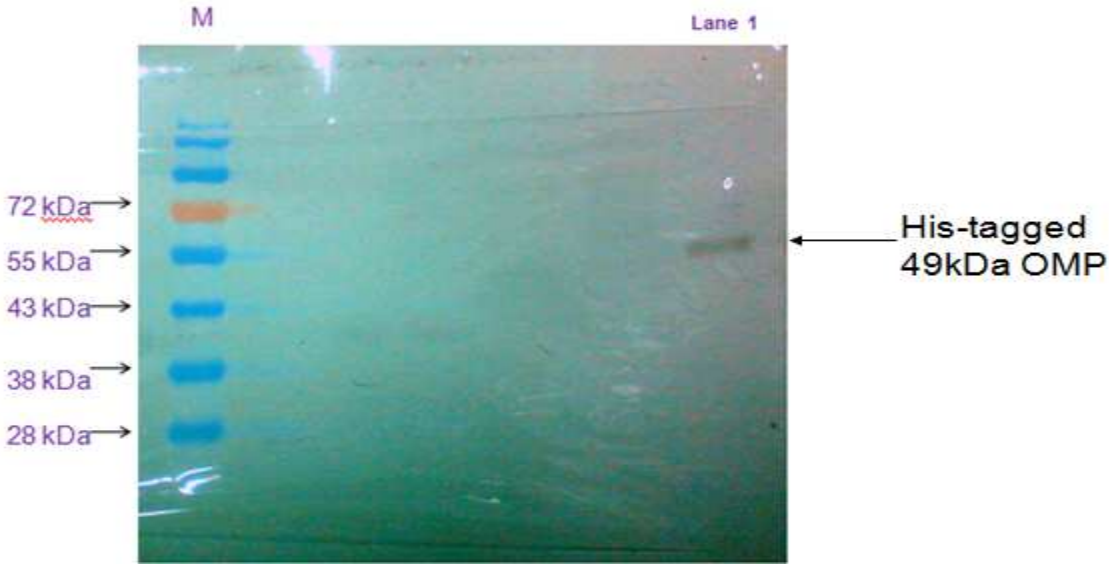

Figure 9: Western blot analysis of the purified r49 kDa OMP of *S. Typhi* detected with anti rabbit polyclonal anti His antibody. Lane M has Protein mol. Wt Marker Lane 1 shows single band of 49 kDa Protein.

DISCUSSION

Salmonella has been around for long and a large number of studies have been carried out in an effort to combat its infections. Salmonella has evolved a remarkable mechanism for adaptation to its intracellular environment. The emergence of multidrug-resistant strains of *S. Typhi* has added a sense of urgency to develop more effective typhoid vaccines [8]. However, none of the current vaccine candidates are likely to be available for several years [39]. Many studies have documented antibody responses to *S. Typhi* proteins, and the major antigenic components include the somatic O antigen (endotoxin, lipopolysaccharide), flagellar H antigen, Vi antigen, and outer membrane protein [40-43].

Salmonella OMPs plays a link between the cell and its external environment and carries important virulence factors that have importance in the bacterial pathobiology and its adaptation. Due to the receptivity to host defense system, such OMPs of gram-negative bacteria are immunologically important. But there are only few reports available regarding the efficiency of OMPs in providing protection against salmonellosis. In earlier studies carried out in our laboratory, OMP of *S.* Typhimurium was identified which is highly immunological, stimulates both humoral and cell mediated immune responses [35]. We later reported that, there is an identical 49 kDa OMP in *S. Typhi* which is also highly immunogenic and elicits humoral as well as cell mediated immune responses and confer protection against *S.* Typhimurium lethal infection in mice [36].

The gene encoding for this 49 kDa OMP of *S. Typhi* was earlier cloned in pQE60 plasmid, expressed in *E. coli* SG13009 cells and purification of r49 kDa OMP was done by Ni-NTA affinity chromatography under different pH conditions [36]. The yield of expressed protein of ~26 mg/L was obtained. We now report cloning of the gene in a different expression vector pET28a, its expression in *E. coli* BL21 and purification by Ni-NTA affinity chromatography using imidazole under denaturing conditions.

The 1.3 kb gene of 49 kDa OMP of *S. Typhi* was amplified by polymerase chain reaction (PCR) using oligonucleotide primers which were designed from 5' and 3'-end regions of this gene (Figure 1). The *Nco* I and *Bam* HI restriction sites were engineered into the 5'-end of forward and reverse primers respectively. The amplified fragment was purified to remove free primers as these may reduce the efficiency of ligation of PCR product to vector during subsequent steps (Figure 2). The identity of the PCR product was confirmed by sequence analysis. Sequence analysis of the purified PCR product revealed a 1300-bp DNA sequence that matched exactly the hypothetical 49kDa gene reported in *S. Typhi* and the homology analysis revealed 100% homology of the PCR amplified gene with the reported gene sequence in *S.* Typhi.

The 1.3kb PCR product was cloned in pET 28a vector between Nco I and Bam HI sites in MCS region in frame with Histidine 6 tag sequence at C terminal end under the control of the IPTG-inducible promoter PT7/lacI. The pET-28a expression system is designed for high-level expression of His-tagged proteins. The pET-28a is of 5.3kb and carries an N-terminal His•Tag/thrombin/T7•Tag configuration in addition to an optional C-terminal His•Tag sequence. The positive transformants was confirmed by restriction enzyme digestion analysis (Figure 4) and PCR amplification screening using inserts specific primers (Figure 5). The recombinants were selected on ampicillin (100μg/ml) and kanamycin (50μg/ml) plates, grown under selection pressure and were confirmed by PCR amplification screening using inserts specific primers (Figure 6). The recombinant plasmids named pJJ, 6.6kb molecular weight that gave strong positive signals were selected for expression studies.

The recombinant plasmids were expressed in *E. coli* expression host strain BL21. Induction of r-protein expression was found to be optimal with the addition of 0.8mM IPTG (Figure 7). It was evident from a comparison of the polypeptide profiles obtained in the absence and presence of IPTG that induction of gene expression results in the appearance of a new (approximately 49kDa) band, which is consistent with the predicted size of recombinant protein. IPTG, an inducer of lac repressor-regulated promoters was used to induce the lac operon because, in contrast to allolactose, which is the natural inducer of the operon, IPTG cannot be hydrolyzed and broken down by the cell. The protein was present in the form of inclusion bodies, urea as denaturant were used for solubilization. Solublization is a critical step towards obtaining maximal amount of the desired protein in solution without inducing any chemical or deleterious modifications to it. Wide panels of detergents are available for the solublization of inclusion bodies including strong denaturants like urea, guanidinium salts and detergents such as sodium dodecyl [44], n-cety trimethylammonium bromide (CTAB) [45] and sarkosyl [46]. In our study 6M urea as denaturant were used for solublization.

To obtain functionally active protein, 49kDa induced OMP was purified from *E. coli* via the 6xHis tag expressed on the carboxy (C) terminus of the protein using nickel-NTA chromatography with 150 mM imidazole and 0.5 M NaCl

(Figure 8). 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 thereby giving a single band of approximately 49 kDa. It was further confirmed by western analysis, as can be seen from figure 9, a band was observed only in eluted fraction suggesting the presence of His tag protein.

Dialysis was performed for removal of excess denaturing agent. Refolding of protein was accomplished by controlled removal of the excess of denaturants, thus creating an appropriate environment where protein can fold spontaneously [47]. We used arginine (50 mM) and glutamic acid (50 mM) that inhibit intermolecular interactions is used for proper refolding of protein and to avoid unwanted aggregation [48].

Thus it became clear that the *S. Typhi* gene for 49kDa protein was expressed in *E. coli* using an IPTG-inducible vector and purified to near homogeneity by a single step Ni-NTA affinity chromatography with a yield of approximately 39mg/liter of culture.

CONCLUSION

In conclusion, this study showed that an amplified 1.3-kb band of 49 kDa OMP of *S. Typhi* was purified and sequenced to confirm its identity. This gene was recloned in a bacterial plasmid pET28a and re-expressed in *Escherichia coli* BL21 successfully. The r-49kDa OMP of *S. Typhi* was purified to near homogeneity by Ni-NTA metal chelate affinity chromatography using 150mM imidazole under denaturing conditions. Therefore, the aim of the present study was successfully achieved as higher yield of expressed of r49 kDa OMP of *S. Typhi* (~39 mg/L) was achieved.

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