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Recombinant OmpC Subunit Vaccine Facilitate Satisfactory Protection Against Challenge Dose Of *Salmonella* Typhimurium In Mice Model

Research Article

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Summary

A study to explore the potential of OmpC (outer membrane protein) of S. Typhimurium E277 in providing protection against mice challenged with Salmonella spp. Open reading frame ompC was amplified using the following designed primers and polymerase chain reaction was performed. 1109 bp size product was digested with BamHI and HindIII, and cloned into the pPROEXHTb vector plasmid. HIS fusion proteins were expressed in bulk using E. coli DH5α cells and a fusion protein were purified and was adjuvanated with Freunds incomplete adjuvant and used for vaccine trial. Adult Swiss albino mice were immunized with purified recombinant OmpC protein with FIA as adjuvant at the dose rate of 50 µg. The control group was inoculated with an equal amount of sterile PBS or FIA. Results revealed that the expressed rOmpC of S. Typhimurium in prokaryotic expression system induced satisfactory protection against virulent challenge study in mice.

I. Introduction:

Salmonella enterica has long been recognized as an important zoonotic pathogen of economic significance in animals and humans (Sinha et al., 1999 and Velge et al 2006). Devising control strategies through vaccine is of immense help in the control of the infections caused by this organism. Vaccination best fulfils the requirement for practical anti-*Salmonella* intervention without many of risks and difficulties associated with other control measures (Zhang-Barber et al., 1999). There is a continuing need for improved *Salmonella* vaccines. In this regard various vaccines like inactivated whole cell vaccines, live attenuated vaccines have been tried, but they have various shortcomings like adverse effects of lipopolysaccharide, short duration of immunity and residual virulence (Yang et al, 2001 and Levine et al., 1989). Thus, development of vaccine based on defined non-toxic components of the bacteria seems desirable. In this view, attention is now focused on the possibility of using outer membrane proteins from *Salmonella* spp in inducing protective immune response.

The outer membrane of *Salmonella* and other Gram-negative bacteria contains family of pore forming proteins called porins (Baalaji et al., 2006). Several workers have successfully demonstrated the induction of high levels of antiporin antibodies and enhanced cell-mediated immunity along with protection afforded by porins against Salmonella (Tufano et al., 1984; Di Donato et al., 1986; Yee et al., 2011). The major porins in Salmonella are OmpC, OmpF, OmpD, out of which OmpC was found to be major surface antigen with unique exposed epitopes expressed in more amounts regardless of the growth condition (Contrearas et al., 1996; Muthukkaruppan et al., 1992 and Singh et al., 1992). Gil-Cruz et al., (2009) observed that Antibodies to OmpD was important because porin immunization did moderate infection with bacteria lacking OmpC and OmpF but not bacteria lacking OmpD. In addition, surface-associated antigens or outer membrane proteins might induce particularly strong cellular immune responses because of superior processing, kinetic advantages compared to internal antigens (Barat et al., 2012). Moreover, ompC gene was found to be highly conserved within different Salmonella serotypes (Prejit et al., 2009). Hence, the present study was designed to explore the potential of OmpC in providing protection against challenge dose of Salmonella spp. in mice model.

II. Materials and Methods:

A. Bacterial strains:

S. Typhimurium E277 (reference strain) was used for cloning, expression and preparation of recombinant protein. The strain was confirmed biochemically and serologically as per (Edwards and Ewing, 1972) and maintained on nutrient agar slant and semisolid buffered agar at 4°C for further study.

B. Molecular biology reagents, kits and analysis:

Taq DNA polymerase, alkaline phosphatase, T4 DNA ligase, restriction endonucleases, molecular biology kits were procured from Bangalore Genei (Bangalore, India), MBI fermentas (Lithuania), Qiagen (Germany), Sigma (USA) and other reputed national and international firms. Oligonucleotides were from procured from Genuine Chemical Corporation (GCC, India). All DNA preparation, plasmid extraction, cloning and expression procedures and blot analysis were done using standard techniques (Sambrook and Russel, 2001).

C. Preparation of adjuvanated recombinant OmpC subunit vaccine:

Open reading frame *ompC* was amplified using the following designed primers as per

genomic approach: Forward, primer 5- CGC GGA TCC GCG GCT GAA ATT TAT AAT AAA GAC GG -3 (35 mer); reverse primer, 5- CCC AAG CTT CC CGC AGG CCC TTT AGC AAC AT – 3. The primer set contained *Bam*HI and *Hin*dIII restriction sites in forward and reverse primer, respectively, at their 5' ends. Genomic DNA from *S*. Typhimurium was extracted and amplification reaction was performed in 25 μl reaction volume each containing 5 μl (~50 ng) of template DNA, 2.5 μl of 10X Taq DNA polymerase buffer [10 mM Tris HCl (pH 9.0), 50 mM KCl], 2.5 mM MgCl2, 1 μl of dNTP mixture containing 10 mM of each dNTP, 1 μl (10 pM) each of forward and reverse primerfor OmpC gene and 1.0 U of Taq DNA polymerase. The PCR programme included initial denaturation at 94oC for 5 min, followed by 34 cycles (94oC for 1 min), annealing (55oC for 1 min.) and extension (72oC for 1:30 min.). Final extension was carried out at 72oC for 10 min.

A single band of approximately 1109 bp size were digested with *Bam*HI and *Hind*III, and cloned into the pPROEXHTb vector plasmid. HIS fusion proteins were expressed in bulk using *E. coli* DH5α cells. Fusion proteins were purified from supernatant using prepacked HIS-sepharose columns in accordance with the manufacturer's instructions (QIAGEN). The purified protein was dialysed extensively in dialysis buffer (25mM Tris-HCl, 25mM NaCl, 2 mM EDTA, pH 7.4) to remove urea. The purity was confirmed using SDS-PAGE (Laemmli, 1970) and concentration of protein was determined by Folin-Lowry method. The protein was adjuvanated with Freunds incomplete adjuvant and used for vaccine trial.

D. Western blot analysis:

Purified His-tagged recombinant OmpC protein was run on 10% SDS-PAGE and separated proteins were electro blotted on to the nitrocellulose paper (NCP) at 0.8 mA/cm2 constant current for an hour. After the transfer, the nitrocellulose paper was blocked overnight by 5% skimmed milk in PBS, washed thrice with PBS-T and then incubated for 1h with Nickel-HRP conjugate (1:1000) at room temperature. After primary antibody interaction, blot was washed thrice with PBS-T. Finally the membrane was dipped in developing solution containing 20 mg diaminobenzidine (DAB), 1.25 ml Tris buffer (PH-7.6), 150 μl of Nickel Chloride and 7.5 μl of 30% H2O2, in 25 ml. The reaction was stopped by rinsing the membrane with autoclaved triple distilled water.

E. Vaccination trial:

Thirty Adult Swiss albino mice were procured from LAR, IVRI, Izatnagar and were reared as per the institutional guidelines. The vaccine group of mice (10) was immunized with purified recombinant OmpC protein with FIA as adjuvant (100 μl) at the dose rate of 50 μg. The adjuvant group of mice (10) received 100 μl of FIA adjuvant and the control group (10 mice) was inoculated with an equal amount (100 μl) of sterile PBS. On 28th day, mice were sacrificed. The protective efficacy of the vaccine preparations and control was analyzed by challenging the mice intraperitoneally by the virulent *S*. Typhimurium strain E-277 (1.5 x 109 cells/mice) and were observed for mortality. The causative organism was reisolated from each group of mice and confirmed biochemically by employing standard methods (Agarwal et al., 2003).

III. Results and discussion

Porins from *Salmonella* Typhimurium and other gram-negative bacteria have been used in diagnostic tests (Contrearas et al., 1995) and in the development of vaccines (Arockiasamy and Krishnaswamy, 2000) as there is experimental evidence suggesting that the host immune response to *Salmonella* porins generates humoral and cell-mediated protective immunity (Sood et al., 2005; Tabaraje et al., 1994 and Zhang-Barber et al., 1999). It would also be of interest to characterize these apparently *Salmonella*-specific exposed epitopes, investigate whether they induce a protective immune response in the host infected with a *Salmonella* spp. This paper reports the amplification, cloning and expression of the ompC encoding gene of *S*. Typhimurium.

A. Adjuvanated recombinant OmpC subunit vaccine:

The DNA fragments of 1,109 bp encoding OmpC gene were amplified in bulk and cloned into pPROEXHTb expression vector and expression was induced using IPTG **(Figure. 1)**. The recombinant plasmids after induction yielded a 43 KDa protein on 10% SDS-PAGE analysis, including 6X- Histidine tag and TEV protease site at the N-terminus.

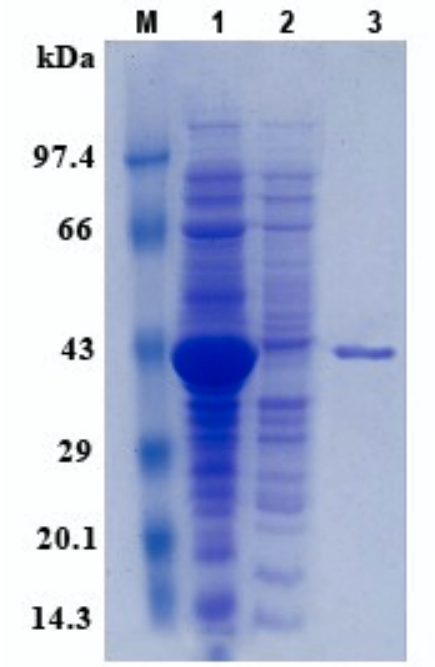

Figure 1: SDS PAGE analysis of the expression profile of recombinant OmpC: Lane M, protein marker. Lane 1: Recombinant fusion protein induced with 1mM IPTG and purified by single step metal affinity chromatography showing a 43 KDa protein; Lane 2: Whole cell lysate. Lane 3: Recombinant ompC eluted protein. The 10% SDS–PAGE was Coomassie stained.

The recombinant fusion protein was purified by single step metal affinity chromatography and on SDS-PAGE analysis, single protein was obtained after purification. The eluted ompC protein was dialyzed against the dialysis buffer, by sequential reduction in the concentration of urea, to enhance the proper re-folding of recombinant ompC. Analysis by SDS-PAGE showed a predominant band of approximately 43 kDa in recombinant clones. This was due to expression of the 36 KDa protein along with some of the sequences of vector such as 6X Histidine tag and TEV protease cleavage site (Schmitt et al., 1993). The protein was collected and concentration was found to be 3.2μg/μl.

B. Western blot analysis and immuno reactivity:

The bacterial lysates of induced cultures of OmpC were separated on 10% SDS-PAGE with a prestained protein molecular mass marker and were transferred onto the nitrocellulose membrane. Western blot analysis with anti-His-tag antibody and Ni-HRP conjugate detected 43 KDa protein in the induced bacterial lysates, representing the recombinant OmpC **(Figure 2)**. On immunoblot analysis the 43kDa band reacted specifically with anti recombinant OmpC hyper immune serum raised in mice, proving immuno reactivity of this purified recombinant protein. The expressed protein was confirmed by using Ni-HRP conjugate by western blot analysis. Immunoblot analysis of recombinant OmpC was analysed with the serum raised in mice against recombinant OmpC, which reacted specifically with recombinant OmpC, crude and native OmpC. Earlier studies have demonstrated that OmpC as the major surface antigen and immunogenic potential with unique surface exposed epitopes as evidenced by Salmonella porin specific monoclonal antibodies (Muthukkaruppan et al., 1992 and Sung et al., 2005).

C. Analysis of protective efficacy of the vaccine:

Results of the challenge study revealed that mice started dying from 24 h till 48 h and the protective efficacies of the vaccines were calculated based on the percentage in each group surviving the virulent *S*. Typhimurium challenge. A higher protection (8/10) was

observed in Group 2 and Group 3 group, as compared to Group 1 (5/10) protection.

These results are in agreement with that of Kannan (2004) who have also reported similar results in poultry birds when challenged with virulent *S*. Gallinarum and Knivett and Stevens (1971), who obtained reduced number of S. Typhimurium in livers of chicken and mice vaccinated with live salmonella vaccine and orally challenged with the organism.

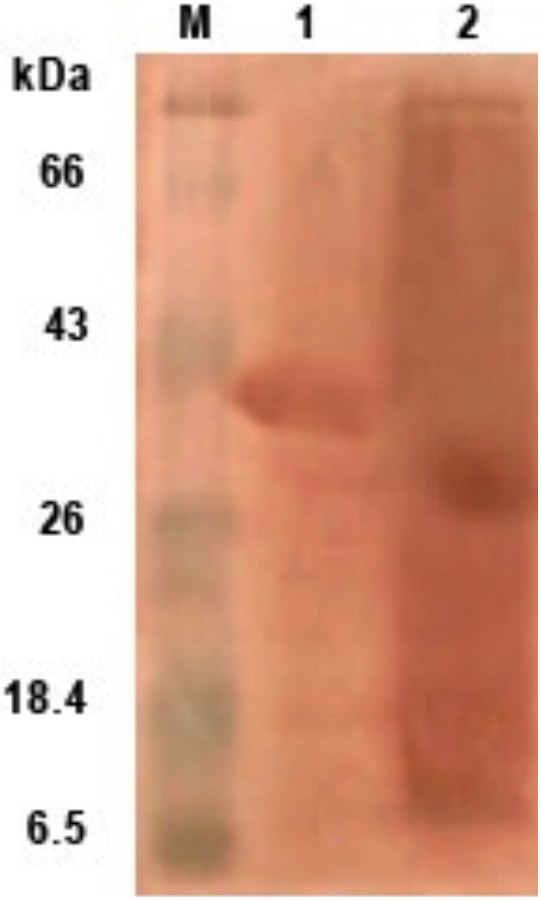

Figure. 2. Immunoblot analysis of anti- 43 kDa porin OmpC using sera from hyperimmunized mice. Molecular size (in kilodaltons) are shown to the left of the panel marked as M. Lanes 1, Immunoblot of purified OmpC extracts; Lane 2: Crude OMP extract. Porin preparations were loaded onto an SDS-10% polyacrylamide gel and electro blotted on to the nitrocellulose paper (NCP) at 0.8 mA/cm2 constant current for an hour.

Group	Control	R+A OmpC	Adj
Mortality	10/10	2/10	10/10
Survival	0/10	8/10	0/10
Protection	0	8/10	0

Table 1. Mortality pattern and protection in vaccinated mice's

Conclusion:

The results of the present work revealed that the expressed rOmpC of S. Typhimurium in prokaryotic expression system induced satisfactory protection against virulent challenge study in mice. Since our preliminary studies proved the effectiveness of the vaccine in controlling Salmonellosis and also since the gene was of conserved nature in different Salmonella serovars, it could be useful for protection against zoonotic serotypes. Therefore, the encouraging outcomes from this study may provide a novel vaccination strategy to pursue stronger OMP-based immune responses against Salmonella infection.

However further studies are needed to elucidate protective response with higher dose of challenge organism and further validate the protective efficacy against heterologous challenges with different Salmonella serovars.

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