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Expression, purification and evaluation of recombinant lipoprotein of *Salmonella typhi* as a vaccine candidate

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ABSTRACT

Lipoprotein has been reported as a vaccine candidate against many pathogenic bacteria, it plays direct role as a virulence-associated function. Here the approach is toward the expression of recombinant lipoprotein of *Salmonella typhi* in prokaryotic host and its evaluation as a vaccine candidate. Lipoprotein gene (*lp1*) was cloned in pET32a expression vector in addition to Bam HI and Hind III restriction sites, and BL21(pLysS) was used as prokaryotic expression host for transformation. Lipoprotein induction was performed by IPTG and 55 kDa (31 kDa of Gene +24 kDa of vector additional protein with His-tag) was analyzed by 12% SDS-PAGE. The recombinant lipoprotein was purified by Ni-NTA affinity chromatography due to the addition of 6X His-tag in recombinant lipoprotein. Western blot analysis using anti-His tag polyclonal antibodies confirmed the specificity of recombinant lipoprotein. Immunogenicity and protection study of recombinant lipoprotein against *S.* Typhi was performed in BALB/c mice. Adjuvants IFA and alum salts were used to enhance the immune response. ELISA results proved that biologically active truncated recombinant lipoprotein (31 kDa) is a suitable immunogen. Alum salts used as adjuvant was effective for long-lasting immunity.

1. Introduction

In humans *S.* Typhi is a causal agent of Typhoid fever. Infection of *S.* Typhi usually acquired by contaminated water and food [1]. Bacterial lipoproteins perform many roles, as signal transduction, nutrient uptake, adhesion, conjugation, sporulation, and participate in antibiotic resistance, transport (such as ABC transporter systems), and extracytoplasmic folding of proteins. Lipoproteins play a direct role in virulence-associated functions, such as colonization, invasion, evasion of host defense, and immunomodulation against pathogens [2]. The bacterium *S.* Typhi causes typhoid fever that is a life threatening. Approximately 5700 cases occur annually in the United States; up to 75% of the cases are acquired during traveling internationally. In the world (predominantly in southern Asia, Africa, and America) it affects about 21.5 million persons each year [3]. The evolutionary history and population genetic structure of *S.* Typhi by mutation discovery within 200 genes reveals 121 housekeeping genes, 50 genes encoding cell surface structures, regulation, and pathogenicity, and 29 pseudogenes [4]. There are three vaccines Ty21a, ViCPS (Vi Capsular polysaccharide) and inactivated typhoid vaccine to control the disease Typhoid fever. Inactivated Typhoid vaccine provides moderate protection with efficacy 51–67%. And the efficacy of other two vaccines (ty21a and ViCPS) was approximately 65–70% [5]. The outer membrane proteins of *Salmonella* have efficacy to protect against Typhoid. Therefore, several outer membrane proteins have been investigated as a vaccine candidate [6]. The molecular structure and function of these proteins have been studied [7,8]. There is an urgent requirement to develop typhoid vaccine to prevent typhoid fever caused by *S.* Typhi. Outer membrane protein of *S.* Typhi has potential as a vaccine candidate and become a health tool for human immunization against typhoid fever because it may elicit a protective immune response [9]. Moreover, lipoprotein as a vaccine candidate is expected to have relatively low production costs and high efficacy compared with many other three vaccines.

Here in this study, the approach is to evaluate the efficacy of lipoprotein of *S.* Typhi against typhoid. The lipoprotein gene was cloned into prokaryotic host using pET32a expression vector, and recombinant lipoprotein were expressed and purified by using metal affinity chromatography. The immunological studies were performed in BALB/c mice to determine the level of protection during experimental challenge.

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2. Materials and methods

The strain of *S.* Typhi was taken from CARI (Central Avian Research Institute) Izatnagar, India in slant form, and was revived in the Hektoen enteric (HE) medium [10].

2.1. Cloning of lipoprotein gene

Overnight grown *S.* Typhi culture was taken to isolate DNA through Nucleo-pore gDNA Fungal, Bacterial mini kit (Genetix Biotech). The Sequence was taken from National Center for Biotechnology Information (NCBI) database to design primers for the PCR amplification of lipoprotein gene. Software Gene Tool Lite (Wishart et al., 1999) [11] was used to design primers. Forward primer 5′ATGCAGGATCCGCGGCCAGCACGCTATTACTGG3′ and reverse primer 5′ATGCAAAGCTTGGGTGTCGCGGTCATCCATTCC3′ were used to amplify the gene sequence. Bam HI and Hind III restriction enzyme sites were incorporated into the primers. The PCR reaction mixture containing 100 ng of template DNA, 10 pmol of each forward and reverse primer, 1.25 U of Taq DNA polymerase, 2.5 μl of 10 X buffer, 1 Mm $MgCl_2$, 1 μl of 10 mM each of dNTPs were used in the final volume of 25 μl reaction mixture. The thermocycler conditions as 95 °C initial denaturation for 4 min, denaturation for 95 °C for 1 min, annealing temperature 62 °C for 45 s, extension temp 72 °C for 75 s, final extension at 72 °C for 10 min were fixed in the programme. PCR amplified product was analyzed by 1% agarose gel electrophoresis. PCR product was purified by Sure-Extract PCR clean-up/gel extraction kit (Genetix Biotech). The purified PCR product was ligated into pTZ57R/T cloning vector (TA cloning vector) for the transformation. The whole procedure was performed according to the protocol of InsTA clone PCR cloning kit (Thermo scientific). The ligation mixture was transformed into DH5α competent cells. After incubation, white colonies were picked up and cultured in Luria-Bertani broth for the selection of recombinant colonies. Plasmid were isolated [12] from different culture tubes, subjected to colony PCR and restriction endonuclease double digestion with the same enzyme for the confirmation of recombinant colonies. Expression vector pET32a (Novagen) was subjected to restriction endonuclease double digestion with Bam HI & Hind III. After restriction digestion, ligation was performed by T4 DNA ligase (Thermo Scientific) at 22 °C for 4 h. After completion of ligation process that ligation mixture was subjected to transform into DH5α competent cells prepared by $CaCl_2$ method. Recombinant colonies screening was performed by ampicillin antibiotic marker, colony PCR and restriction endonuclease double digestion. After confirmation, recombinant vector was transformed into BL21 (pLysS), a compatible host for the expression. The gene was sequenced in the sequencing facility, CDFD Hyderabad using same primer used for PCR to check the mutation.

2.2. Protein expression

Recombinant colonies were cultured into fresh sterile Luria-Bertani broth containing (100 μg/ml) ampicillin and culture was incubated at 37 °C until cells reached (OD_{600} 0.5). For induction, the culture was incubated at 32 °C in the rotatory shaker at 180 rpm in addition of 1.0 mM IPTG concentration. Cultures were collected at different time intervals 0, 2, 4, 6, 8, 10 h and collected cultures were stored at −80 °C.

2.3. Purification of recombinant lipoprotein

The expression of lipoprotein was initiated using 1.0 mM IPTG concentration at OD_{600} 0.5. The recombinant lipoprotein was expressed with 6X His tag to enable immobilized metal affinity chromatography. Because 6X His tag was used in the purification process. Recombinant lipoprotein purification was performed according to (Hesaraki et al., 2013) with some modifications [13]. Recombinant cells harboring the lipoprotein were centrifuged, the formed pellet was suspended in TE buffer (10 mM Tris, 1 mM EDTA, at pH 8.0). The suspension of cells was subjected to sonication for 7 cycles, 45 s each with 2.5 min of intervals for the incubation period on ice. The cell suspension was centrifuged at 5000 rpm for 25 min. The pellet achieved after centrifugation was again suspended in TE buffer containing 10 mM $MgCl_2$, 0.7 M NaCl and 0.1% Triton X-100 and again centrifuged at 5000 rpm for 25 min. The pellet was suspended in TE buffer, centrifuged, and solubilized by overnight agitation on ice in loading buffer (60 mM phosphate, 0.3 M NaCl, 2 M urea pH 8.0). The preparation was again centrifuged at 10000 rpm for 10 min. The final supernatant was applied onto a column with Ni-NTA agarose (Qiagen, USA) and wash with washing buffer (100 mM NaH_2PO4, 10 mM Tric Cl, 8 M urea, pH 6.3) containing 20 mM imidazole. Recombinant lipoprotein was eluted with elution buffer (100 mM NaH_2PO4, 10 mM Tric Cl, 8 M urea, pH 4.5) containing 200 mM imidazole. The concentration of the purified protein was estimated with Bradford method [14].

2.4. Western blotting analysis

Western blotting was performed for purified recombinant lipoprotein. The gel was transferred to polyvinylidene fluoride membrane (PVDF, pore size of 0.45 m) (Invitrolon, Thermo Fisher Scientific) using a semidry electro blotter (Bio-Rad Laboratories) at 200 mA for 30 min in transfer buffer (0.2 M glycine; 24 mM Tris; 10% methanol [pH 8.3]). PVDF membrane was placed in blocking buffer overnight at room temperature. Blocking buffer contains 3% skimmed milk and 0.15% Tween-20 in 10 mM phosphate-buffered saline (PBS) [pH 7.4]). The membrane incubated with horseradish peroxidase (HRP) conjugated with rabbit anti-His-tag polyclonal antibody (1:10000 dilution) (Invitrogen, Thermo Fisher Scientific) in PBS—Tween-20 for 1 h at room temperature. Blots were washed and incubated in a solution of hydrogen peroxide and 4-chloro-1-naphthol, at room temperature in the dark for color development.

2.5. Immunization and protection studies in mice

All mice were treated according to institutional guidelines for the treatment of animals. Eight to nine-week-old BALB/c mice (18 ± 1 g) were separated in eight randomized groups of thirteen animals and immunized intraperitoneally (i.p.) with recombinant lipoprotein, recombinant lipoprotein + FIA and recombinant lipoprotein + alum salts as mentioned in Table 1. Mice were bled from the tail vein to collect pre-immune sera before immunization. The recombinant lipoprotein was injected intraperitoneally on days 0, 7, 21, 28 and they were bled on day 40. The collected sera were kept at-20 °C. The control mice were injected with PBS (Phosphate Buffered Saline) at the same immunization schedule.

2.6. Antibody response (ELISA)

After immunization sera were analyzed by Enzyme Linked Immunosorbent Assay according to the Engvall and Perlman [15]. The 96-well polypropylene microwell ELISA plates were coated with 2.5 μg of antigen (recombinant lipoprotein) at 250 μl/well. Antigens were prepared in 50 mM sodium carbonate buffer having pH 9.6. Plates were incubated overnight at 4 °C and washed with 1 X PBST. Three percent Bovine Serum Albumin was used to block at 37 °C for 2.5 h incubation period. The 250 μl of serially diluted serum (1:50, 1:100, 1:200, 1:400, and so on) in Phosphate-Buffered Saline were added to antigen-coated wells. Plates were incubated