



Generation of Recombinant GroEL (HSP60) of *Bacillus Anthracis* in Heterologous System *E. coli* and Evaluation of Its Humoral Immune Response in Mouse Model

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Abstract

Anthrax, a disease of public health as well as bio-warfare potential is caused by Gram positive, non-motile and spore forming *Bacillus Anthracis*. Bacteraemia and toxemia of *B. anthracis* is responsible to cause lethality in anthrax-infected host. In this study, GroEL (HSP60) gene was cloned in pET28a+ vector and expressed in heterologous system *E. coli*. Over expressed recombinant GroEL was purified by IMAC chromatography and analysed on 10% SDS-PAGE. In western blot analysis, the rGroEL reacted with mouse anti-his antibody and anti-*B. anthracis* Sterne mouse serum. The immunogenicity of GroEL with aluminium hydroxide was evaluated in BALB/c mouse model. The endpoint titer of anti-rGroEL circulating antibodies in mouse serum was found high by indirect ELISA after first and second booster doses. IgG1 and IgG2b isotypes were found dominating in comparison to other isotypes. Humoral immune response generated by rGroEL indicated that it is immuno-dominant antigen of *B. anthracis*, which could be further evaluated as a subunit vaccine candidate against anthrax.

Keywords: Anthrax; *Bacillus Anthracis*; GroEL; HSP60; Vaccine

Introduction

Bacillus Anthracis is a Gram-positive spore forming bacterium, which is the causative agent of deadly disease anthrax. In 19th century, Robert Koch for the first time isolated *B. anthracis* and explained its microbial and pathological etiology [1,2]. *B. anthracis* has potential to form rigid spores under adverse environmental conditions. *B. anthracis* spores are highly resistant to dehydration, high temperature, chemicals, high pressure and UV radiation. *B. anthracis* spores can maintain viability for decades in dormant state, and resume germination and rapid growth on getting favourable environment [3-5]. Anthrax is an endemic disease, mainly in several agrarian countries. Anthrax is primarily a disease of domestic and wild animals, mainly grazing animals, and human is infected on coming in contact directly or indirectly with the diseased animals [6]. Clinical manifestations of human anthrax depend on the route of exposure of *B. anthracis* spores, which may be cutaneous, gastrointestinal, inhalational or injectional. Cutaneous anthrax causes up to 20% mortality, if not treated with antimicrobial agents. Ingestion of spore-contaminated food can cause gastrointestinal anthrax, which accounts about 25%-60% mortality. The most fatal and fulminant manifestation of anthrax is due to inhalation of *B. anthracis* spores called as inhalational anthrax. Inhalational anthrax has the highest mortality even after antibiotics therapy. Injectional anthrax is caused by taking drugs with anthrax spores contaminated needles [1,6-8]. The pathogenesis of *B. anthracis* depends primarily on the two large virulent plasmids, pXO1 and pXO2, which harbour genes to encode anthrax toxins and a poly-D-glutamate capsule, respectively. Tripartite anthrax toxin i.e. Protective antigen (PA), Lethal factor (LF) and Edema factor (EF) are encoded by pXO1. PA is a central component of anthrax toxins and makes lethal toxin with LF, and edema toxin with EF. Zinc metalloprotease LF inactivates the MAPKK that impairs the signalling pathway and calmodulin dependent EF catalyzes the formation of cAMP from ATP which induces edema [9,10].

Due to ease of aerosolization, *B. anthracis* has been categorized as one of the top priority bio-warfare agents [11,12]. Anthrax has been used as bioweapon during World War-I and World War-II [6]. Since 2009, a new threat of anthrax has emerged throughout the Europe in the form of anthrax spore contaminated heroin needles in drug users [8].

In 1881, Louis Pasteur first developed live attenuated anthrax vaccine Pasteur I and Pasteur II. Afterwards, Max Sterne developed non-capsulated live attenuated *B. anthracis* Sterne spore vaccine [13]. Anthrax Vaccine Adsorbed/Biothrax (AVA) and Anthrax Vaccine Precipitated (AVP), acellular vaccines have been licensed in USA and UK for human use under the animal rule. AVA and AVP acellular vaccines are prepared from culture supernatant of pXO1+/pXO2⁻ *B. anthracis* strains (V770-NP1-R or Sterne 34F2) which contains mainly PA with small amount of other bacterial proteins [14,15]. Despite of severe adverse effects of AVA and AVP vaccines, both vaccines induce long durable humoral and cellular immune response with annual boosters. Several studies have established that PA based vaccines generate immunity against toxin, but not against *B. anthracis* bacterium [16,17]. Several immunodominant antigens of *B. anthracis* have been identified to develop effective anthrax subunit vaccines. These immunodominant antigens of *B. anthracis* were evaluated as vaccine candidates either alone or in combination with PA [17-20].

Membranous and secreted antigens have been considered as potent vaccine candidate due to first line interaction between host and pathogen [21]. In the era of next generation subunit vaccine development, researchers are looking towards use of membrane component as well as secreted proteins to elicit humoral and cellular immune response. The heat shock protein, GroEL (HSP60) is present on the spore surface as well on bacterial membrane and in the secretome of bacteria [21-24].

Therefore, in the current study, GroEL (BA0267) gene of *B. anthracis* was cloned and expressed in heterologous system *E. coli*.

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Recombinant GroEL protein was purified, and administered with aluminium hydroxide in BALB/c mice via subcutaneous route to study the humoral immune response. Immunological response elicited by GroEL indicated that GroEL is a potent immunodominant antigen of *B. anthracis*.

Materials and Methods

Ethical statement

All animal care and experimental procedures were conducted as per the approved protocols of Institutional Animal Ethics Committee (IAEC) of Defence Research and Development Establishment (Reg. No.37/Go/C/1999/CPCSEA) and Institutional Biosafety Committee (Protocol no. IBSC/12/BT/AKG/22). All the experimental mice were maintained in pathogen free facility.

Bacterial strains and vector

Strain of *B. anthracis* bearing plasmids pXO1 and pXO2 was isolated from cutaneous anthrax sample from Andhra Pradesh, India [25]. Glycerol stock of *B. anthracis* was streaked and grown on Brain Heart Infusion (BHI) agar plate at 37°C for 16 h. Genomic DNA of *B. anthracis* was isolated by using QIAamp DNA Mini Kit (Qiagen, USA). Bacterial strains viz. *E. coli* host strains BL-21 (DE3) and DH5 α , and vector pET 28a+ were outsourced from Invitrogen and Novagen, USA.

Construction of recombinant plasmid of GroEL

The gene of GroEL (1635 bp) was amplified from genomic DNA of *B. anthracis* using forward primer (5'-ATTCCATGGGCATGGCAAAAGATATTAATTTAGTGAAG -3') and reverse primers (5'-TTACTCGAGCATCATTCGCGCCCATACC -3') containing NcoI and XhoI restriction site, respectively. The PCR amplicon of GroEL and pET28a+ vector were digested individually using the respective aforementioned restriction enzymes. Ligation of digested inserts and vector was performed with T4 DNA ligase at 16 °C for overnight. Ligated plasmids were transformed into chemically competent *E. coli* DH5 α cells and grown on Luria Bertani (LB) agar plate containing 50 μ g/ml kanamycin. The positive clones of transformed *E. coli* DH5 α were selected and recombinant plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, USA).

Expression and localization of rGroEL

Recombinant plasmid GroEL-pET28a+ were transformed into chemically competent cells of *E. coli* BL21(DE3) and isolated colonies of GroEL were grown in LB broth containing 50 μ g/ml kanamycin and induced at 0.6 optical density (OD) of cells with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma, USA) for 4 h at 37°C. Induced and un-induced culture pellets were harvested and analysed on 10% SDS-PAGE. Expression of recombinant GroEL was confirmed with Western blot using anti-His HRP conjugated antibody (Qiagen, USA). For localization, induced culture pallet was re-suspended in native lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole) and physically lysed by using sonicator (BioLogics Inc., USA). Soluble fraction and pallets were analysed on 10% SDS-PAGE.

Purification of rGroEL

Purification of His-tagged recombinant GroEL was performed by immobilized metal affinity chromatography (IMAC) in native conditions. Pallet of GroEL was re-suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole; pH-8) and lysed by sonicator. Soluble fraction of GroEL was added to equilibrate Ni-NTA

column for binding followed by column washing with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl and 20 mM imidazole; pH-8). After washing, the protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole; pH-8). The purified rGroEL was analysed on 10% SDS-PAGE, dialysed and estimated by BCA method (Sigma, USA). The level of Lipo-polysaccharide (endotoxin) was measured by Limulus Amebocyte Lysate assay, and endotoxin free rGroEL antigen was used for immunizations.

Interaction of rGroEL with serum generated against *B. anthracis* Sterne

Sub-lethal dose of *B. anthracis* Sterne spores was injected intraperitoneally on 0th day and 12th days in BALB/c mice (n=5). After second dose, serum was collected on 7th day. Purified rGroEL antigen was separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane, treated with 1:1000 pooled anti-*B. anthracis* Sterne mouse serum, and probed with rabbit anti-mouse HRP conjugate antibody (Sigma, USA) followed by development with HRP substrate 3,3'-diaminobenzidine (DAB).

Mouse immunization

Six to eight weeks old female BALB/c mice were received from animal facility, DRDE, Gwalior and randomly grouped. Recombinant antigen GroLE was adsorbed on 0.35% aluminium hydroxide (Alhydrogel® adjuvant, InvivoGen, USA). One group of BALB/c mice (n=6) was immunized subcutaneously with 10 μ g of aluminium hydroxide adsorbed GroEL. The second group of mice was kept as control and administered with PBS only (Figure 1A). Blood of animals was collected at day 0 (before immunization) and prime dose was given followed by two boosters on day 14 and 21. The serum of immunized mice was collected on day 21 (1st booster serum) and 28 (2nd booster serum, Figure 1A). Level of anti-GroEL IgG antibodies and its isotypes were determined in serum collected after first and second booster.

Determination of IgG and IgG subtypes antibodies

IgG and IgG subtypes of anti-GroEL were measured in hyper immune sera of first and second booster by indirect ELISA. Briefly, 96 well ELISA plate (Nunc-Immuno Plate, Denmark) were coated with 0.1 μ g/well of rGroEL antigen in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated at 4 °C for overnight. Coated plates were washed thrice with 0.05% Tween-20 containing PBS (PBS-T) and blocked with 250 μ l/well of 5% skim milk powder (Sigma, USA) in PBS for 1 h at room temperature (RT). Following three washes, 100 μ l of mouse serum at different dilutions were added in triplicates and incubated for 1 h at RT. Unbound antibodies were removed by washing and 100

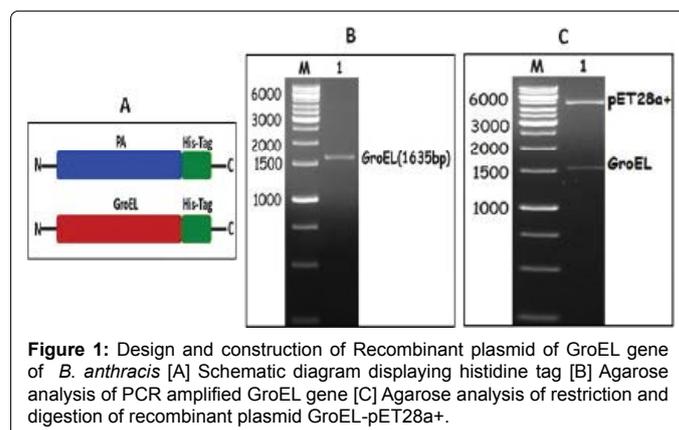


Figure 1: Design and construction of Recombinant plasmid of GroEL gene of *B. anthracis* [A] Schematic diagram displaying histidine tag [B] Agarose analysis of PCR amplified GroEL gene [C] Agarose analysis of restriction and digestion of recombinant plasmid GroEL-pET28a+.

μ l of rabbit anti-mouse HRP conjugate (Sigma, USA) was added at a dilution of 1:10000.

For the subtyping of anti-GroEL antibodies (serum after second booster), sera were serially diluted from 1:1000 to 1:512000 and added in triplicates and incubated for 1 h at RT. The plates were washed and incubated with rabbit anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Mouse monoclonal antibody isotyping reagents, Sigma, USA) as per manufacturer's instructions. After 1 h of incubation, five times washed plates were incubated with 100 μ l of o-phenylenediamine dihydrochloride (OPD; Sigma, USA) as HRP substrate for 10 min at RT and the reaction was stopped by 2N H₂SO₄. The absorbance was taken at 490 nm by ELISA reader (BioTek instruments, USA).

Statistical analysis

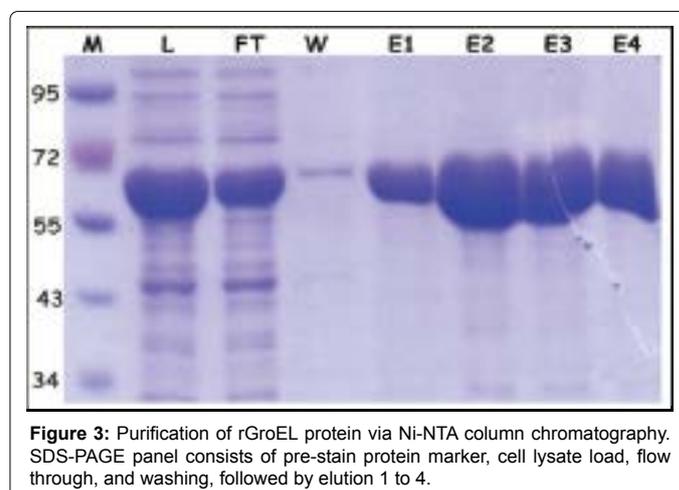
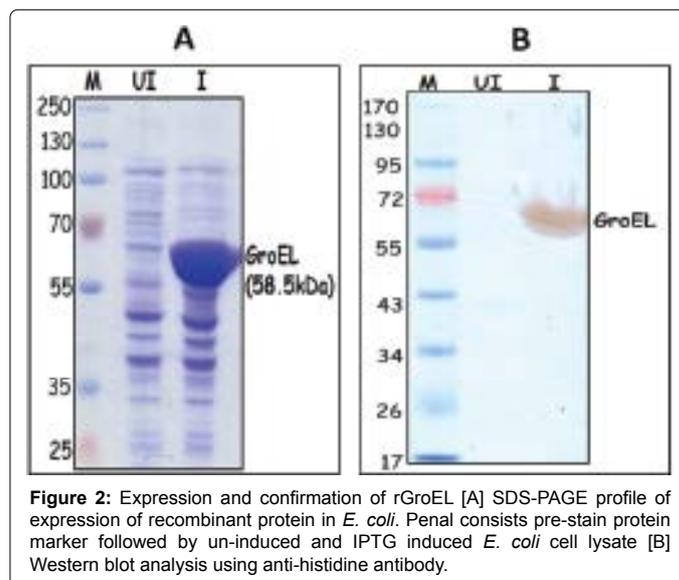
Statistical analysis of data and graphical representation was performed by GraphPad Prism 5 software. T-test method was used for statistical comparisons of data of different groups. Statistical significance was settled by the value of p (*p \leq 0.05, **p \leq 0.001).

Results and Discussion

Anthrax caused by *Bacillus Anthracis* is a highly devastating disease, which is mainly found in agrarian countries. *B. anthracis* is considered as a bioterrorism agent due to its aerosolization and dissemination properties. Besides, it poses challenges to public health system also in the form of cutaneous anthrax. Several types of post-exposure prophylactic therapies are recommended for anthrax treatment including antibiotics and antitoxins. However, for pre-exposure therapy, only AVA or AVP vaccines are available. *B. anthracis* causes pathogenesis by two factors viz; toxins and bacterial sepsis. Current licensed anthrax vaccines (AVA and AVP) are effective mainly against anthrax toxins but probably provides limited protection against *B. anthracis*. So, it is desirable to include a component in vaccine which could provide protection against spores and vegetative cells to restrict the anthrax infection. Spore antigens and vegetative components of *B. anthracis* enhance the protective efficacy of PA based subunit vaccines. In order to develop next generation subunit vaccines, several recombinant antigens viz. Lethal Factor, Edema Factor, Extractable Antigen 1, Poly-D-glutamic acid, and BclA of *B. anthracis* have been evaluated for their ability to enhance protective efficacy of PA based vaccines [23]. In this study, we cloned and expressed the rGroEL (HSP60) of *B. anthracis* in *E. coli* for its immunological characterization in mouse model.

To prepare recombinant antigen, PCR amplified GroEL gene of *B. anthracis* was cloned in pET28a+ vector (Figures 1B and 1C). The location of histidine tag and open reading frame of GroEL has been shown in Figure 1A. The expressed recombinant GroEL protein (~58 kDa) was analysed on SDS PAGE and by Western blot with anti-His antibodies (Figures 2A and 2B). The recombinant GroEL was purified from soluble fraction of cell lysate using IMAC affinity chromatography (Figure 3). The yield of purified GroEL was approximately 15 mg/L (Figure 4A). The LPS contents in purified protein, as determined by Limulus Amebocyte Lysate assay were below 5EU/ml. Purified rGroEL reacted with anti-Sterne serum on PVDF membrane (Figure 4B). Thus, the reactivity of rGroEL with anti-Sterne serum corroborated that it is a potent immunogenic antigens of *B. anthracis*. Therefore, the immunogenicity of rGroEL was evaluated in mouse model to determine the humoral immune response. Immunological characteristics of rGroEL could be important parameters to develop subunit vaccine either alone or in combination with other antigens of *B. anthracis* against anthrax.

Specific immunological response elicited by subunit vaccine candidates is necessary to provide protection against pathogens. Humoral and cellular immune response relies upon the IgG and its isotypes secreted by plasma B cells, which efficaciously neutralize toxins and opsonize the free extracellular pathogens [26-28]. Aluminium hydroxide adsorbed rGroEL was administered in mice via subcutaneous route and evaluated its immunogenicity. In order to determine the inductive anti-GroEL antibodies, mice sera were collected separately from all the individual immunized mice (Figure 4C). The cut-off value of IgG titer was calculated from the mice sera of control group as the mean OD plus 2SD at 1:200 dilutions. Anti-GroEL antibodies in individual mouse serum of immunized animals were measured on 7th days after first and second boosters. All immunized animals generated significantly high endpoint IgG titers. The endpoint titers of IgG antibody in GroEL immunized animals were observed up to 1:48000 (Log 4.74 \pm 0.230) and 1:170000 (Log 5.31 \pm 0.20) after first and second boosters (Figure 5A). Significant IgG endpoint titer elicited by GroEL indicated that GroEL antigen evokes strong humoral immune response. Among IgG subclasses, higher titers of IgG1 and IgG2b than the titers of IgG2a and IgG3 indicate the Th2 type responses to a particular antigen [28]. In the present study, the titre of IgG1 and IgG2b subtypes were dominating over IgG2a and IgG3 (Figure



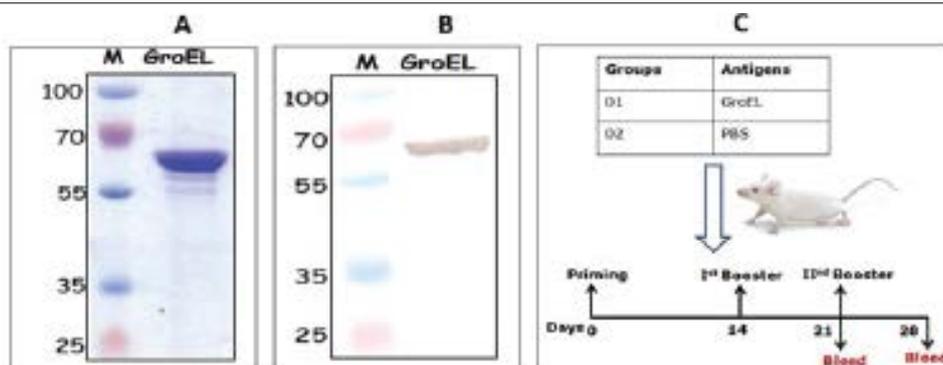


Figure 4: Purified rGroEL, reactivity with anti-sterne serum and animal schedule [A] SDS-PAGE analysis of IMAC purified rGroEL [B] Western blot analysis of purified rGroEL against anti-Sterne serum [C] Animal of Immunization groups and schedule for evaluation of humoral immune response.

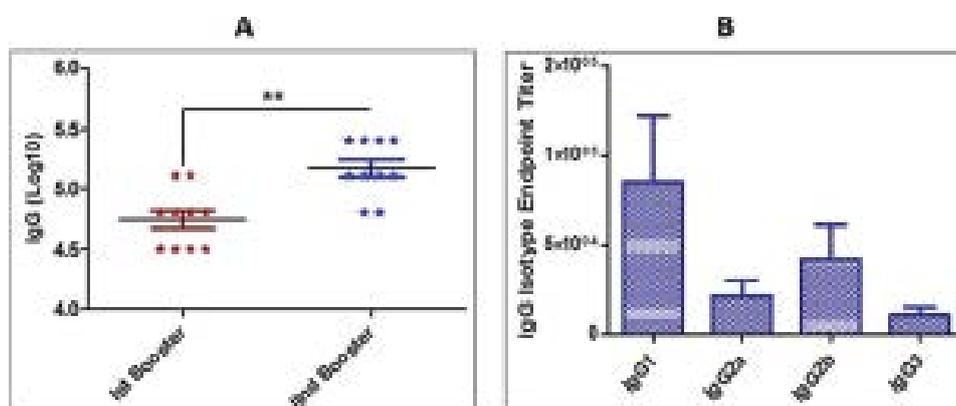


Figure 5: Determination of IgG and IgG isotypes antibodies elicited rGroEL antigen. Indirect ELISA based end point titers of IgG determined in each mouse of immunized groups and IgG isotyping in pooled sera [A] IgG endpoint titer [B] IgG isotypes endpoint titer.

5B). IgG antibodies tires and IgG sub-class prolife against GroEL has established Th2 type immune response, which may play an important role in stimulation and maintenance of B cells.

Conclusion

In conclusion, the humoral immune response elicited by rGroEL indicated that it is potent immunodominant antigen of *B. anthracis*. Therefore, the GroEL could be used further for evaluation as subunit vaccine candidate either alone or in combination with other immunodominant antigens of *B. anthracis* against anthrax.

Conflict of Interest

The authors declare that they have no conflict of interest.

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