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# Cloning and Expression of Heat Shock Protein 60 kDa Gene from *Brucella melitensis* as Subunit Vaccine

Research Article

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## ABSTRACT

Brucellosis is caused by the bacterium *Brucella* and affects various domestic and wild species. GroEL (Heat Shock Protein 60kDa) as one of the major antigens that stimulate the immune system, increases *Brucella* survival. The aim of the current study was to clone and express GroEL in *Escherichia coli* in order to design subunit vaccine. Amplifying was performed using specific primers. The full-length open reading frame of this gene was cloned into the expression vector pET-32a(+) and expressed in BL21 (DE3). The expressed antigen was purified and the molecular weight of the recombinant protein was about 70 kDa. Sequencing results along with SDS-PAGE and Western analysis confirmed the expression of recombinant GroEL in the heterologous *Escherichia coli*. The results of colony polymerase chain reaction (PCR), enzyme digestion and sequencing showed that the GroEL antigen has been successfully cloned and subcloned into pET-32a(+). The results showed that *Escherichia coli* was able to express GroEL protein appropriately. This protein was expressed by induction with isopropyl β -D-thiogalactoside (IPTG) at concentration of 1 m*M* and it was confirmed by Ni-NTA column, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting electrophoresis. The results of this study showed that *Escherichia coli* can be used as an appropriate host to produce the recombinant GroEL protein. This recombinant protein may be useful to simulate immune system, to produce recombinant vaccine and diagnostic kit in future studies after it passes biological activity tests *in vivo* in animal model and or other suitable procedure.

KEY WORDS expression, GroEL, immunogenicity, recombinant vaccine.

## INTRODUCTION

Brucellosis, a common zoonotic disease, concerns veterinarians as well as public health authorities in developing countries. Brucellosis is caused by *Brucella*, a gram negative, facultative intracellular, partially acid fast coccobacillus lacking capsule or flagellea (Pappas, 2010). This disease is characterized by abortion and reduced fertility in animals, and also by chronic infections with symptoms such as undulant fever, arthritis, and osteomyelitis in human (Pappas *et al*. 2006). The genus of *Brucella* consists of more than ten species which *B. abortus*, *B. melitensis* and *B. suis* cause most of the animal and human diseases. *B. melitensis* that mainly infects goats and sheep is considered as the most pathogenic species of *Brucella* to humans (Franco *et al*. 2007). Many researchers demonstrated that the correct identification of pathogens is critical for epidemiological studies and for the development of effective preventative measures, including vaccination (Ahsani *et al*. 2010a; Ahsani *et al*. 2010b; Ahsani *et al*. 2011; Zandi *et al*. 2014; Shahdadnejad *et al*. 2016). Vaccination against diseases is very important as the main prophylactic to reduce losses

due to the disease or minimize its severity (Ahsani *et al*. 2010a; Ahsani *et al*. 2010b; Ahsani *et al*. 2011; Zandi *et al*. 2014). In animals, immunization against *Brucella* infections is usually performed by administration of the live attenuated smooth *Brucella* strains like *B. abortus* S19, *B. melitensis* Rev.1, and non-smooth strain *B. abortus* RB51 (Corbel, 2006). However, live attenuated vaccines have the limitations of causing abortion in immunized pregnant animals, being pathogenic for humans, inducing resistant to antibiotics and interfering with the lipopolysaccharides-based serological tests (Cassataro *et al*. 2005). Up to now, there is no commercially available vaccine against human brucellosis and the disease is prevented by immunization of uninfected animals and elimination of the infected ones (Seleem *et al*. 2010). To develop a human *Brucella* vaccine, those *Brucella* proteins that exist in *Brucella* strains pathogenic to humans but absent in *Brucella* strains and not pathogenic to humans would be ideal for vaccine development (Golshani *et al*. 2015). GroEL gene encodes an inner membrane protein of *Brucella* with about 60 kDa molecular weight. This protein belongs to the heat shock protein family (HSPs) and can function as a chaperon, so it has an important role in the structure and folding of other proteins. *Brucella* produces a GroEL antigen in response to macrophage phagocytosis in order to increase its survival. GroEL is one of the major antigens that stimulate the immune system. Furthermore, this antigen has an important role in the disease cycle in humans and animals (Al Dahouk *et al*. 2007). In a comparative study on two different strains of *Brucella*, the GroEL antigen has been introduced as a good candidate for vaccine production and also for development of diagnostic kits (Amirmozafari *et al*. 2008). Polymerase chain reaction (PCR) is the most modern practical technology in diagnosing infectious diseases and compared with classical techniques, it has been shown to be more rapid, with results obtained in a few hours, and also more reliable (Ahsani *et al*. 2010a; Mohammadabadi *et al*. 2004; Mohammadabadi *et al*. 2011). Moreover, PCR allows a faster bacterial identification directly from clinical samples (Ahsani *et al*. 2010b). Genotyping, which is based on a more stable marker, DNA, is not dependent on gene expression. Another advantage of genotyping methods is that the discriminatory power of DNA-based methods is generally superior to that of phenotypic methods. The ability to distinguish between genomes is important to several disciplines of microbiological research, for example in studies on population genetics and microbial epidemiology (Ahsani *et al*. 2011; Zandi *et al*. 2014; Shahdadnejad *et al*. 2016) of great importance when choosing a method for genotyping are the typing ability, reproducibility, discriminatory power and also the ease and cost of performing the analysis. With PCR, selected segments of any DNA molecule can be amplified exponentially. The aim of the present study was to clone and express GroEL antigen from the *B. melitensis* strain *Rev 1*. Finally, the recombinant GroEL was purified to use as recombinant vaccine to stimulate the immune system in further studies.

## MATERIALS AND METHODS

### *Bacterial strains*, growth conditions and isolation

*B. melitensis* strain *Rev1* was obtained from the *Brucella* culture collection (Razi Institute, Mashhad, Iran) and was cultured as previously described (Delpino *et al*. 2007). DNA was extracted using a DNA extraction kit (Bioneer, Korea). The quality and quantity of the extracted DNA were analyzed by agarose gel electrophoresis and Nano-Drop ND-100 spectrophotometer (Thermo, USA). T/A cloning vector pTZ57R/T (Thermo, USA) were used for cloning and sequencing of the amplified gene. *E. coli* strain TOP10F` was used as host for cloning, sequencing and maintenance. The prokaryotic expression vector pET-32a(+) (Novagene, USA) and *E. coli*, BL21 (DE3) (Stratagene, USA) were used for rGroEL protein production.

### PCR amplification

*B. melitensis Rev1* genomic DNA was used as template for amplification full length open reading frame of GroEL gene using *EX Taq* DNA polymerase (Takara, Japan). The specific primers with restriction sites at the 5′ end were designed using primer Premier 5, according to the available nucleotide sequences in the NCBI GenBank database (Table 1). Polymerase chain reaction (PCR) was carried out by the Personal Cycler™ thermocycler (Biometra, Germany) with the reaction mixture containing 2.5 µL of 10X PCR buffer, 2 µL $MgCl_2$ and 2 µL dNTPs, 0.3 µL of the DNA solution (50 to 100 ng/µL), 1.5 µL of mix primer (5 pmol/µL) and 0.125 U/µL of *EX Taq* DNA polymerase, and deionized water up to final volume of 25 µL. The PCR program was performed with an initial denaturation step at 94 ˚C for 6 min followed by 27 cycles of denaturation at 94 ˚C for 30 sec, annealing at 58 ˚C for 30 sec and extension at 72 ˚C for 45 sec, and a final extension at 72 ˚C for 10 min.

### Cloning and nucleotide sequences analysis

The PCR products were purified from the agarose gel by Ron`s Agarose Gel Mini prep Kit (BioRon, Germany) according to the instructions provided by the manufacturer. The purified PCR products were ligated into pTZ57R/T cloning vector by T/A cloning strategy according to the manufacturer's instruction. Competent cell preparation and transformation steps were followed as described by Sambrook and Russell (2001).

**Table 1** The specific primers with restriction sites

| Primer sequences (5' → 3') | Restriction enzyme | Length (bp) |
|---|---|---|
| F:5`-A**CCATGG**ATGGCTGCAAAAGACGTAAAATTCG-3` | *NcoI* | 1641 |
| R:5`-A**GAGCTC**TTATTAGAAGTCCATGCCGCCCATGC-3` | *SacI* | |

The recombinant vectors were transformed into competent *E. coli* TOP10F`. The bacterial clones harbouring recombinant plasmid DNA were screened based on their ampicillin resistance. The fidelity of *E. coli* TOP10 F` transformants was verified by PCR reaction using M13 universal primers. The plasmids were purified using the Ron`s Plasmid Mini Kit (BioRon, Germany) and were confirmed by restriction sites enzyme digestion and sequencing (Bioneer, South Korea).

In order to sub-clone the recombinant GroEL into the expression vector, recombinant plasmid was subjected to digestion with *NcoI* and *SacI* restriction enzymes (Thermo, USA). After digestion, the digestion product was electrophoresed on low melting agarose gel 0.8% and then purified. The purified digested product was cloned into the expression vector pET-32a(+) in which the recombinant protein is included a six-Histidine tag (His-tag) at the N-terminal end for easier purification. Standard techniques for these steps such as ligation, competent cell preparation and transformation were followed as previously described (Sambrook and Russell, 2001). Recombinant vectors were transformed into competent *E. coli* BL21 (DE3). The recombinant clones harbouring plasmid DNA with inserts were screened based on their ampicillin resistance. The fidelity of *E. coli* BL21 (DE3) transformants was verified by PCR reaction using T7 universal primers. Recombinant plasmids were purified using the Ron`s Plasmid Mini Kit (BioRon, Germany) and confirmed by restriction enzyme digestion. Purified plasmids were subjected to sequencing (Bioneer, South Korea).

**Expression, confirmation, and purification**

The positive recombinant construct was cultured in LB ampicillin medium. Protein synthesis was induced using 0.1 m*M* isopropyl β -D-thiogalactoside (IPTG) in recombinant bacterial culture with OD 600. Bacteria were incubated for 5 h at 37 ˚C then harvested by centrifugation (3000 g, 20 min, 4 ˚C) and stored at -80 ˚C. The pellet from a 100 mL bacterial culture was suspended in lysis buffer (Tris 50 mM, EDTA 5.0 m*M*, urea 8.0 M, pH=8.0) and was lysate with sonication. Lysed cells were centrifuged at 9000 g for 15 min at 4 ˚C to separate the supernatant containing soluble materials from the pellet. Both the supernatant and the pellet were evaluated on SDS-PAGE 10% to analysis the expression of rGroEL.

Expressed protein was purified by chromatography through Ni-agarose (Thermo, USA) from insoluble phase of lysate using guanidine hydrochloride 6 M to dissolve the pellet according to the instructions provided by the manufacturer.

The quality and identity of the purified rGroEL protein were analysed by SDS-PAGE (10%) and western blotting assay. For western blotting, the SDS-PAGE gels were electro blotted onto nitrocellulose. The blotted nitrocellulose was then blocked with skim milk for 3 h. The membranes were washed three times and then Anti Poly-Histidine-HRP (Sigma) (1:2000 diluted in BSA 1%) was added. After 1 h incubation at room temperature and washing, diaminobenzidine (DAB) as chromogen was employed for visualization. Finally, the quantity of the recombinant protein was estimated using Bradford assay. The purified rGroEL protein was stored at -20 ˚C for further evaluation of immunogenicity and protective efficacy in mice.

## RESULTS AND DISCUSSION

**PCR amplification and cloning**

The GroEL gene from *B. melitensis Rev1* was amplified and the accuracy of this fragment was visualized on agarose gel electrophoresis (Figure 1).

Consequently, the amplified products were successfully ligated into cloning vector and transformed into competent *E. coli* TOP10F` cells. After screening positive colonies using colony-PCR, the integrity of the recombinant plasmids was confirmed by restriction digestion.

**Expression and purification of recombinant protein**

Expression of the rGroEL gene in *E. coli* cells bearing recombinant plasmids was analyzed by SDS-PAGE and Western blotting (Figure 2). The best induction time was reached by selecting the induced bacteria samples at time T1, T2 and T3 and the highest expressing colony was in T3 (Figure 2.A). Ni-agarose chromatography was used to achieve proteins purification and the quality of purified protein was confirmed by SDS-PAGE and Western blotting with the Anti Poly-Histidine-HRP.

Recently, many efforts have been made to identify new immunogens in *Brucella* using immune approaches. However, not all of these new targets showed *in vivo* protective efficacy (Yang *et al.* 2013). Resistance to facultative intracellular bacterial pathogens mainly depends on acquired cell-mediated resistance which activates specific T lymphocytes followed by macrophages activation for increasing killing of such organisms (Zhan *et al.* 1996).

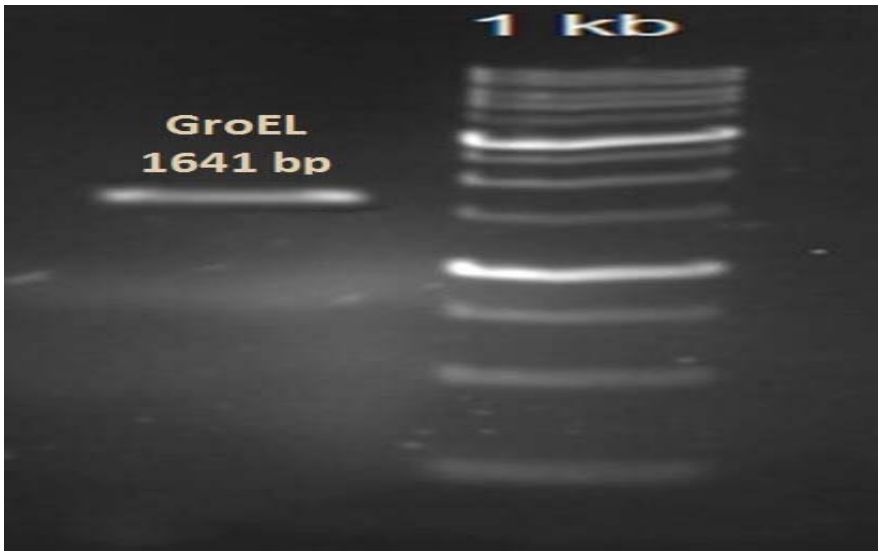


**Figure 1** PCR products of GroEL gene with 1641 bp length

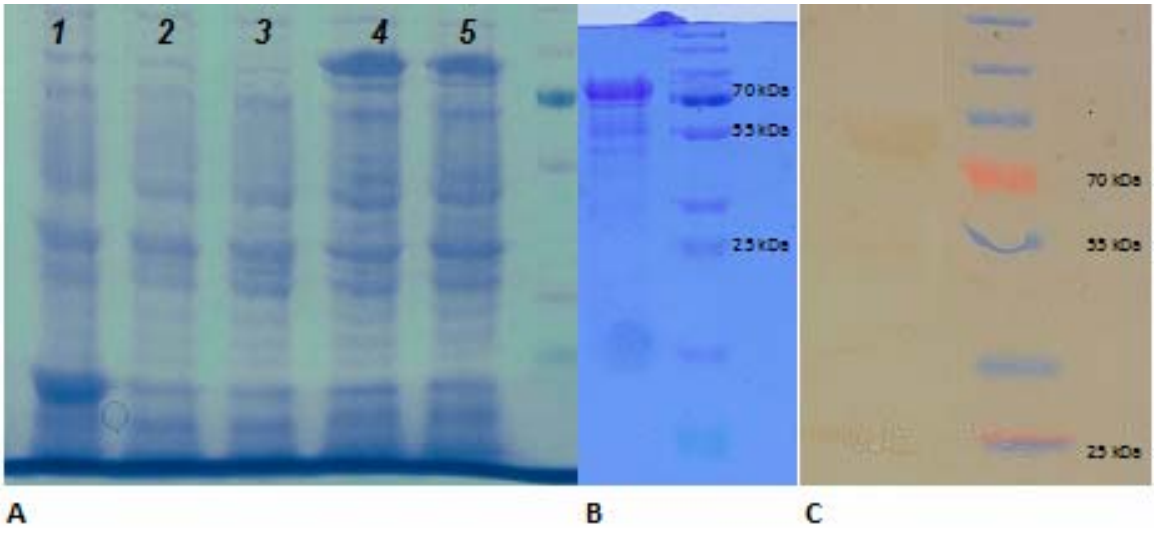


**Figure 2** **A**: SDS-PAGE analysis of the recombinant protein
Line 1: pET-32a as negative control; 2: total cell lysate of *E. coli* BL21 (DE3) containing pET32a-GroEL showing the expression of before induction; Line 3, 4 and 5: total cell lysate of *E. coli* BL21(DE3) containing pET-32a- GroEL showing the expression in different time point (after 1, 2, and 4 hours) by IPTG
**B**: purified protein
**C**: Western blotting profile of the rGroEL protein
Pre-stained protein marker (Thermo, USA) with 9 bands

An important protection activity inducer in monocytes and macrophages is the interferon gamma (Ritchie *et al.* 2012). Th1 CD4 + T cells which producing interferon gamma are responsible for macrophage activation and attraction of inflammatory effector cells, and also play a role in acquired cellular resistance. Identification and characterization of specific antigens that induce preferentially a Th1 subset response would be important for the development of protective recombinant vaccines against *Brucella*. HSPs are major antigens which eliciting humoral and cell-mediated immune responses against different bacterial pathogens (Bae and Toth, 2000).

GroEL as a common heat-shock protein present in different type of pathogens induces humoral and cellular immune responses in host models (Silva, 1999). In this study, GroEL gene as a dominant *B. melitensis Rev l* antigen was candidate for cloning and expression in order to use as efficient subunit vaccine.

Due to the importance of high-level production of recombinant protein in immunological studies, the fusion was cloned in pET-32a(+) expression vector. The pET is the most powerful system has so far been developed for the cloning and expression of the recombinant proteins in *E. coli*.

The pET32 series is designed for cloning and high-level expression of peptide sequences fused with the 109aa Trx•Tag™ thioredoxin protein. Cloning sites are available for producing fusion proteins also containing cleavable His•Tag® and S•Tag™ sequences for detection and purification. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals where expression is induced by providing a source of T7 RNA polymerase in the host cells. T7 RNA polymerase is so active that when fully induced, almost all of the cell's resources are converted to the target gene expression (LaVallie *et al*. 1993). Sequencing of the cloning product confirmed the integrity of the cloning. The plasmid construct pET-32a-GroEL was transferred into *E. coli* BL21 (DE3) containing T7 RNA polymerase and the expression was induced by the addition of IPTG. Successfully induced expression by different concentration of IPTG and high level production of the fusion demonstrated the high efficiency of our fusion construct.

## CONCLUSION

Brucellosis is a common zoonotic disease that can infect domestic animals and till today there is no recombinant vaccine for it. Protein recombinant vaccines are feasible than live or poor bacterial vaccine because it can be produced in large scale and also are safe for recipient host. Therefore, the aim of the present study was to clone and expression of one candidate *B. melitentis* antigen GroEL in order to designing suitable recombinant vaccine**.** In order to produce rGroEL the cloning and expression were done successfully and results of sequencing and also SDS-PAGE and Western blotting confirmed rGroEL production. The evaluation of humoral and cellular immune responses of this antigen against *Brucella melitensis* infection in mice has already initiated in our laboratory.

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