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Original Article

Cloning, expression and molecular analysis of Iranian *Brucella melitensis* Omp25 gene for designing a subunit vaccine

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Abstract

Brucellosis is a well-known domestic animal infectious disease, which is caused by Brucella bacterium. The outer membrane protein 25 kDa (Omp25) gene plays an important role in simulating of TNF-α, IFN-α, macrophage, and cytokines cells. In the current study molecular cloning and expression analysis of Omp25 gene for designing a subunit vaccine against Brucella was investigated. Amplifying the full length of candidate gene was performed using specific primers. Sub-cloning of this gene conducted using pTZ57R/T vector in TOP10F`strain of *Escherichia coli(E.coli)* as the host. Also, pET32(a)$^+$ vector used for expression in BL21 (DE3) strain of *E.coli*. Omp25 gene with 642 bp size was amplified and cloned successfully. The expression results were confirmed by sequencing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses which showed 42 kDa protein band correctly. Also, phylogenic analysis showed this gene has a near genetic relation with other Brucella strains. According to our results we can propose this gene as a candidate useful for stimulation of cell-mediated and humoral immunity system in future study.

Keywords: *Brucella melitensis* Rev1; Omp25; Phylogenic analysis; Gene expression

INTRODUCTION

Brucellosis is a well-known domestic animal infection which survives within a broad range of eukaryotic cells as a small gram-negative coccobacillus. 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 humans (1,2). The genus of Brucella consists of more than ten species, *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 (3). 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 (4). 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 (5). 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 (6). 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 (7). Therefore, Brucella outer membrane proteins (OMPs) are cell specific surface antigens which have remarkable immunogenicity characteristics. OMPs are excellent candidates in production of recombinant vaccines against Brucellosis (8,9). These cell surface antigens are classified into two main groups consisting of OMP2a and OMP2b, and OMP25 and OMP31 (9). OMP25 antigen is one of the virulent factors and the major antigen involved in survival of Brucella and found to be highly conserved among different Brucella species.

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Brucella species lacking Omp25 have been shown to be attenuated in mice as well as cattle (10).

Also, Omp25 has been shown to inhibit TNF-α (is an important component of the cytotoxic immune response) production in *B. suis* infected human macrophages (11,12).

The objectives of the present study were cloning, expression and molecular analysis of Omp25 gene for designing subunit vaccine against Brucella infectious disease.

MATERIALS AND METHODS

Bacterial strains, growth conditions and Isolation

B. melitensis strain Rev 1 was obtained from the Brucella culture collection (Razi Institute, Mashhad, Iran) and cultured as described (13). DNA was extracted using a DNA extraction kit (Bioneer, Korea). The quality and purity of the extracted DNA were analyzed by agarose gel electrophoresis and NanoDrop ND-100 spectrophotometer (Thermo, USA), respectively. *Escherichia coli (E. coli)* strain TOP10 F` was used as host for gene cloning, sequencing and maintenance of different DNA fragments. T/A cloning vector pTZ57R/T (Thermo, USA) was used for cloning and sequencing of the amplified gene.

PCR amplification

B. melitensis Rev1 genomic DNA was used as template for amplification of full length open reading frame of Omp25 (642 bp) gene using *EX Taq* DNA polymerase (Takara, Japan). The specific primers were designed according to the *B. melitensis* 16M as template using Primer Premier 5, according to the available nucleotide sequences on the NCBI GenBank database (Table 1).

Polymerase chain reaction (PCR) was carried out using the Personal Cycler™ thermo cycler (Biometra, Germany) with the reaction mixture containing 2.5 µL of 10× PCR buffer, 2 µL $MgCl_2$ 10 mM, and 2 µL dNTPs 10 mM, 0.5 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 some deionized water up to a final volume of 25 µL. The PCR program was performed with an initial denaturation step at 94 °C for 6 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 62 °C for 30 sec and extension at 72 °C for 45 sec, and a final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose gel and visualized by Gel Red (Biotium, England).

Cloning and subcloning

PCR products were purified from the agarose gel by Ron`s Agarose Gel Mini Prep Kit (BioRon, Germany) according to the manufacturer's instruction. The purified PCR products were ligated into pTZ57R/T cloning vector by T/A cloning strategy according to the manufacturer's instructions (Thermo, USA). Competent cell preparation and transformation steps were performed (13). Recombinant vectors were transformed into competent *E. coli* TOP10F`. The recombinant vectors were transformed into competent *E. coli* Top10F` cells. The bacterial clones harboring recombinant plasmid DNA were screened based on their colony PCR and restriction sites enzyme digestion. PCR was used for verification fidelity of *E. coli* Top10F` transformants. The plasmids were purified using the Ron`s Plasmid Mini Kit (BioRon, Germany) and confirmed by restriction enzyme digestion. The purified plasmids were subjected to sequencing (Bioneer, South Korea).

In order to subcloning recombinant Omp25 in to expression vector ($pET32a^+$), recombinant plasmids which purified using the Ron`s Plasmid Mini Kit (BioRon, Germany) and confirmed by restriction enzyme digestion and sequencing was subjected to digestion with *Nco* I and *EcoR* I restriction enzymes (Thermo, USA). After digestion, the product was electrophoresed on low melting agarose gel 1% and then purified.

Table 1. The specific primers with restriction sites

Gene	Primer sequences	Restriction enzyme	Length (bp)
Omp25	F: 5`- *CCATGG*ATGCGCACTCTTC -3`	*NcoI*	
	R:5`- *GAATTC*GAACTTGTAGCCGATGCC -3	*EcoRI*	642

The purified digested product was cloned into the expression vector in which the recombinant protein includes a six-Histidine tag (His-tag) at the C-terminal end for easier purification. Standard techniques for these steps such as ligation, competent cell preparation and transformation were followed, as described previously (14). Recombinant vectors were transformed into competent *E.coli* BL21 (DE3). The recombinant clone(s) harboring 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 and purification

For expression of Omp25, the positive pET32(a)$^{+}$ Omp25 construct was cultured on LB ampicillin medium. Protein synthesis was induced with 0.1 mM IPTG (isopropyl β -D-thiogalactoside) in a culture of bacteria with an OD = 0.6. 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 mM, urea 8.0 M, pH = 8.0) and lysed by sonication. Cell lysate was subjected to centrifugation 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 10% to analyse the expression of rOmp25.

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

Quality and identity of the purified rOmp25 protein was analyzed by SDS-PAGE (10%) and western blotting assay, respectively. 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 rOmp25 protein was stored at -20 °C for further evaluation of immunogenicity and protective efficacy in mice.

Phylogenic analysis

As we mentioned in our previous study (14), the obtained sequence of Omp25 gene was analyzed by homology search and aligned with other reported Omp25 gene species using Basic Local Alignment Search Tool (BLAST) and CLC Main workbench 5.5 software (CLC bio), respectively. Phylogenic tree was drawn using Neighbor-Joining procedure of MEGA5.1 software with 1000 repetitions and genetic distance determined using Create Pairwise Comparison procedure of CLC Main workbench 5.5 software**.**

RESULTS

Amplification, cloning and nucleotide acid sequencing of OMP25 gene

The Omp25 gene with 642 bp was amplified and the accuracy of this fragment visualized on agarose gel electrophoresis (Fig. 1). Amplified products were successfully ligated into pTZ57R/T cloning vector and transformed into competent *E. coli* TOP10F` cells. After selection of positive colonies using Colony-PCR, the integrity of the recombinant plasmids were confirmed by restriction enzyme digestion (*Nco* I and *EcoR* I). The sequencing of pET32a-Omp25 performed with specific primers as well as universal T7 and T terminator primers (appearance of 1354 bp band confirmed the appropriate cloning).

Expression and purification of recombinant protein

The expression of Omp25 recombinant protein was induced with 0.1, 0.2 and 0.4 mM IPTG. The results of induction with different

concentrations of IPTG were the same. Further, rOmp25 protein was expressed with 0.1 mM IPTG at OD = 0.6 for 4 h at 37 °C. Expected rOmp25 protein size of 42 kDa (Omp25 with 25 kDa and Pet32 with 17 kDa) was detected after induction of the culture with IPTG and most of it was found to be localized inside the inclusion bodies in the cells, after sonication. Purification of the rOmp25 was performed by Ni-NTA affinity chromatography using denaturing method. SDS-PAGE (10%) analyzing of the lysate from the induced *E. coli* BL21 (Fig. 2A) and the purified protein (Fig. 2B) revealed the expected recombinant protein with the molecular mass of approximately 42 kDa. The expression yield of the purified protein was estimated by Bradford assay to be 220 µg/mL. Western blotting with antibodies revealed the specific reactivity with purified rOmp25 produced in *E. coli* cells and showed the functional expression in the prokaryotic system (Fig. 2C).

Phylogenic analysis

In our previous study (14), we found that the Omp25 gene sequence was found to be identical with the *B. melitensis* 16 M and had lower similarity observed between Omp25 of *B. melitensis* Rev1 and *B. canis* (Table 2).

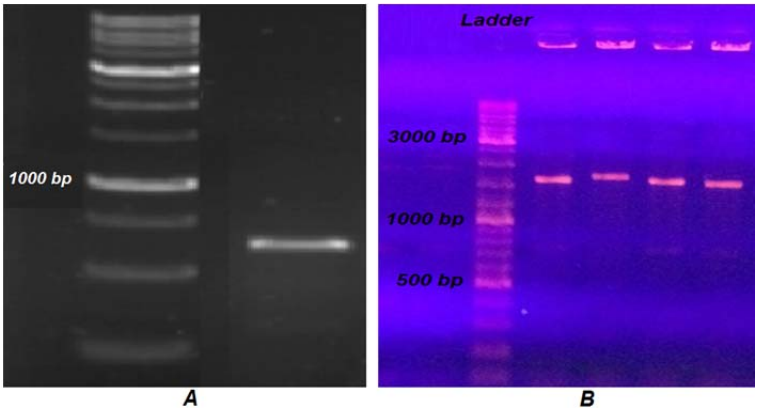

Fig. 1. (A) Electrophoresis of PCR products on agarose gel 1%, (B) the insertion of Omp25 gene fragment into pET32(a)$^{+}$ was confirmed using colony PCR.

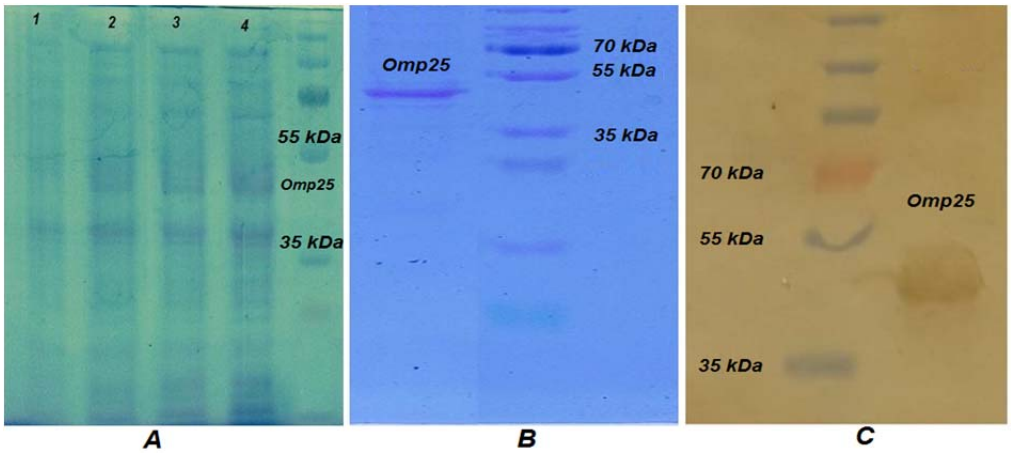

Fig. 2. (A) SDS-PAGE analysis of the recombinant protein. Line 1: total cell lysate of *E. coli* BL21(DE3) showing the expression of before induce; Lines 2, 3, 4: total cell lysate of *E. coli* BL21(DE3) containing pET32(a)$^{+}$ Omp25 which showing the expression after induce IPTG. (B) Purified protein with the molecular mass of around 42 kDa. (C) Western blotting profile of the rOmp25 protein. Ladder is a pre-stained protein marker size (Thermo, USA).

Table 2: Pairwise comparison between candidate gene and other Brucella species. Upper triangle shows percent identity and down triangle shows nucleotides differences in each matrix. Omp25 refer to Omp25 gene of *B.melitensis Rev1*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Omp25	**1**		99.38	99.38	99.22	99.53	99.53	99.38	99.38	99.07	99.84	99.84	99.84	99.84	99.84	100.0
B.suis bv, 1 str S2,CP006961.1	**2**	4		100.00	99.84	99.84	99.84	99.69	99.69	99.38	99.22	99.22	99.22	99.22	99.22	99.38
B.suis VB122,CP003128.1	**3**	4	0		99.84	99.84	99.84	99.69	99.69	99.38	99.22	99.22	99.22	99.22	99.22	99.38
B.canis strCliveri,HG803175.1	**4**	5	1	1		99.69	99.69	99.53	99.53	99.22	99.07	99.07	99.07	99.07	99.07	99.22
B.pinnipedialis B2/94,CP002078.1	**5**	5	1	1	2		100.00	99.84	99.84	99.53	99.38	99.38	99.38	99.38	99.38	99.53
B.suis ATCC23445,CP000911.1	**6**	5	1	1	2	0		99.84	99.84	99.53	99.38	99.38	99.38	99.38	99.38	99.53
B.abortus A13334,CP003176.1	**7**	4	2	2	3	1	1		100.00	99.69	99.22	99.22	99.22	99.22	99.22	99.38
B.abortus S19,CP000877.1	**8**	4	2	2	3	1	1	0		99.69	99.22	99.22	99.22	99.22	99.22	99.38
B.abortus X79284.1	**9**	6	4	4	4	3	3	2	2		98.91	98.91	98.91	98.91	98.91	99.07
B.melitensis str 0331.JX627633.1	**10**	1	5	5	6	4	4	5	5	7		100.00	100.00	100.00	100.00	99.84
B.melitensis Nl, CP002931.1	**11**	1	5	5	6	4	4	5	5	7	0		100.00	100.00	100.00	99.84
B.melitensis M5-90, CP001851.1	**12**	1	5	5	6	4	4	5	5	7	0	0		100.00	100.00	99.84
B.melitensis M28, CP002459.1	**13**	1	5	5	6	4	4	5	5	7	0	0	0		100.00	99.84
B.melitensis ATCC 23457, CP001488.1	**14**	1	5	5	6	4	4	5	5	7	0	0	0	0		99.84
B.melitensis bv,1 str 16M AE005917.1	**15**	0	4	4	50	3	3	4	4	6	1	1	1	1	1	

DISCUSSION

Recently many efforts have been made to identify new immunogens in Brucella proteome using immune proteomic approaches. Selection and production of new vaccine candidates are the primary practical steps toward introducing new vaccines. Major OMPs of Brucella species have been defined as immunogenic and protective antigens (15). Omp25 is one such major OMP of Brucella *spp.* Omp25 has been shown to be involved in virulence, as *B. abortus* is attenuated when infected with *B. abortus* Omp25 mutant (9). Omp25 has been shown to inhibit TNF-α production in *B. suis* infected human macrophages (11). There are reports which have shown that DNA vaccine of Omp25 of *B. meltensis* is protective against the virulent *B. melitensis* challenge in mice (16)**.** In this study, Omp25 gene as a dominant *B.melitensis* Rev 1 antigen was candidate for cloning, expression and molecular analysis as a first step in order to design a recombinant vaccine against *B.melitensis* Rev1.

Due to the importance of high level production of recombinant protein in immunological studies, the fusion was cloned in pET32(a)$^{+}$ 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 available for producing fusion proteins also containi 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 (17).

Sequencing of the cloning product confirmed the integrity of the cloning. The plasmid construct pET32(a)$^{+}$ Omp25 was transformed 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. In addition, results indicated that multiple sequence alignment of different Brucella Omp25 sequences shows the protein is highly conserved among Brucella pathogens (99% - 100% sequence similarity) indicating that it could be a potential vaccine target against Brucella infection. The major difference was seen in Omp25 gene of *B. ovis* and *B. melitensis.* There was a short deletion of 36 nucleotides at 3' end of gene in *B. ovis* and absences of *EcoR* V site in *B. melitensis* (18).

CONCLUSION

The aim of the present study was to clone, express and molecularly analyze one candidate *B. melitentis* antigen Omp25 in order to design a suitable recombinant vaccine**.** In order to produce rOmp25 the cloning and expression were done successfully and results of sequencing and also SDS-PAGE and western blotting confirmed our rOmp25. In addition, phylogenetic analysis showed that selected gene was nearly similar in different Brucella species. The evaluation of humoral and cellular immune responses of this antigen against Brucella *melitensis* infection in mice has already been initiated in our laboratory.

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