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Cloning and Expression of *Brucella abortus* Omp19 an Immunogenic Minor Outer Membrane Protein

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***Brucella*e species are important bacterial zoonotic pathogens which cause brucellosis, an infection with a worldwide distribution. Prevention and diagnosis of infected cases are challenging researchers. Surface proteins are valuable antigens as they are most exposed to the host immune system. Many of outer membrane proteins are expected to be capable of eliciting immune response; one of them is an 18kDa immunogenic protein (Omp19). The aim of present study is producing recombinant Omp19 in a way to prevent brucellosis. Using Gene Runner software to design primers. Omp19 amplified by PCR method. After purification, pJET1.2 and pET28a (+) were used as cloning and expression vectors respectively. *Escherichia coli* $DH_5\alpha$ had the role of unexpression host with usual aim of cloning. BamHI and HindIII were applied as restriction enzymes and *E. coli* BL21 (DE3) as expression host. Recombinant Omp19 devoid of other *Brucella* antigens will allow determination of their potential protective activity against brucellosis.**

Vaccination against animal brucellosis is usually performed by using living attenuated Brucella strains. These vaccines have numerous disadvantages due to basic infections in both animals and mankind. They also elicit antibodies against the Lipopolysaccharide (LPS) which interfere in the differential diagnosis between vaccinated and infected animals. So, accessing to the method or technique to improve new vaccines is an aim in brucellosis investigation. Production of recombinant 18kDa outer membrane lipoprotein of Brucella abortus (Omp19) gained a new vision for preventional researches. Sequencing results of the cloned plasmid vector confirmed the cloning procedure. Gene fragment was subsequently subcloned in expression system pET28a(+). Expression of recombinant protein was induced by adding 1mM IPTG to the growing culture of OD 0.6. Cloning and expression of recombinant 18kDa outer membrane lipoprotein of *Brucella abortus* (Omp19) allow the evaluation of the potential protective activity and the possibility for the development of subunit vaccines in further investigations.

Key words: Brucellosis, *Brucella abortus*, Omp19, Cloning, Expression.

*Brucella*e species are gram-negative, facultative intracellular bacteria that can survive within host cells causing a chronic infectious disease in a lot of animal species and humans. The most important *Brucella* species are *B. abortus* and *B. melitens*is. *Brucella abortus* is a global zoonotic agent that primarily infects cattle and causes brucellosis[1,2]. *Brucella* infection can also lead to reduced fertility,

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weight loss and reduced milk yield showing the economic importance of *Brucella*. Brucellosis remains endemic in many developing countries including India.and Iran[3]. It has been nearly eradicated from livestock following control plans[4]. Human brucellosis is known to have nonspecific manifestations such as undulant fever, osteomyelitis, and arthritis[5,6]. The immunodominant antigen of smooth *Brucellae* is LPS, the principle agent of sero diagnosis of the infection[7]. Live attenuated vaccines are licensed to prevent animal brucellosis. They give short term immunity and interfere with serological tests, also there is an ever being problem with immunoglobulins to *Brucella* LPS which cross react with that of certain Gram negative bacteria. These general vaccines are the sole choices which are available now[8-10]. There is no safe brucellosis human vaccine[11-13]. The specific properties of *Brucella* outer membrane seem to be vital to *Brucella* virulence. OMPs are another surface proteins acting as protective antigens. OMPs categorize according to apparent molecular mass, 88-94kDa, 36-38kDa and 25-27kDa. They are resistant to nonionic detergents and EDTA. In addition, there are several minor OMPs with low molecular weight in *B. abortus* (Omp19, Omp16.5 and Omp10) [14,15]. *Brucellae* antigens are of great importance for vaccine design, diagnostic or pharmaceutical objectives. A proper vaccine candidate is an 18-kDa immunoreactive lipoprotein (Omp19)[16]. This outer membrane protein has structural features of bacterial lipoprotein presents in all eight Brucella species and their biovars. Cloning and Expression of recombinant 18kDa outer membrane lipoprotein of *Brucella abortus* (Omp19) may promote the future investigations in brucella vaccines. In this study, we report the cloning, sequencing and expression of the gene coding for *B. abortus* rOmp19 which is anticipated to show a protective activity.

MATERIAL AND METHODS

Bacterial strains, growth condition and plasmid vectors

B. abortus S19 was routinely cultured on *Brucella* agar and incubated in 37°C for 72 hours. pJET1.2 (Fermentas) and pET28a(+) (Novagen) were applied as cloning and expression vectors respectively. *Escherichia coli* $DH_5\alpha$ and *E. coli* BL21(DE3) were used as prokaryotic hosts for cloning and expression respectively.

Primers design

NCBI site (ACCESSION U35742) used to access the sequence of the gene coding for *Brucella abortus* Omp19 using Gene Runner software to design sense and anti sense sequences. A primer pair was designed to amplify the whole coding region of Omp19 consisting of FB18F 5'-AACGGATCCATGGGAATTTCAAAAG CAAGTCTGCTC-3'as forward and FB18R 5'-AAAAAGCTTTCAGCCCAACAGCGTCAC GGCCTGC-3' as reverse including restriction sequences of *BamHI* and *HindIII* respectively. They were designed based on nucleotide target sequence of Omp19

PCR

The polymerase chain reaction (PCR) was done with *Taq* DNA polymerase including Denaturation: at 95°Cfor 30s, Annealing at 61?C for 45s, Extention at72°C for 45s, Final Extention at 72°C for 10min. 61°C was considered as optimum gradiant of annealing tempreture with *Taq* DNA polymerase. Omp19 complete ORF amplified by PrimSTAR® HS DNA polymerase. (TaKaRa). Reaction mixtures of 50 µl contained 10µl 5xPrimSTAR™Buffer, 0.2mM of each dNTP Mixture, 0.2 µM of each primer, 50ng genomic DNA, and 1.25U PrimSTAR® HS DNA polymerase, up to 50µl sterilized distilled water.

Cloning of the gene encoding the 18-kDa protein in a PET28a (+) vector

PrimSTAR® HS DNA polymerase product purified by AccuPrep®GEL Purification Kit (Cat.No. K-3035,k-3035-1)(Bioneer) and The amplified Omp19 gene was cloned in pJET1.2/blunt cloning vector. 20µl total volume contained T4 DNA ligase 1µl, 2x reaction buffer 10µl, PCR product 1-2µl, pJET1.2 1µl and water, nuclease-free up to19µl, using clonJET® PCR cloning Kit (fermentas). Vertification of recombinant pJET1.2 was performed by screening via colony PCR with pJET1.2 specific primers . pJET1.2 containing the gene coding for *B. abortus* Omp19 was double digested with BamHI and HindIII and vertified by sequencing and aligned with refrence sequence. DNA fragments purified by gel and subcloned in pET28a(+) between BamHI and HindIII sites.

Recombinant pET28a(+) in the right orientation for expression from the lac promoter was used to transfer *E. coli* BL21(DE3) competent cells. Recombinant clones were grown in LB medium with kanamycin at 37°C, to reach an OD 0.6. At this point the culture was induced by 1M IPTG and incubated for 3 h at 37°C . This part describes the expression of the 18-kDa protein of *Brucella abortus* in a recombinant form.

RESULTS

The 18-kDa protein of *Brucella abortus* was successfully cloned in pJET1.2. Alignment of recombinant pJET1.2 sequencing results with reference sequence showed complete identity and no mutation in cloned ORF. omp19 was expressed in the PET28a(+) vector as inclusion bodies in BL2 1 (DE3). PCR and anzymatic digestion assured the accuracy of procedure. *E. coli* BL2 (DE3) containing the plasmid encoding the 18-kDa protein were grown and induced to express recombinant protein. Expression vector and expression host which applied, were suitable for expressing high levels of protein. SDS-PAGE analysis of induced culture samples compared to non-induced ones and non-transformed *E. coli* BL21(DE3) showed the expression of a protein of approximately 20kDa. This was consistent with our expected protein of 17.53 kDa plus pET28a(+) added amino acids (Fig. 3). The applied method, offers this lipoprotein in a recognizable level to immune system.

ORIGIN·····

·····1·ctgcagtcat·gccccagaac·aaacgccaat·atcggggaaa·ctgtggcctt·cctgtgccag

·····61·tttcagaatt·atcaggtggt·ttcaagcatt·cttgaagggc·tttggaggca·gaaatgaaag

·····121·gtgctggtgt·tgctttttta·accatatagc·cattttatcg·gtccaatata·tcatattgca

·····181·tggcttcggc·atgtcgcccg·gggcgcgccc·ccacccctga·tgagaaatgc·gcaaatggag

·····241·aacctgatgg·gaatttcaaa·agcaagtctg·ctcagcctcg·cggcggctgg·cattgtcctg

·····301·gccgggtgcc·agagctcccg·gcttggtaat·ctcgataatg·tttcgcctcc·gccgccgcct

·····361·gcaccggtca·atgctgttcc·ggcaggcacg·gtgcagaaag·gcaatcttga·ttctcccaca

·····421·caattcccca·atgcgccctc·cacggatatg·agcgcgcaat·ccggcacaca·ggtcgcaagc

·····481·ctgccgcctg·catccgcacc·ggacctgacg·cccggcgccg·tggctggcgt·ctggaacgcc

·····541·tcgcttggtg·gtcagagctg·caagatcgcg·acgccgcaga·ccaaatatgg·ccagggctat

·····601·cgcgcaggcc·cgctgcgctg·ccccggtgaa·ctggctaatc·ttgcctcctg·ggccgtcaat

·····661·ggcaagcaac·tcgtccttta·cgatgcgaac·ggcggtacgg·ttgcctcgct·ctattcttca

·····721·ggacagggcc·gcttcgatgg·ccagaccacc·ggcgggcagg·ccgtgacgct·gttgggctga

·····781·tctgacccgg·aaacgatgac¶

Fig. 1. *Brucella abortus* 18 kDa immunoreactive antigen gene, complete cds.
The Starting and ending codons are ATG and TGA which produce 18 kDa immunoreactive antigen.
The whole jene is used to design for cloning. BamHI designed on the first primer and
Hind III on the second primer, before ATG and after TGA respectively

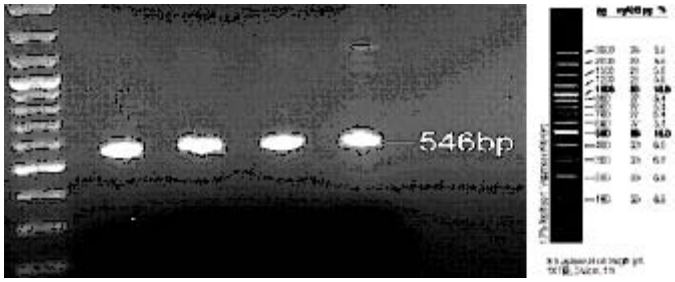

Fig. 2. PCR product with PrimSTAR® HS DNA polymerase

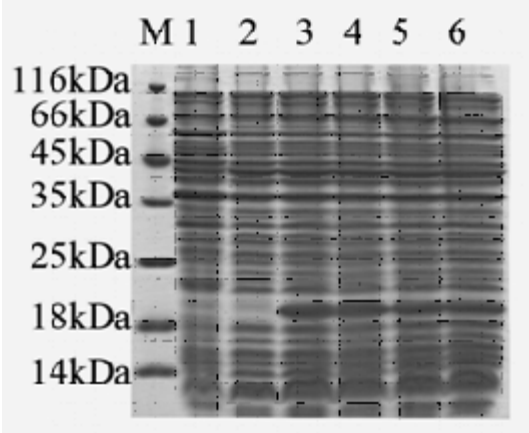

Fig. 3. Expression of recombinant Omp19 in the *E. coli* BL21. (M) marker, (1) *E. coli* BL21, (2) noninduced strain, (3-6) samples of recombinant *E. coli* BL21 1-4 hours after induction

DISCUSSION

There are important secondary facts of the signiûcance of brucellosis over human history. It's one of the world's most extensive zoonotic diseases causing abortion in domestic animals and infection of human[17,3]. At present, three vaccine strains (*Brucella* abortus S19 and RB51 strains and *Brucella* melitensis Rev1) are used for the control of brucellosis in livestock , However, these vaccines can be pathogenic for man[8-10]. While efficient treatment of humans depends on long periods of antibiotic therapy due to the intracellular nature of *Brucella*, prevention would be a better option[18-21]. Because of their side effects or lack of efficacy, existing vaccines cannot be used for human[11-13,22]. With no safe human vaccine in sight, despite suggestion, control of human brucellosis remains dependent on control of animal brucellosis. Live attenuated S19 had been used since the early 1930s to prevent brucellosis in cattle[23-27] until it was replaced by strain RB51 during the 1990s [28-30]. Vaccination with *B. abortus* strain 19 increases resistance to infection, but protection may not be complete and some vaccinated cattle may become infected. Also some vaccinated cattles may develop antibodies that interfere with diagnostic test results[23,24]. The RB51 vaccine can cause infection[28]. Even humans can be at risk for infection with RB51 if they have contact with aborted infected tissue and blood, without wearing gloves, masks[11,12]. Rev 1 retains a certain virulence, making it inappropriate for vaccinating pregnant animals and representing a possible risk to man[31,32]. Further optimization of S19 will be necessary to develop a human vaccine strain, Such modification could result from the expression of additional vaccine candidate proteins to enhance vaccine efficacy. Although the major antigen is lipopolysaccharide of smooth *Brucella*, outer membrane proteins are

important in host immune responses to the pathogens[14,15]. As a diagnostic or an immunization component, these proteins are important to be serologically evaluated and must be producible in laboratory in a pure form. Conventional protein purification procedures are labor intensive especially in the case of *Brucellae* which are potent pathogens. Thus, this strategy with the recombinant protein is a preferred one, but the resultant protein should retain its immunological properties. Omp19 is an important outer surface protein of *Brucellae*[16]. Here we produced the recombinant Omp19 in common host *E. coli* BL21 successfully. We used Protective antigen (Omp19) extracted from *Brucella abortus* cloned and expressed at high levels that can present it properly to the immune system. Some advantages of the method used, are applying PrimSTAR® HS DNA polymerase with high proof reading power which produced blunt ended insert without adding or deleting any bases.

The other one is using pJET1.2/blunt vector with eco47IR gene which expresses a fital restriction enzyme so, only recombinant colonies including inserts are observed on the plates, another genetical element is ampicilin resistant ß-lactamase gene with the role of selection of recombinant *E. coli* cells. Specific primers help us to recognize the insert, after primer attachment, the cloning insert, will be the exact gene. Another insert evaluation is double digestion of cloned pET28a (+) with BamHI and HindIII. Proper level of mentioned protein was produced which can present it appropriately to the immune system. Recombinant Omp19 of *Brucella abortus* predicts a potential antigen as vaccine for further investigations in serologic diagnosis programs, the design of specific chemotherapeutic agents and development of an acellular human vaccine.

As Omp19 is present in the all six *Brucella* species and all their biovars, brucella abortus Omp19 antigen will allow determination of their potential for the development of a subunit vaccine that is without the drawbacks of the living attenuated vaccine, protein offered in the identifiable level to immune system by *E. coli* BL21 as expression vector. The expression reached the maximum rate two hours after induction and remained unchanged in next two hours. The used expression system, resulted in a good yield of the recombinant protein, So, the method to produce and express rOmp19 in a level to be recognizable to immune system, is considerable for further vaccine researches.

CONCLUSION

In conclusion, the studies presented in the present work, indicated cloning and expression of rOmp19, in a level to be identifiable to immune system, which make it available as interesting candidate for design of specific chemotherapeutic agents for further investigations in vaccines and serologic diagnosis programs.

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