

Design and Production of a Novel Polypeptide with Immunogenic Potentials for Immunoassay of *Brucella Melitensis*

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HIGHLIGHTS

- Prediction of antigenic epitopes for *Brucella* membrane proteins.
- Production of designed polypeptide in *E. coli* BL21 (DE3).
- Interaction of produced polypeptide with *Brucella* specific antibodies.

ABSTRACT

Keywords:

Brucellosis

Diagnosis

Antigenic epitopes

Recombinant

polypeptides

Brucellosis is one of the most common diseases in humans and it has a worldwide spread. The design and production of newly synthesized proteins can be served as a goal for the rapid and accurate detection of brucellosis. To this aim, finding the antigenic epitopes is the first step to design a diagnostic method. In this study, the epitope mapping procedure was carried out by IEDB analysis resource using Flagellin and Porin amino acid sequences. The selected sequences were linked by GS linkers and cloned into pET26b vector. After confirmation of the expressed recombinant polypeptide by western blotting, it was immunologically analyzed by gel diffusion assay. The SDS PAGE and western blot analysis confirmed the 27 KDa polypeptide production and observing an arc in gel diffusion test demonstrated the precipitation of serum antibodies and the presence of specific antigen complexes. The results showed that the recombinant polypeptide produced in *E. coli* BL21, could interact with antibodies present in *Brucella* immunized sheep serum.

Introduction

Brucellosis is one of the most common human diseases and it has a worldwide spread. This bacterium is transmitted from human to human and from animal to human beings, mainly through infected dairy crops. Among the species of *Brucella*, four species of *Brucella melitensis*, *B. Abortus*, *B. Suis* and *B. Canis* are the

most important causes of human disease (Franco et al., 2007). Brucellosis is considered as a health and economic problem in many countries of all parts of the world. It is an endemic infection worldwide and is prevalent in developing countries (Moreno and Moriyón, 2002). Human brucellosis is also distributed equally around Iran. *Brucella*, as an intracellular microbial pathogen prefers monocytes and macrophage cells. The bacterium contains aggressive genes that are important in host interaction and pathogenesis. Dependent secretion encoding genes (BAB_RS 31620) of the Secretion systems III, IV, and V are involved in binding, infectivity, and hemolysis

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of *Brucella*. In addition, the availability of the genome sequence of the *Brucella* species provides an opportunity for aggressive mechanisms. Some features of outer membrane proteins in *Brucella* are quite distinct and play an important role in determination of its characteristics. *Brucella* membrane proteins structure are divided according to their size. The outer membrane protein (OMP) with a molecular mass of 36 kDa is a porin which is the first candidate for protection against the infection (Pathak et al., 2017). In addition, the membrane protein antigens in *Brucella* can induce both cellular and humeral immunity systems (Van De Verg et al., 1996; He, 2012).

The production of engineered newly synthesized proteins from several antigenic and exposed structural proteins can be served as a goal for the rapid and accurate detection of brucellosis (Seco-Mediavilla et al., 2003; Yin et al., 2016). Identification and evaluation of the different antigens of *Brucella* species have a key role in promotion of the accurate and timely diagnosis programs. In the present research, the production and purification of antigenic epitopes of the porin and flagellin proteins of *Brucella melitensis* were carried out which are the most important antigenic structural markers of this bacterium and will be evaluated in ELISA test for sheep and human Brucellosis in future by the present researchers.

Materials and Methods

Prediction of immunodominant epitopes and construction of the Polypeptide sequence

Initially, amino acid sequences of the Flagellin and Porin of *Brucella melitensis* were found from ExPASy and NCBI data Banks (Porin BAB/RS19100, membrane protein BAB/RS23045). The epitope mapping procedure was employed with IEDB analysis Resource (<http://tools.immuneepitope.org/main/>) and linear epitopes from Flagellin and Porin protein sequences were predicted. Antigenicity, solubility, and accessibility of both proteins were the most important factors for analysis. The predicted Flagellin and Porin epitopes sequences were linked by flexible linkers (marked sequences). Then, the fragment was optimized with *E. coli* codon usage and the sequence of interest with start, stop, and S tag peptide codons was synthesized and constructed in to pET 26b vector (Bioneer, Korea).

Pofla polypeptide sequence

MTSNNRHDGQYGDFFSDDRVDAGGVSTGSGGG
LLLSAAALVAASGAQAADAIV APEPEAVEYVRV
CDAYGAGYFYGGSTITPEVSYTKFGGEWKDTVAE
DNAWGGGSEQGGEDVDN DGGGSSDDRVDAGG
GGGSTNNSRHDGQGGGSLEQGGEDVDNNGGSR
DGQYGDFFSGGSDTVAEDNAWGS GGG

Pofla sequence after reverse translation and codon optimization

ATGAAAGAAACCGCTGCTGCTAAATTCGAACGC
CAGCACATGGACAGCACCTCTAACAACTCTCGT
CACGACGGTCAGTACGGTGACTTCTCTGACGAC
CGTGACGTTGCTGACGGTGGTGTCTTCTACCGGTT
CTGGTGGTGGTCTGCTGCTGGGTCTGCTGCTGC
TCTGG TTGCTGCTTCTGGTGCTCAGGCTGCTGAC
GCTATCGTTGCTCCGGAACCGGAAGCTGTTGAAT
ACGTTCTGTTTTCGACGCTTACGGTGCTGGTTA
CTT CTACGGTGGTCTACCATCACCCCGGAAGTT
TCTTACACCAAATTCGGTGGTGAATGGAAAGAC
ACCGTTGCT GAAGACAACGCTTGGGGTGGTGGT
TCTGAACAGGGTGGTGAAGACGTTGACAACGAC
GGTGGTGGTCTTCTGACGACCGTGACGTTGCTG
ACGGTGGTGGTGGTGGTCTACCTCTAACAACTC
TCGTCACGACGGTCAGGGTGGTGGTCTCTGGA
ACAGGGTGGTGAAGACGTTGACAACGGTGGTGG
TTCTCGTCACGACGGTCAGTACGGTGACTTCTCT
GGTGGTGGTCTGACACCGTTGCTGAAGACAAC
GCTTGGGGTCTGGTGGTGGT

Recombinant production of designed polypeptide

E. coli BL21 strain was transformed with the recombinant plasmid. The single colonies of *E. coli* BL21(DE3) containing the recombinant plasmids were grown in LB medium with 30 µg/ml kanamycin at 37°C to reach the density of OD₆₀₀=0.6. Then, IPTG 1 mM was added to induce the expression of the recombinant protein. After 3 hours of cultivation at 37°C, the bacterial cells were harvested via centrifugation at 8,000 rpm for 5 min.

The recombinant protein with the S-tag peptide was subjected to SDS-PAGE on a 10% polyacrylamide gel. The samples were mixed with a 2X loading buffer (125 mM Tris, 20% Glycerol, 4% SDS and 0.01% bromophenol blue at pH 6.8). The gels were stained with Coomassie brilliant blue R-250. For analysis of the polypeptide by western blotting, the recombinant proteins were separated on a 10% polyacrylamide gel and were electrophoretically transferred to a nitrocellulose membrane (Whatman, UK). The membrane was incubated with a 1:2000 dilution of the ALP (alkaline phosphatase)-labeled anti-Stag monoclonal antibody (Abcam, UK). The NBT/BCIP substrate solution (Roche, Germany) was used as the ALP substrate to visualize the immune reactivity.

Purification of the recombinant polypeptide

After confirmation of the produced recombinant S-tagged polypeptide, it was purified by affinity chromatography using S-tag resin (Novagen, USA). The bacterial pellet was suspended in 5 ml of denaturing buffer (6 M Urea, 20 mM NaH₂PO₄, and 500 mM NaCl at pH 8.0) and lysed with

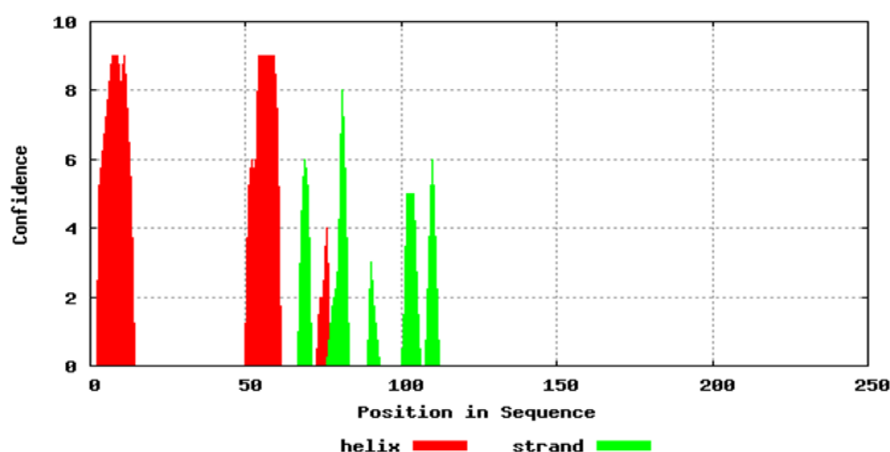


Figure 1. The predicted secondary structure of the polypeptide by PSIPRED server.

sonication on ice. After carrying out the chromatography steps, the bounded protein was eluted with 1 ml of elution buffer (3M thiocyanate, pH 2.0) (Sankian, Yousefi et al. 2007) and dialyzed in PBS at room temperature for two hours.

Immunological evaluation of the pofla polypeptide

The recombinant polypeptide was evaluated by Ouchterlony test (Gel Diffusion) for polypeptide confirmation and specific interaction with infected serum antibodies (Lord, Rolo et al. 1989). A total of 100 positive sheep sera suspect to brucellosis (or with abortion) which had positive coombs tests and 100 healthy sheep sera from the veterinary organization were prepared. Gel diffusion was set up with agarose 1% and 1X PBS.

Results and Discussion

Design and characterization of Pofla polypeptide

Designed amino acid sequence of Pofla polypeptide by Epitope mapping in IEDB server, together with upstream and downstream parts has the molecular weight of 27 kDa. The Pofla obtained sequence was analyzed by several servers like Jpred4, PSIPRED, and ProtParam which showed that the secondary structure of the polypeptide had both alpha helix and beta stranded sheets, proper stability, and good solubility in water. pI of recombinant polypeptide was calculated as 3.84 (Fig.1).

Pofla polypeptide production

The designed 658bp DNA fragment coding the Pofla polypeptide was synthesized into pET26b vector. The desired plasmid sequence was confirmed using PCR

(Fig. 2).

The designed polypeptide was expressed in *E. coli* and approved by the SDS-PAGE (Fig. 3a), and Western blot analysis with S tag monoclonal antibody (Fig. 3b).

Polypeptide-antibody interaction analysis by gel diffusion test

Antigen-antibody complex precipitation was assessed by Ouchterlony test (Fig. 4). These results indicated that there are serum antibodies against of these *Brucella* specific immune-determinant epitopes which could recognize the bacterial antigens, specifically. Also, it has confirmed the efficient folding of pofla polypeptide produced in *E. coli*.

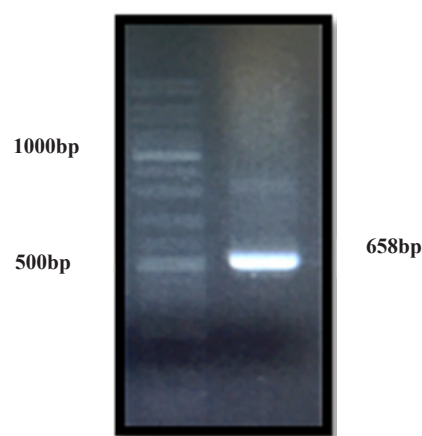


Figure 2. PCR product electrophoresis on 1% agarose gel. 658bp band indicated the presence of desired gene in plasmid construct.

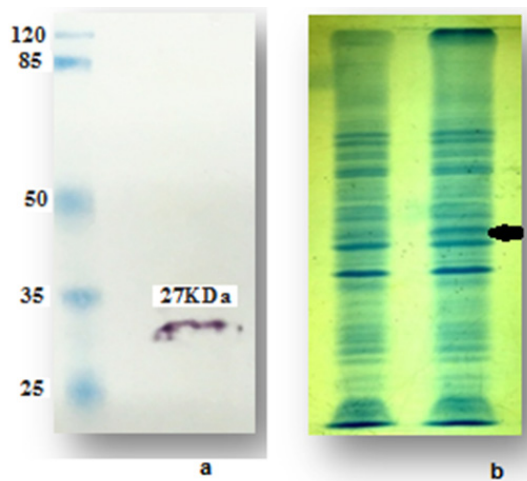


Figure 3. The Western blot (a) and SDS PAGE (b) analysis of 27KDa pofla polypeptide.

Conclusion

Brucella species are gram-negative bacteria, the optional intracellular bacteria that can cause infection in many animals and human species that is economically and hygienically important (Guilloteau et al., 1999). *Brucella* in animals is preferably placed in reproductive organs and embryonic tissues, which causes abortion and infertility, followed by significant economic losses (Corbel, 2006). The important concept, in the way to achieve efficient diagnostic, preventive, and therapeutic method, is the finding of effective antigenic epitopes to timely and accurately detect the brucellosis. To this end, an immunological diagnosis and evaluation of various antigens of the *Brucella* cell should be performed. From the preceding research, it can be concluded that Flagellin and porin proteins of *Brucella* could be a good candidates to design a laboratory diagnostic kit. The results obtained from this study showed that *Brucella* external membrane proteins, widely identified as immunogenic and antigenic epitopes, can be useful targets in generating diagnostic and vaccine-producing antigens. In traditional methods for the production of *Brucella* natural proteins, a bulk culturing is prepared and the proteins are purified from the whole culture, whose both processes are very laborious and dangerous procedures. Also, the obtained amount of specific proteins of the bacteria outer membrane is not enough for following process and thus, is not cost-effective. To control brucellosis, vaccine production and detection of infection are the most important disease control programs. Therefore, the prevention of human brucellosis is dependent on the disease control in animals. Vaccines for the prevention of the animal brucellosis

containing live strain are available, but there is no approved recombinant vaccine for human protection. Therefore, identification of *Brucella* antigens for the production of a laboratory diagnostic kit and also, an efficient vaccine is essential (Bhattacharjee et al., 2002; Schurig et al., 2002; Olsen and Stoffregen, 2005; McDermott et al., 2013). In the present study, we obtained the antigenic epitopes of flagellin and porin proteins of *Brucella melitensis* and designed a recombinant fused polypeptide with S-tag sequence. In order to produce this protein, the plasmid encoding the desired gene successfully transferred into *E. coli* BL21 (DE3), and a protein with the molecular weight of 27 kDa was expressed being confirmed on the SDS-PAGE and verified by Western blotting using a conjugated monoclonal S-tag antibody. Finally, the purified recombinant polypeptide was confirmed by the gel diffusion against the infected sheep serum that confirmed the specificity of the selected epitopes in the designed polypeptide. Therefore, as a final conclusion, this polypeptide which has been designed by IEDB server from porin and flagellin proteins of *Brucella melitensis* can be proper candidate for setting a diagnostic ELISA kit.

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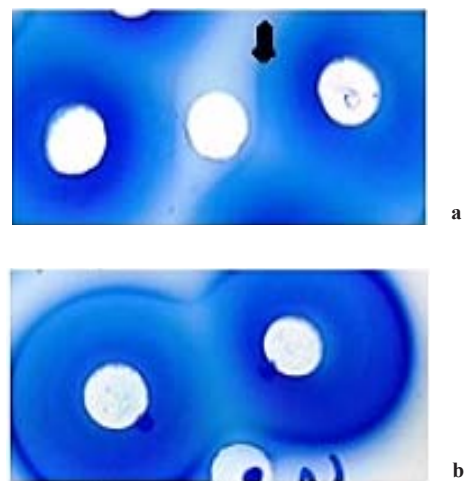


Figure 4. Antigen-Antibody complexes precipitation in Gel Diffusion test (a), Negative control (b).

Competing Interests

The authors declared no conflict of interest.

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