Archives of Razi Institute, Vol. 73, No. 3 (2018) 199-206

Original Article

Design and Production of a Novel Recombinant Chimeric IL2-Omp31 Antigen against Brucella Infection

Naghavi, M., Sekhavati *, M.H., Tahmoorespur, M., Nassiri, M.R.

Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

Received 07 May 2017; Accepted 13 July 2017
Corresponding Author: Sekhavati@um.ac.ir

ABSTRACT

Brucellosis is a zoonotic disease in human and animals. *Brucella melitensis* is one of the most pathogenic species of Brucella in goat and sheep. Omp31 is an outer membrane protein of Brucella that acts as an immunogenic protein. Cytokines are glycoproteins with low molecular weight that play the role of an immune adjuvant and regulate immune responses. Interleukin-2 is one of the most important cytokines, which are secreted by the white blood cells and involved in T cell immune responses. In the present study, a chimeric Omp31-Interleukin2 recombinant protein was generated by means of genetic engineering techniques. This chimeric coding sequence was amplified by using specific primers and using Splicing Overlap Extension (SOE) PCR technique. The fusion of the two mentioned proteins was accomplished using a rigid linker. The generated chimeric IL2-Omp31 fragment was TA cloned, and then subcloned into pEt22b vector as an expression vector. The chimeric protein was successfully expressed in E. coli BL21 (DE3) and confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and also Western-blotting analysis. Finally, in order to assess the antigenic features of the recombinant chimeric IL2-Opm31 protein, its secondary structure and antigenicity were predicted in silico.

Keywords: *Brucella melitensis*, Interleukin-2, Omp31, Cytokines

Conception et Production d'Un Nouvel Antigène Chimique IL2-Omp31 Recombinant contre l'Infection à Brucella

Résumé: La brucellose est une maladie zoonotique chez l'homme et les animaux. *Brucella melitensis* est l'une des espèces les plus pathogènes de Brucella chez les chèvres et les moutons. Omp31 est une protéine de membrane externe de Brucella qui agit comme une protéine immunogène. Les cytokines sont des glycoprotéines de faible poids moléculaire qui jouent le rôle d'un adjuvant immunitaire et régulent les réponses immunitaires. L'interleukine-2 est l'une des cytokines les plus importantes, sécrétées par les globules blancs et impliquées dans les réponses immunitaires des cellules T. Dans la présente étude, une protéine recombinante chimère Omp31-Interleukine2 a été générée au moyen de techniques de génie génétique. Cette séquence de codage chimérique a été amplifiée en utilisant des amorces spécifiques et en utilisant la technique de PCR par Extension de Chevauchement d'Épissage" (ECE). La fusion des deux protéines mentionnées a été réalisée en utilisant un connecteur rigide. Le fragment chimérique d'IL2-Omp31 généré a été TA cloné, puis sous-cloné dans le vecteur pEt22b en tant que vecteur d'expression. La protéine chimère a été exprimée avec succès dans *E. coli* BL21 (DE3) et confirmée par électrophorèse sur gel de dodécylsulfate

de sodium polyacrylamide (SDS-PAGE) et également par analyse par transfert Western. Enfin, afin d'évaluer les caractéristiques antigéniques de la protéine chimérique IL2-Opm31 recombinante, sa structure secondaire et son antigénicité ont été prédites in silico.
Mots-clés: *Brucella melitensis*, Interleukine-2, Omp31, Cytokines

INTRODUCTION

Brucellosis is one of the main zoonotic diseases caused by Gram-negative intracellular bacteria (Olsen, 2013). The main complications associated with this pathogen are usually abortion and reduced fertility in animals and such symptoms as undulant fever, arthritis, and osteomyelitis in human (Olsen, 2013). The genus of Brucella consists of at least ten species, among which B. melitensis, B. abortus, and B. suis pathogens are often isolated from both animals and humans (Cloeckaert et al., 2001; Galinska and Zagorski, 2013; Gwida et al., 2010; Haag, 2010). Brucella melitensis was isolated for the first time from the spleen of a British soldier who had died in the island of Malta by David Bruce in 1886 (Bruce, 1887; Pappas, 2005). The main immunogenic proteins in Brucella bacterium are cell-specific membrane antigens, which are called outer membrane proteins (OMPs) (Yousefi et al., 2016; Abbassi-Daloii et al., 2017). Omp31, as a dominant antigen, is present in all Brucella species, except for B. abortus (Kittelberger et al., 1995; Cloeckaert et al., 2002). The alignment of Omp31 sequence of B. melitensis Rev 1 revealed that this antigen has a close similarity to other Brucella species (Yousefi et al., 2016). Omp31 is also known as the most exposed OMP on the smooth strains of B. melitensis (Bowden et al, 1995). Regarding this, Omp31 is usually considered as a favorable subunit vaccine candidate against brucellosis (Vahedi, 2011; Yousefi et al., 2018). One of the best vaccines accessible for the brucellosis infection in sheep and goats is live attenuated strain of B. melitensis Rev.1 (Cloeckaert et al., 2002). This vaccine is less effective in preventing infection in animals or resulting in proper response after exposure to lethal field strains (Olsen, 2013). However, subunit vaccines are not frequently efficient in prompting strong immunity; furthermore, the vaccines consisting of only one bacterial antigen could not typically protect against Brucella infection (Yang et al., 2005). Adjuvants are frequently suggested in many studies as helpers for increasing the immunogenicity of the vaccine and improving immune responses (Schijns, 2000). Cytokines are the powerful regulators of immune responses and also have immune adjuvant activity. In this regard, interleukin-2 (IL-2) acting as one of the main cytokines is essential for the stimulation and improvement of immune responses (Wales et al., 2005). This cytokine is secreted by CD4+ T cells in response to antigenic stimulation, evokes the proliferation of T lymphocytes, and also enhances cellular and humoral immune responses (Leonard, 2001; Lin et al., 2004). The primary objective of the present study was the construction, cloning, and expression of a novel chimeric mouse IL-2 and Omp31 (IL2-Omp31) antigen for designing a subunit vaccine against Brucella infectious disease.

MATERIALS AND METHODS

Polymerase chain reaction. Omp31 (723bp) and codon optimized IL-2 (500 bp) sequences, which were separately cloned into T-vector (Yousefi et al., 2016), were used as templates in polymerase chain reaction (PCR). In order to build the chimeric sequence, to initialize the PCR (i.e., PCR I; Figure 1), IL-2 and Omp31 sequences were amplified using specific primers (Table 1). The PCR I was carried out using the Personal Cycler™ thermocycler (Biometra, Germany) with the reaction mixture. The final mixture for each reaction consisted of 2.5 µL of 10× PCR buffer, 2 µL

dNTPs (2.5 mM), 2 µL MgCl2 (10 mM), 1.5 µL of mix primer (5 pmol/µL), 0.5 µL of the template DNA (T vector harboring Omp31 or IL-2 sequence, 50-100 ng/µL), 0.125 U/µL of Ex Taq DNA polymerase (Takara, Japan), and finally deionized water up to a volume of 25 µL. The PCR program was accomplished with 6 min at 94 °C as an initial denaturation step, followed by 30 cycles of 94 °C for 30 sec (denaturation step), at 62°C for 30 sec (annealing step), and at 72 °C for 45 sec (extension step), and at 72 °C for 10 min (final extension). The PCR products were separated and analyzed by electrophoresis on 1% agarose gel.

Table1. List of primers used in this study Restriction enzyme

primer	Sequence	
Reverse IL2-R	5`CCATGGGAATGTATAGCATGCAGCTGGC 3`	NcoI
Forward IL2-F	5` TTTAGCCGCTGCTTCTTTTGCCGCAGCTTCCGGGCTGGTGCTAATAAT 3`	-
Reverse Omp31-R	5`GAAGCTGCGGCAAAAGAAGCAGCGGCTAAAATGAAATCCGTAATTTTG 3`	-
Forward Omp31-F	5` CTCGAGGAACTTGTAGTTCAGACCGA3	XhoI

Bold letters are used to highlight the coding sequence of the helical linker (Ping and Ming-Guang, 2008) at the end of 5`-IL2-R and Omp31-F primers. The restriction sites were also underlined at the end of 5´-IL2-F and Omp31-R primers. The chimeric antigen was produced by linking IL-2 and Omp31 sequences together using the overlap extension polymerase chain reaction (OE-PCR) (Figure 1). The final mixture for next reaction was similar to that of the PCR I products, except that the Omp31 and IL-2 PCR products were used as templates (this step was performed without primers, Figure 1). The temperature program was set by initial denaturation for 5 min at 94°C, 15 cycles of 30 sec at 94 °C (denaturation step), 30 sec at 60 °C (annealing step), and 30 sec at 72°C (extension step). Final extension was conducted for 5 min at 72 °C. The last step of (i.e., PCR II) was "PCR amplification" by IL2-F and Omp31-R primers. The PCR programs for this step was performed in 25 cycles of denaturation for 30 sec at 94 °C, 30 sec at 60 °C (annealing step), and 30 sec at 72°C (extension step). The initial denaturation and final extension were 5 min at 94°C and 72 °C, respectively.

Gene Cloning. The chimeric fragment (i.e., IL2-Omp31), which was purified from the agarose gel using a gel purification kit (GeNet Bio, Korea), was ligated into T/A cloning vector based on the manufacturer's instructions (Thermo, USA). Competent cell preparation and transformation were accomplished as previously described in the literature (Sambrook and Russell, 2001). Therefore, the recombinant plasmids were transformed into competent E. coli DH5α. Ampicillin-resistant colonies, harboring recombinant plasmid DNA, were grown over night in Luria broth (LB) medium containing 50 µg/mL of ampicillin at 37 °C. Colony PCR was used for confirming the fidelity of E. coli DH5α transformants by M13 universal primers.

Expression and purification. For expressing the fragment, chimeric IL2-Omp31 (rCIL2-Omp31) open reading frame was isolated by NcoI and XhoI restriction enzymes. The excised fragments were purified from agarose gel using a commercial kit (BioRon, Germany). The rCIL2-Omp31 fragments were sub-cloned into pET22 (b) vector, namely E. coli BL21 (DE3). The harboring transformed bacteria were spread in LB agar containing 50 µg/mL ampicillin (Sigma, Germany), and then incubated at 37 °C overnight. One positive BL21 clone was selected using the colony PCR with T7 universal primers. The presence of rCIL2-Omp31 coding sequence was confirmed by restriction endonuclease analysis and sequencing. The positive colonies were cultured on a selective LB medium at the optical density (OD) of 0.6. In addition, isopropyl β-D-thiogalactoside (IPTG) was added at the final concentration of 0.1 mM to induce the expression of the chimeric protein at 37 °C. The harvested cells were suspended and lysed using lysis buffer and sonication (Yousefi et al., 2016).

Subsequently, the cell lysate was centrifuged at 9000 g for 15 min at 4 °C to separate the supernatant containing soluble materials from the pellet. To analyze the expression of rCIL2-Omp31 protein, both supernatant and pellet were evaluated on sodium dodecyl sulfate polyacrylamide gel electrophoresis 10% (SDS-PAGE). Expressed protein was purified from the insoluble phase of lysate using guanidine hydrochloride 6 M to dissolve the pellet using Ni-agarose chromatography (Thermo, USA). The quality and identity of the recombinant rCIL2-Omp31 protein was analyzed using the 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-polyhistidine (HRP; Sigma; 1:2000 diluted in 1% BSA) was added. After 1 h of incubation at room temperature and washing, diaminobenzidine was employed as chromogen for visualization. Finally, the quantity of the recombinant protein was estimated using the Bradford assay (Yousefi et al., 2016). The purified recombinant Brucella lumazine synthase protein was stored at -20°C for further studies.

Prediction of the secondary structure and antigenicity. Improved self-optimized prediction method (SOPMA) software was applied for analyzing the secondary structures of rCIL2-Opm31 protein (http:// npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page= /NPSA/npsa_sopma.html) (Golshani et al., 2014). Helices, sheets, turns, and coils were in four conformational states, which were predicted in this procedure. In addition, tertiary structures were anticipated using an online ligand-binding site prediction server (http://zhanglab.ccmb.med.umich. edu/I-TASSER/) (Gwida et al., 2010). Furthermore, antigenicity was measured by the VaxiJen 2.0 server (http:// www. ddg - pharmfac. net / vaxijen/ VaxiJen/-VaxiJen.html) for the alignment-independent prediction of protective antigens.

RESULTS

The chimeric IL2-Omp31 was successfully amplified using the SOE-PCR process as shown in Figure 1. In this procedure, the rCIL2-Omp31 coding sequence (1270 bp) was amplified using Omp31 (723bp) and mouse codon-optimized IL2 (500 bp) sequences as templates (Figure 2. a1 and a2, respectively) in the SOE-PCR. Moreover, it was visualized using 1% agarose gel, horizontal electrophoresis (Figure 2. a3). The chimeric coding sequence was successfully ligated into T/A

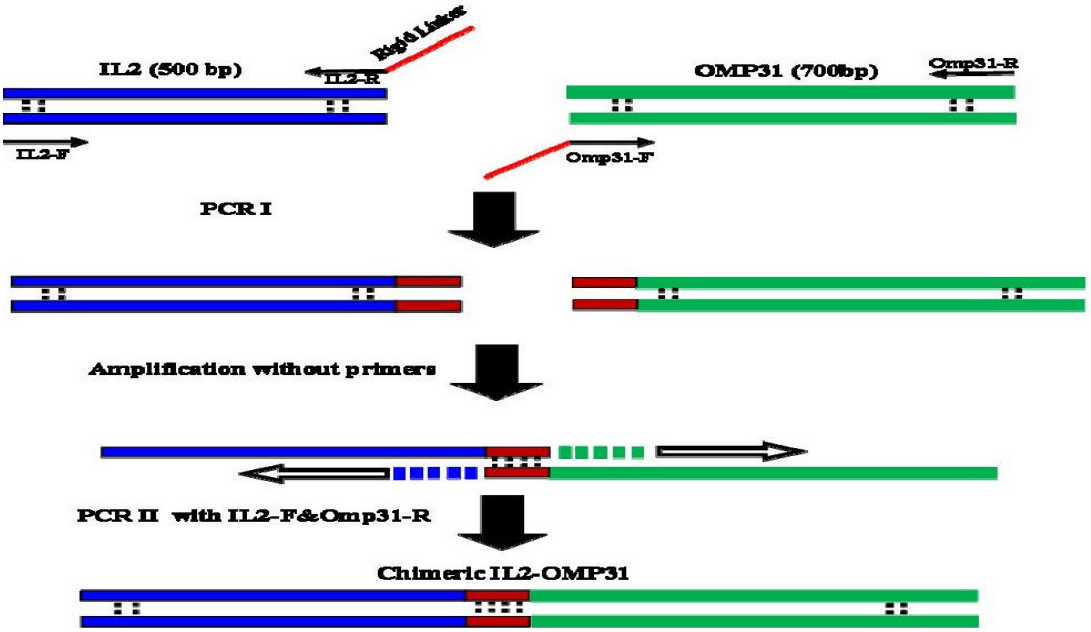

Figure 1. Schematic presentation of different steps to design chimeric interleukin II-Omp31 construct

cloning vector and then sub-cloned into pET22 (b) vector. The results of sequencing exhibited the accuracy of the frame without any mutation in the coding sequence (Figure 2B).

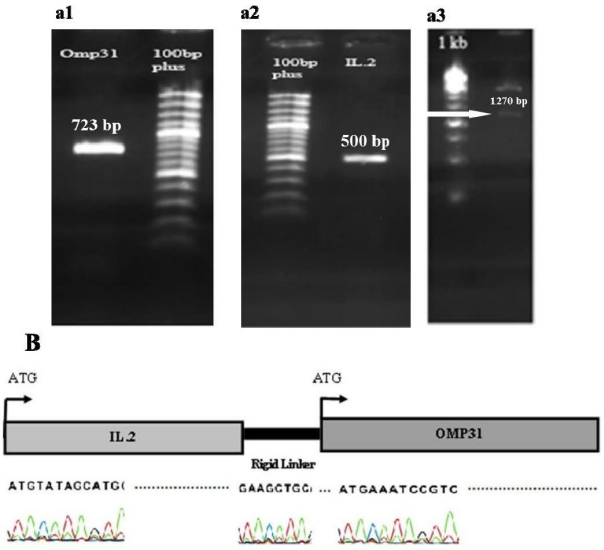

Figure 2. a) Agarose gel electrophoresis of polymerase chain reaction products for Omp31 (a1), murine codon-optimized interleukin-2 (a2) and chimeric interluekin2-Omp31 (a3) coding sequences on 1% agarose gel, b) schematic figure of chimeric interleukin2-Omp31 construct and the results of partial sequencing for some regions of this construct

Expression and purification of recombinant protein. The expression of rCIL2-Omp31 protein was induced with IPTG (0.1 mM) at the OD of 0.6 for 4 h at 37°C. The 46 kDa expected protein size was assessed after inducing the culture with IPTG (Fig.3). The SDS-PAGE (10%) analysis of the lysate from the induced E. coli BL21 (Figure 3A) and also the purified protein (Figure 3B) showed the expected chimeric recombinant protein with the approximate molecular weight of 46 kDa. The expression yield of the purified protein (120 µg/mL) was better 16 h after IPTG induction. Western blotting with anti-HRP antibody revealed the specific reactivity with purified rCIL2-Omp31 produced in E. coli cells.

Prediction of secondary and tertiary structures and antigenicity. To determine the antigenic features of rCIL2-Opm31, its secondary structure was anticipated by the aid of the SOPMA server software. The results indicated no interaction between the two protein domains (i.e., IL2 and Omp31) in rCIL2-Opm31, which was separated by the rigid linker (Figure 4). Moreover, the results of antigenicity prediction for different combinations of chimeric rCIL2-Omp31 parts showed no significant difference in antigenicity score between rCIL2-OMP31 and Omp31 (Table 2).

DISCUSSION

Currently, antibiotic therapy is not considered as the best approach. Vaccine development is a cost-effective option for the management of bacterial infections (Zhang et al., 2014). However, the accessible vaccines against B. melitensis applied for the livestock are not effective in animals. On the other hand, currently, there is no human vaccine against any species of Brucella (Olsen, 2013). Subunit vaccines, which consist of components or single bacterial proteins instead of the entire pathogens, are usually less effective in inducing long-lasting immunity and bear a low risk of causing adverse reactions (Yang et al., 2005; Ghasemi et al., 2015). Protective immunity against Brucella is mediated by cell-mediated immunity, particularly via Th1 immune responses, which is characterized by the production of gamma interferon, IL-2, and tumor necrosis factor beta (Avila-Calderon et al., 2013). These cytokines encourage both macrophage activation and antibodies (Fowell et al., 1991; Mossman and Coffman, 1989). In the present study, we designed and constructed a chimeric recombinant protein consisting of Omp31 as an immunogen and IL-2 as a Th1 cytokine to increase the potential of specific and protective immunity against subsequent B. melitensis infection in mice. In this regard, Zhang et al. (2008) designed a cis-expression plasmid DNA encoding two genes of human IL2 and multiple epitopes of foot-and-mouth disease virus. They showed that IL2 upregulated a specific immunological response and provided

protection against the homologous virus (Zhang et al., 2008). Their results suggested that IL-2 could be a suitable adjuvant for enhancing the robustness of their DNA vaccine. In the present study, a rigid linker (EAAAK)2 was used to fuse two different coding sequences. The results of the secondary structure prediction showed that the rigid linker could effectively separate two protein domains.

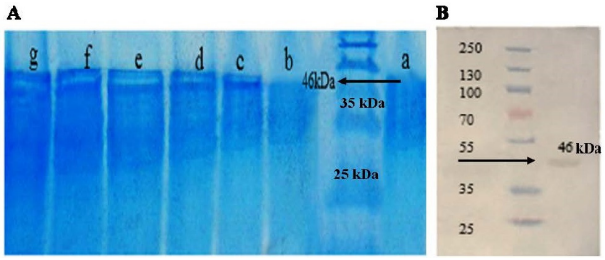

Figure 3. a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of rCIL2-Omp31 protein with different sampling times after induction by 0.1 mM isopropyl β-D-thiogalactoside (column a is negative control [BL21 cell lysate without recombinant vector] and columns b, c, d, e, f, and g refer to BL21 cell lysate containing recombinant expression vector 0, 2, 4, 8, 12, and 16 h after isopropyl β-D-thiogalactoside induction, b) western blotting profile of recombinant proteins (pre-stained protein marker [Thermo, USA] with 9 bands)

Moreover, the comparison of the predicted antigenicity suggested that fusion by rigid linker did not significantly affect the score of OMP31 antigenicity. As a vital component of recombinant fusion proteins, linkers have been shown to increase stability and bioactivity (Chen and Shen, 2012; Chen and Shen, 2013). Moreover, some studies have reported that EAAAK motif can be used to effectively separate two antimicrobial proteins and produce a fusion protein with a higher antimicrobial activity (Arai et al., 2001; Lee et al., 2013).

In conclusion, our results showed that this chimeric protein could be used as a candidate subunit vaccine against B. melitensis infection in a mouse model. However, it is essential to conduct further studies using pre-clinical experiments, including the evaluation of antigenicity and protection efficiency.

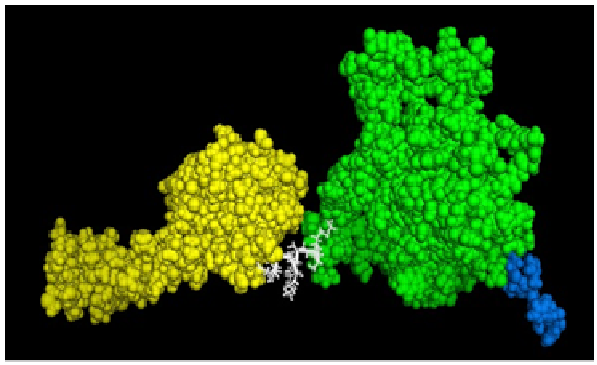

Figure 4. Schematic presentation of the secondary structure of the chimeric rCIL2-Opm31 protein predicted by SOPMA server software (mus musculus interleukin-2 [yellow], rigid linker [white], Brucella melitensis OMP31 antigen [green], and His-Taq sequence [blue])

Table 2. Antigenicity of chimeric rCIL2- Omp31

Protein sequence	Antigenicity score
Interleukin2-Linker-Opm31-HisTaq	0.622
Interleukin2-Linker-Omp31	0.625
Linker-Omp31	0.682
Omp31	0.667
Omp31-His Taq	0.659

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

The present study was funded by INFS (infs.gov.ir) of I.R.I with grant No. 92041813.

Acknowledgment

The authors would like to express their gratitude to INFS and also Ferdowsi University of Mashhad, Mashhad, Iran, for their support.

References

Abbassi-Daloii, T., Yousefi, S., Sekhavati, M.H. and Tahmoorespur, M., 2018. Impact of heat shock protein 60 KD in combination with outer membrane proteins on immune response against Brucella melitensis. Apmis, 126(1), pp.65-75.

Arai, R., Ueda, H., Kitayama, A., Kamiya, N., Nagamune, T., 2001. Design of the linkers which effectively separate domains of a bifunctional fusion protein. J Protein Engin 14, 529-532.

Avila-Calderon, E.D., Merino, A.L., Sriranganathan, N., Boyle, S.M., Contreras-Rodríguez, A., 2013. A History of the Development of Brucella Vaccines. J BioMed Research Int 2013, Article ID 743509

Cloeckaert, A., MaggyGrayon, B., Olivier Grépinet, B., 2002. Identification of Brucellamelitensis vaccine strain Rev.1 by PCR-RFLP based on a mutation in the rpsL gene. J Vaccine. 20, 2546–2550.

Bowden, R.A., Cloeckaert, A., Zygmunt, M.S., Dubray, G., 1995. Outer-membrane protein and rough lipopolysaccharide-specific monoclonal antibodies protect mice against Brucellaovis. J Med Microbiol 43, 344-347.

Bruce D., 1887. Note on the discovery of a microorganism inMalta fever. J Practitioner 39, 161–170.

Chen,X., Zaro, J., Shen, W.C., 2013. Fusion Protein Linkers: Property, Design and Functionalit. J. NIH-PA 65, 1357–1369.

Chen, X., Zaro, J.L., Shen, W.C., 2012. Fusion protein linkers: Property, design and functionality. J Adv Drug Deliv Rev 65, 1357-1369.

Cloeckaert, A., Verger, J.M., Grayon, M., Paquet, J.Y., Garin-Bastuji, B., Foster, G., Godfroid, J., 2001. Classification of Brucella spp. isolated from marine mammals by DNA polymor-phism at the Omp2 locus. J Microbes Infect 3, 729–738.

Cloeckaert, A., Vizcaino, N., Paquet, J.Y., Bowden, R.A., Elzer, P.H., 2002. Major outer membrane proteins of Brucella spp. past, present and future. J Vet Microbiol 90, 229-247.

Delpino, M.V., Estein, S.M., Fossati, C.A., Baldi, P.C., Cassataro, J.,2007. Vaccination with Brucella recombinant DnaK and SurA proteins induces protection against Brucellaabortus infection in BALB/c mice. J Vaccine 25, 6721-6729.

Fowell, D., McKnight, A.J., Powrie, F., Dyke, R., Mason, D., 1991. Subsets of CD4+ T cells and their role in the induction and prevention of autoimmunity. J Immunol Rev 123,37–64.

Galinska, E.M., Zagorski, J., 2013. Brucellosis in humans – etiology, diagnostics, clinicalforms. J Ann Agric Environ Med 20,233–238.

Geourjon, C., Deléage, G., 1995. SOPMA. Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. J Comput Appl Biosci 11, 681-684.

Ghasemi, A., Jeddi-Tehrani, M., Mautner, J., Salari, M.H., Zarnani, A.H.,2015. 'Simultaneous immunization of mice with Omp31 and TF provides protection against Brucellamelitensis infection'. J Vaccine1333, 5532-5538.

Golshani, M., Zandi, P., Bouzari, S., 2014. In silico Design of Truncated Omp31 Protein of Brucellamelitensis: Its Cloning and High Level Expression in Escherichia coli. J Vaccine Res 1, 16-21.

Gwida, M., Al Dahouk, S., Melzer, F., Rosler, U., Neubauer, H., Tomaso, H., 2010. Brucellosis– regionally emerging zoonotic disease? J Croat Med 51,289–295.

Haag, A.F., Myka, K.K., Arnold, M.F., Caro-Hernandez, P., Ferguson, G.P., 2010. Importance of lipopolysaccharide and cyclic beta-1,2-glucans in Brucella-mammalian infections. J Microbiol 124509

Kittelberger, R., Hilbink, F., Hansen, M.F., Ross, G.P., de Lisle, G.W., Cloeckaert, A., de Bruyn, J., 1995. Identification and characterization of immunodominant antigens during the course of infection with Brucellaovis. J Vet Diagn Invest 7, 210-218.

Lee, M., Bang, K., Kwon, H., Cho, S.,2013.Enhanced antibacterial activity of an attacincoleoptericin hybrid protein fused with a helical linker. J Molecul Biol 40, 3953-3960.

Leonard, W.J., 2001. Cytokines and immunodeficiency diseases. J Nat Rev Immunol 1, 200–208.

Lin, Y., Ya-Li, Q., Wei-Cheng, B., Xiao-Lan, Y., Hong-Liang, H., Huan-Chun, C., 2004. Expression of an interleukin-6 - interleukin-2 fusion protein (pIL-6-IL-2) in P. pastoris. J Cytokine Netw 15, 240–246.

Mossman, T.R., Coffman, R.L., 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. J Annu Rev Immunol 7,145–73.

Olsen, S.C., 2013. Recent developments in livestock and wildlife brucellosis vaccination. J Rev Sci Tech 32, 207-217.

Pappas, G., Akritidis, N., Bosilkovski, M., Tsianos, E., 2005. Brucellosis. J Engl Med 352, 2325–2336.

Ping, L., Ming-Guang, F., 2008. Bifunctional enhancement of a β-glucanase-xylanase fusion enzyme by optimization of peptide linkers. Microbiol Biotechnol 79, 579–587.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, in Cold SpringHarbor Laboratory Press, pp. 203-206.

Schijns, V.E., 2000. Immunological concepts ofvaccine adjuvant activity. J Curr Opin Immunol 12, 456–463.

Sekhavati, M.H., Tahmoorespur, M., Abbassi-Daloii, T., Yousefi, S., Khabiri, A.A., Akbari, R., Shekari, E., 2015. Dual Promoter Vector Construction for Simultaneous Gene Expression Using SOE-PCR Technique. Iran J Appl Anim Sci 5, 491-495.

Vahedi, F., Talebi, A.F., Ghorbani, E., Behroozikhah, A.M., Shahriari-Ahmadi, F., Mahmoudi, M., 2011. Isolation, cloning and expression of the Brucellamelitensis Omp31 gene. J Vet Res Shiraz Uni 12, 156-162.

Vizcaino, N., Cloeckaert, A., Zygmunt, M.S., Dubray, G., 1996. Cloning, nucleotide sequence, and expression of the Brucellamelitensis omp31 gene coding for an immunogenic major outer membrane protein. J Infect Immun.64, 3744–3751.

Wales, G.R., Baird, M.A., Davies, N.M., Buchan, G.S., 2005. Fusing subunit antigens to interleukin-2 and encapsulating them in liposomes improves their antigenicity but not their protective efficacy. J Vaccine 23, 2339–2341.

Wass, M.N., Sternberg, M.J., 2009. Prediction of ligand binding sites using homologous structures and conservation at CASP8. J Proteins 77, 147-151.

Yang, X., Hudson, M., Walters, N., Bargatze, R.F., Pascual, D.W., 2005. Selection of pro-tective epitopes for Brucellamelitensis by DNA vaccination. J Infect Immun 7297–7303.

Yousefi, S., Tahmoorespur, M., Sekhavati, M.H., 2016. Cloning, expression and molecular analysis of Iranian Brucellamelitensis Omp25 gene for designing a subunit vaccine. J Res Pharma Sci 11, 405-411.

Yousefi, S., Abbassi-Daloii, T., Sekhavati, M.H. and Tahmoorespur, M., 2018. Evaluation of immune responses induced by polymeric OMP25-BLS Brucella antigen. Microbial pathogenesis, 115, pp.50-56.

Zhang, H., Qiu, Y., Zhao, Y., Liu, X., Liu, M., Yu, A., 2014. Immunogenicity of oral vaccination with Lactococcuslactis derived vaccine candidate antigen (UreB) of Helicobacter pylori fused with the human interleukin2 as adjuvant. J Molecular Cell Prob 28, 25-30.

Zhang, H.Y., Sun, S.H., Guo, Y.J., Zhu, W.J., Shi, K., Xu, G.X., Wang, J., 2008. Optimization strategy for plasmid DNAs containing multiple-epitopes of foot-and-mouth diseasevirus by cis-expression with IL-2. J Vaccine 26, 769-777.