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ORIGINAL ARTICLE

Immunoreactivity evaluation of a new recombinant chimeric protein against *Brucella* in the murine model

Abbas Abdollahi[1], Shahla Mansouri[1*], Jafar Amani[2*], Mahdi Fasihi-Ramandi[3], Mohammad Moradi[1]

[1]Department of Microbiology, Kerman University of Medical Sciences, Kerman, Iran
[2]Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[3]Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background and Objectives: Brucellosis is an important health problem in developing countries and no vaccine is available for the prevention of infection in humans. Because of clinically infectious diseases and their economic consequences in human and animals, designing a proper vaccine against *Brucella* is desirable. In this study, we evaluated the immune responses induced by a designed recombinant chimera protein in murine model.

Materials and Methods: Three immunodominant antigens of *Brucella* have been characterized as potential immunogenic and protective antigens including: trigger factor (TF), Omp31 and Bp26 were fused together by EAAAK linkers to produce a chimera (structure were designed *in silico*), which was synthesized, cloned, and expressed in *E. coli* BL21 (DE3). The purification of recombinant protein was performed using Ni-NTA agarose. SDS-PAGE and anti-His antibody was used for confirmation purified protein (Western blot). BALB/c immunization was performed by purified protein and adjuvant, and sera antibody levels were measured by ELISA. otted.

Results: SDS-PAGE and Western blotting results indicated the similarity of *in silico* designing and *in vitro* experiments. ELISA result proved that the immunized sera of mice contain high levels of antibodies (IgG) against recombinant chimeric protein.

Conclusion: The recombinant chimeric protein could be a potential antigen candidate for the development of a subunit vaccine against *Brucella.*

Keywords: *Brucella,* Vaccine, Immunity, Recombinant

*Corresponding author: Shahla Mansouri, Ph.D, Department of Microbiology, Kerman University of Medical Sciences, Kerman, Iran.
Tel: +98 343 325 7665, +98 913 142 3384
E-mail: smansouri@kmu.ac.ir

*Corresponding author: Jafar Amani, Ph.D, Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.
Tel: +98 912 406 6964
E-mail: jafar.amani@gmail.com

INTRODUCTION

Brucella is facultative intracellular pathogens that infect humans and many domestic animals such as cows, sheep, and goats. Infection causes abortion and infertility in the animals and undulant fever in humans (brucellosis) and is endemic in many developing countries. Brucellosis is a zoonotic infection, leading to clinically infectious diseases and economic consequences (1, 2). The efforts of disease eradication and infection prevention have been made

through the use of vaccines and health guidelines (3, 4). The control of brucellosis is attempted by vaccine administration using *B. abortus* strain 19, *B. melitensis* Rev1, and *B. abortus* RB-51 vaccines. Despite the efficacy of vaccination, these vaccines have some disadvantages, such as the ability to cause disease in humans and abortion in pregnant animals, and difficulty in the diagnostic validation of infection stages in vaccinated animals (5-7). Recombinant subunit vaccines have predetermined compositions with suitable homogeneity; they can be controlled to ensure good production and are completely inert. Because of the problems derived from the utilization of attenuated and killed vaccines in humans and animals, similar to other infectious diseases vaccines, development of a beneficial subunit vaccine against brucellosis is desirable. However, the success of subunit vaccines to stimulate the immune response depends on the optimization of the antigen and adjuvant (s) and selection of the delivery system (8).

Intracellular and cell surface components have recently been considered as protective antigens, but only few antigenic components have suitable immunogenic activity, for example, *Brucella* lumazine synthase, BLS (Cytoplasm); ribosomal protein L7/L12 (Cytoplasm); sugar-binding 39-kDa protein, p39 (periplasm); Bp26 periplasmic immunogenic protein, Bp26 (periplasm); molecular chaperone, DnaK (cytoplasm); outer membrane protein, Omp16,19,25,31 (outer membrane); Cu/Zn superoxide dismutase, SodC (periplasm); SurA Peptidyl-prolyl cis-trans isomerase, SurA (periplasm) and Trigger factor, Tig/TF (cytoplasm). Despite the immunogenicity of these antigens, the desirable protection against bacteria could be improved using a multiple subunit vaccine. Omp31, TF, and Bp26, have been characterized as potential immunogenic and protective antigens and have been previously studied in whole and portion form to determine their protective immunogenicity (9, 10).

In this study, we developed a new structural model containing three putative antigenic determinants of *Brucella*, Omp31, TF, and Bp26, and evaluated the irimmunoreactivity and sero response against a chimeric recombinant protein encoding these *Brucella* antigens, in the murine model.

MATERIALS AND METHODS

According to previous researches (11-19), we chose three antigenic determinants of TF, 485 amino acids, Bp26, 25 amino acids (87-111) and Omp31, 27 amino acids (48-74), fused together by EAAAK rigid linkers to avoid the construction changes in final composition; also these rigid linker maintain the conformation of protein by lowest changes in structure. The segment arrangement of chimera was determined by changing the three antigenic determinants to construct the best structure *in silico*. Codon of this chimera was optimized to best efficiency of expression. Restriction enzyme (RE) site were added at 5' and 3' ends (20).

In silico Prediction

Databank collection, antigen designing and physicochemical parameters. The identification and analysis of gene sequences and gathering information was carried out by searching the literature from the NCBI PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) & and http://www.uniprot.org). The alignment and sequences identity of component to identify a conserved region in all the required sequences were performed using BLAST software (http://www.uniprot.org/blast/) and ClustalW software (http://www.ebi.ac.uk/Tools/clustalW2). Chimeric gene optimization to cloning and expression in *Escherichia coli*, were used by Swissprot reverse translation (http://www.bioinformatics.org/sms2/rev_trans.html) and Codon Optimization online service (https://eu.idtdna.com/CodonOpt). DNA/RNA GC Content Calculator (http://www.endmemo.com/bio/gc.php) were used to calculate G/C% before and after optimization. Antigenicity, linear epitope, beta-turn, surface accessibility and flexibility of chimeric designed antigen were predicted by IEDB Analysis Resource (http://tools.immuneepitope.org/tools/), Vaccine Design server (http://www.violinet.org/vaxign/index.php) and The Proteome Binders Epitope Choice Resource (http://bioware.ucd.ie/epic/). Chimeric antigen physicochemical parameters: total number of residues, solvent accessibility, aliphatic index, theoretical isoelectric point (pI), extinction coefficient, half-life, molecular weight, grand average hydropathy and instability index, were computed using Expasy's Protparam (http://us.expasy.org/tools/protparam.html), Protein Calculator v3.4 (http://protcalc.sourceforge.net/) and Recombinant Protein Solubility Prediction (http://www.biotech.ou.edu/). Protein solubility of different residues

was predicted by DSSP (http://www.cmbi.ru.nl/dssp.html) and VADAR (http://vadar.wishartlab.com/).

Antigenic and allergenic epitope prediction. T-cell epitopes prediction parameters; binding both MHC class I- and MHC class II were analyzed by GPS-MBA Prediction of MHC-binding system Version 1.0 (http://mba.biocuckoo.org/links.php) and Immune Epitope Database, IEDB-Analysis Resource (http://tools.immuneepitope.org/). Chimeric antigens were analyzed for continuous B-cell epitopes using Bcepred (http://www.imtech.res.in/-raghava/bcepred/). The discontinuous B-cell epitopes were predicted with Discotope server (http://www.cbs.dtu.dk/services/DiscoTope/). Conformational B-cell epitope was predicted with web server CBTOPE (http://www.imtech.res.in/raghava/cbtope/) and The PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/). Presence of possible allergenic sites, based on the similarity of known epitopes with any region of antigen, was predicted using AlgPred (http://www.imtech.res.in/raghava/algpred/) and SDAP-Structural Database of Allergenic Proteins (https://fermi.utmb.edu/).

RNA secondary structure. Analysis of the secondary structure of messenger RNA of the chimera was predicted using the 'mfold' Web Server (http://mfold.rit.albany.edu/?q=mfold/RNA-Folding), RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and RNA structure Web Servers for RNA Secondary Structure Prediction (http://rna.urmc.rochester.edu/RNAstructureWeb/).

Protein secondary and tertiary structure. Secondary structure prediction, sequence analysis and functional parameters of protein were computed with GOR IV secondary structure prediction method (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) and Predict Protein server (https://www.predictprotein.org/). Tertiary structure-3D and stability prediction of protein were performed by DeepView - Swiss-PdbViewer (http://spdbv.vital-it.ch/). 3D structure was simulated and modeled by using Rasmol-Molecular Graphics Visualisation Tool (http://rasmol.org/). Recombinant chimeric protein modeling was performed using I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER). The C-score (confidence score for estimating the quality of model) and TM-score (measuring scale of the structural similarity) were given in the I-TASSER result page. Tertiary structure to recognize faults in the generated models, energy plot, Z-score (overall model quality) and 3D structures were validated using ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php). The stereochemical quality of protein structure was validated by Ramachandran plot (Z-score) in PROCHECK (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/).

***In vitro* experiments and optimization.** After *in silico* design and prediction, the chimeric gene was synthesized and subsequently cloned into pET-28a (+) to construct pET-chimeric protein (pET-CP) plasmids (Biomatik, Ontario, Canada).

Gene expression and purification of recombinant protein. The pET-CP was transformed into *E. coli* BL21 (DE3) strain (Novagen, Merck KGaA, Germany). Preparation of competent *E. coli* (BL21) and transformation of it was performed using calcium chloride and heat shock method respectively. The transformed clones were inoculated into 5 ml Luria Bertani (LB) medium (Merck, Germany), containing 50µg/ml kanamycin (Sigma-Aldrich, Germany) and overnight growth at 37 °C. The culture was used to inoculate 1000 ml LB medium-kanamycin. The incubation was continued with agitation (300 rpm) to 0.5 OD value at 600 nm; Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) (Sigma-Aldrich, Germany) was added (final concentration of 1mM) to induce the gene expression at 37 °C for duration of 4 hours; the concentration of IPTG was optimized by adding various amounts of IPTG to determine the best concentration (IPTG gradient). The culture was harvested by centrifugation at (10000×g, 10 min, 4 °C), then resuspended in lyses buffer (8 M urea, 0.1 M NaH-2PO4, and 0.01 M Tris, pH=8.0) containing protease inhibitors (Sigma-Aldrich, Germany). Recombinant chimeric protein was purified using Nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, UK). Proteins were eluted in 1ml buffer containing 200 mM imidazole (Sigma-Aldrich, Germany) and 500 µl MES buffer (20 mM) (Sigma-Aldrich, Germany). The purified protein was monitored by SDS-PAGE (Bio-Rad, USA) and its concentration was estimated by Nanodrop-Biowave II analyzer (biochrom, UK) and Bradford protein method measurement. The protein elution was dialyzed against 0.1 M phosphate buff-

ered saline (PBS, pH=7.4) for 72hrs in cold room to remove urea and then stored at -70 ºC for future use. Molecular weight (MW) of protein was determined using prestained protein ladder marker (SM0671), with 10 bands (approx. 10, 15, 25, 35, 40, 55, 70, 100, 130, 170 kDa) (Fermentas, USA). To evaluate of accuracy of *in silico* data, protein solubility were performed by culturing in two different temperatures 37 °C and RT, with above procedures.

Western blot analysis. SDS-PAGE protein bands were transferred into nitrocellulose membrane (Sigma-Aldrich, Germany). The membrane was then blocked in 3% bovine serum albumin (BSA), phosphate buffered saline, overnight at 4 °C. The membrane was then washed three times in phosphate buffer saline, tween-20 (PBST) and was incubated anti His-Tag antibody (Sigma-Aldrich, Germany), 1h at 37 °C. Membrane was then washed three times with PBST and incubated with anti-mouse IgG-peroxidase (Sigma-Aldrich, Germany) for 1h at 37 °C. The membrane was again washed three times and developed in diaminobenzidine (DAB) solution (Sigma-Aldrich, Germany). By visualizing of the protein band, the reaction was stopped adding distilled water. This procedure was repeated to patient pulled serum (with brucellosis) to confirm the reactivity of antibody with recombinant protein.

Evaluation of immunogenicity of chimeric antigen. Female BALB/c mice (Pasteur Institute of Iran, Tehran, Iran) in each study group (n=10) were immunized subcutaneously (s.c.) with 30 µg chimeric purified protein with/out Freund's Adjuvants. On day 0, Complete Freund's Adjuvants (CFA) and on day 14 and 28, Incomplete Freund's Adjuvants (IFA) (Sigma-Aldrich, Taufkirchen, Germany) was mixed in equal amounts with recombinant protein, respectively; the blood samples were collected and stored at -20 °C. As negative group, ten mice without any injection were regarded (11, 14, 18). The injection procedure and blood collection was performed at days 0, 14, 28; blood samples were collected by tail vein at day 38. Serum was separated and the specific antibody titer was determined by ELISA, using chimeric protein molecule pre-coated micro plates; the high binding 96 well ELISA plates (Nunc, Denmark) were coated with purified protein (µ10g/ml). Goat anti-mouse IgG-peroxidase (Sigma-Aldrich, Germany) was used as secondary antibody. The ELISA results of immune mice were compared with the titer of antibody in serum controls; the cut off value for the assay was calculated as the mean specific OD plus 3 standard deviation (SD) for 10 sera from non-immunized mice (control group); the absorbance was measured at 450 nm. The titer of each serum was calculated as the reciprocal of the highest serum dilution yielding a specific optical density higher than the cut off value. All experimental procedures and animal care were performed in compliance with the institutional animal care guidelines of ethics committee of Kerman University of Medical Sciences (Ethical Approval Code-K/93/193, 9.8.2014).

Circular Dichorism (CD) analysis. To evaluate the secondary structures prediction, recombinant chimeric protein were analyzed by Circular dichroism (CD). The data were assayed using the JASCO J-810 CD spectrometer (USA). To analyze the secondary structure, the concentration of 0.25 mg/ ml of chimera protein in phosphate-buffered saline was used.

Statistical analysis. The experimental data between groups were analyzed using the t-test and one factor analysis of variance (ANOVA) in SPSS 20.0 (Statistical significance was assumed at the $P < 0.05$ level). Violin plot was used to show the differences between results of the groups.

RESULTS AND DISCUSSION

Bioinformatics analysis. Blast and alignment Sequence comparison, illustrated the highly conserved sequences among chimera amino acid sequences and strains of *Brucella* spp. Final construction of chimera, 1-485aa (TF), 486-495aa (EAAAKEAAAK-Linker), 496-520aa (Bp26), 521-525aa (EAAAK-Linker) and 526-552aa (Omp31), was made by fusing the C terminal of TF, middle portion of $Bp26^{87-111}$ and N terminal of $Omp31^{48-74}$, used of two hydrophobic-rigid amino acid linkers. Gene optimization to expression in *Escherichia coli* was improved by changing the GC count from 51% in native form to 55% in reforming nucleotide to best expression in *Escherichia coli* according to results of data bases analysis. Prediction of antigenicity and linear epitope of antigen, showed the antigenic determinant and epitopes in several different sequences in chimeric antigen. MHC I and

II classes binding sites, were determined in protein structure, in several positions, described previously. According to databank analysis, there was no presence of possible allergenic sites, based on the similarity of known epitopes with any region of antigen. Physicochemical parameters prediction of protein, was computed approximately: molecular weight: ~ 65 kDa, Number of amino acids: 552, theoretical pI: ~ 5.0, extinction coefficient: ~ 24780 M^{-1} cm^{-1}, estimated half-life: >10 hours (*Escherichia coli*, *in vivo*), the N-terminal of the sequence considered: M (Met), instability index: ~ 45.00 (regarding that chimeric protein was stable), aliphatic index: ~ 80.00 (a positive factor for the increase of thermostability), grand average of hydropathicity-GRAVY (sum of hydropathy values): ~ -0.680 and ~ 100% chance of solubility when over expressed in *E. coli*.

RNA secondary structure analysis was indicated that there was no disorder in mRNA conformational structure and normal folding was formed. Optimal secondary structure with a minimum free energy of ~ -480 kcal/mol prepared a suitable ΔG in nucleotides of mRNA and there was not have a hairpin or pseudo knot in first nucleotides (Fig. 1).

Protein secondary structure analysis showed that 58.70%, 7.07% and 34.24% of protein sequences were alpha-helix, extended strand and random coil, respectively. As we expect, there were two helixes in positions 485-495 and 520-525 that correlated with the position of linkers. There was no signal peptide cleavage site in protein sequence, described previously. Tertiary structure of the protein showed a construction with three determined domains (Fig. 2), which linked together with two linkers. Comparison of chimera protein with native domain structures illustrated that the chimera protein had acceptable stability (~ -14000 Kcal/mol). This data was confirmed by Ramachandran plot.

Expression and purification of recombinant protein. Expression condition was evaluated using the gradient change in temperature, IPTG concentration and growth time. Transformed pET-CP into *E. coli* BL21 (DE3) strain over expression was performed in IPTG final concentration of 1mM at 37 °C for duration of 4 hours (Fig. 3). Solubility of protein was adjusted by culturing in two different temperatures 37 °C and RT and then running the supernatant and precipitant of bacteria lysate by SDS-PAGE to demonstrate the protein existence (Fig. 4); the purified protein was absolutely soluble and illustrated in supernatant of bacteria lysate gel electrophoresis of both temperature. This result was similar to *in silico* prediction. Recombinant chimeric protein elution was purified using Ni-NTA resin. The purified pro-

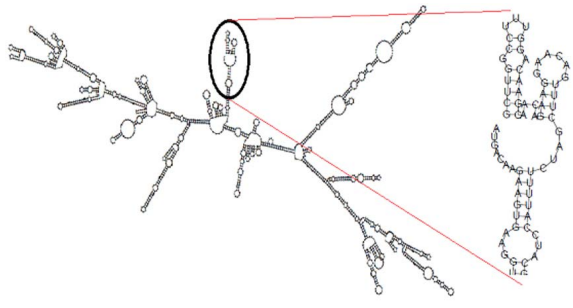

Fig. 1. mRNA secondary structure analysis with Secondary Structure Prediction (http://rna.urmc.rochester.edu/RNAstructure.html/). mRNA with a minimum free energy of ~ -480 kcal/mol. There is no hairpin or pseudo knot in first nucleotides, provide a suitable structure.

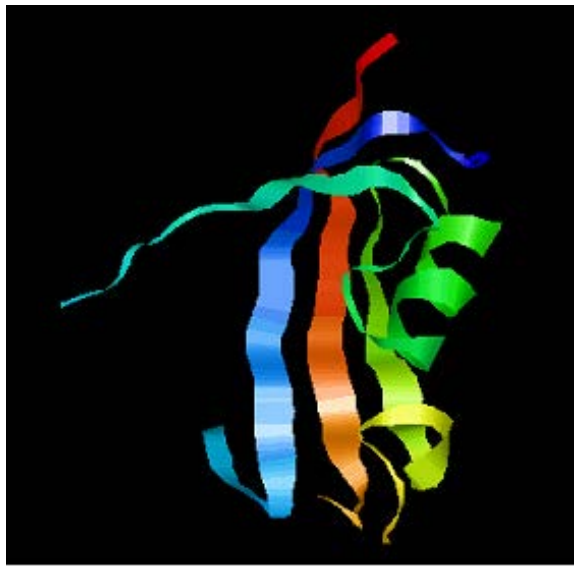

Fig. 2. 3D structure was simulated and modeled using Rasmol-Molecular Graphics Visualisation Tool (http://rasmol.org/). Tertiary structure of the protein shows a final construction of chimera with three determined domains; TF, green segment (in first position), Bp26, red-orange segment (middle) and terminal Omp31, blue segment. EAAAK-Linkers promote the formation of construction, with folding in among three segments. This structure designed according the ideal immunogenicity and conformational structure *in silico*.

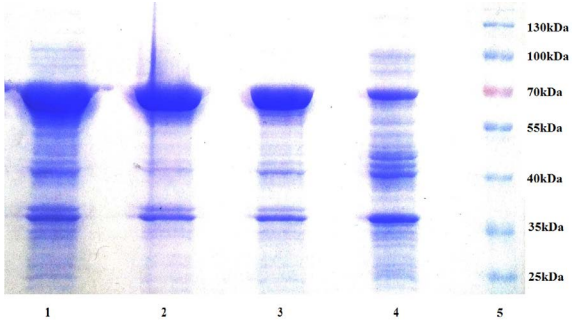

Fig. 3. Presented expressed recombinant protein in SDS-PAGE; lane 5, prestained protein size marker (70kDa), lane 4, Negative control cells (non-induced BL21 with pET-CP); lane 3, pellet of IPTG induced bacteria, 2hrs; lane 2, pellet of IPTG induced bacteria, 3hrs; lane 1, pellet of IPTG induced bacteria, 4hrs (concentration: 700µg/ml).

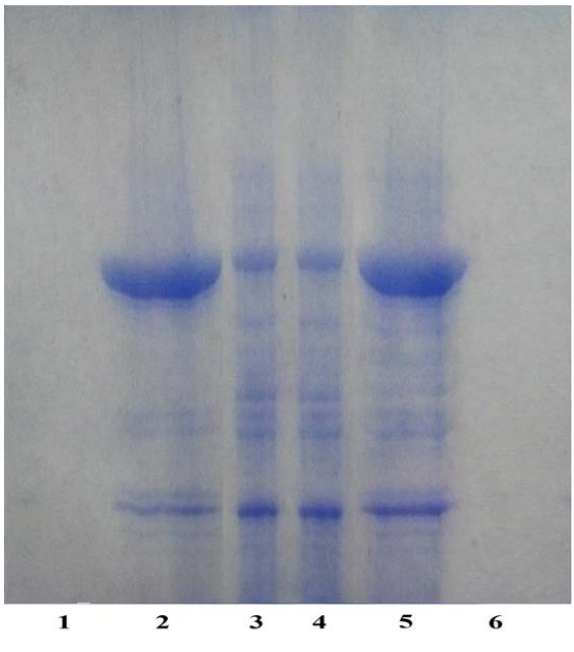

Fig. 4. Protein solubility were performed by culturing in two different temperatures 37 °C and RT and then running the supernatant and precipitant of bacteria lysate by SDS-PAGE; lane 1 and 2, pellet and supernatant of IPTG induced bacteria in RT, lane 3 and 4, uninduced bacteria in RT and 37 °C, lane 5 and 6, supernatant and pellet of IPTG induced bacteria, in 37 °C, respectively. According to figure, there is no obvious expression of protein in pellet of bacteria lysate (lane 1 and 6) in compare with lane 2 and 5 (supernatant); that indicate the solubility of protein in supernatant of bacteria lysate.

tein was monitored by SDS-PAGE and its concentration was estimated; The concentration of eluted protein after dialysis against 0.1 M phosphate buffered saline (PBS, pH=7.4) for 72 hrs in cold room was 700µg/ml. MW of protein was ~ 70kDa in compare with protein ladder bands; because of addition the amino acid residues of pET28a, MW of the purified protein, was higher (~ 5kDa) than bioinformatics prediction (~ 65kDa) (Fig. 5).

Western blot analysis. The results of western blot assay using anti-His Tag antibody confirmed that major band observed in SDS-PAGE (~ 70kDa) was recombinant protein (Fig. 5).

Circular Dichroism (CD). Circular Dichroism analysis of protein was evaluated to determine the physicochemical parameters; alph-helix: 55.8%, extended strand: 9.9% and random coil: 34.3%, approximately similar to bioinformatics prediction.

ELISA. The Immunogenicity of chimeric antigen was evaluated by ELISA after injection of protein at 0, 14, 28 and 38 days. Using ELISA, 1/250 diluted sera from healthy control group yielded ODs

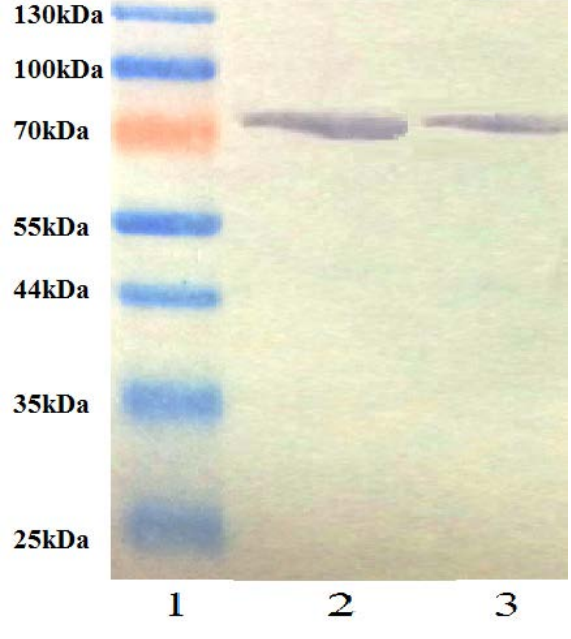

Fig. 5. Western blotting analysis of purified protein showed a single band, corresponding to the expected size of recombinant protein: protein size marker, lane 1; anti-His Tag antibody, lane 2 and pulled sera from infected sera, lane 3.

between 0.203 and 0.359 (mean, 0.278; SD, 0.049), resulting in a cutoff value of 0.425 (Fig. 6). Results of sera samples from injected recombinant protein with/out adjuvant yielded days 14, 28 and 38, ODs mean and SD, showed in Figs 6 and 7 in detail. There was no significant difference between OD results of injected group with/out adjuvant ($p < 0.05$).

Safe elimination of infectious diseases by the effects of recombinant subunit vaccines, which are well defined, avirulent, noninfectious, nonviable, and safe, is an important advantage in compare with live hazardous vaccines because of remnant virulence and infectious potential of viable microorganisms, such as *Brucella* (4, 6-7). Concurrence of results obtained through bioinformatics approach in laboratory experiments demonstrates that *in silico* analysis can be utilized for vaccine design in a safe manner, in contrast with live vaccines (21-24). In brucellosis infection, the ability of induction both B/T-cell responses is important in a new vaccine candidate; therefore, mapping of B/T-cell antigenic determinants by *in silico* approaches is an important method for designing a successful vaccine (25-28).

There are several components in *Brucella* with immune stimulation activity; among these antigens, immune response of B/T-cells to TF (acts as a chaperone by maintaining the newly synthesized protein in an open conformation), Bp26 (26 kDa periplasmic immunogenic protein), and Omp31 (major outer membrane protein associated with peptidoglycans)

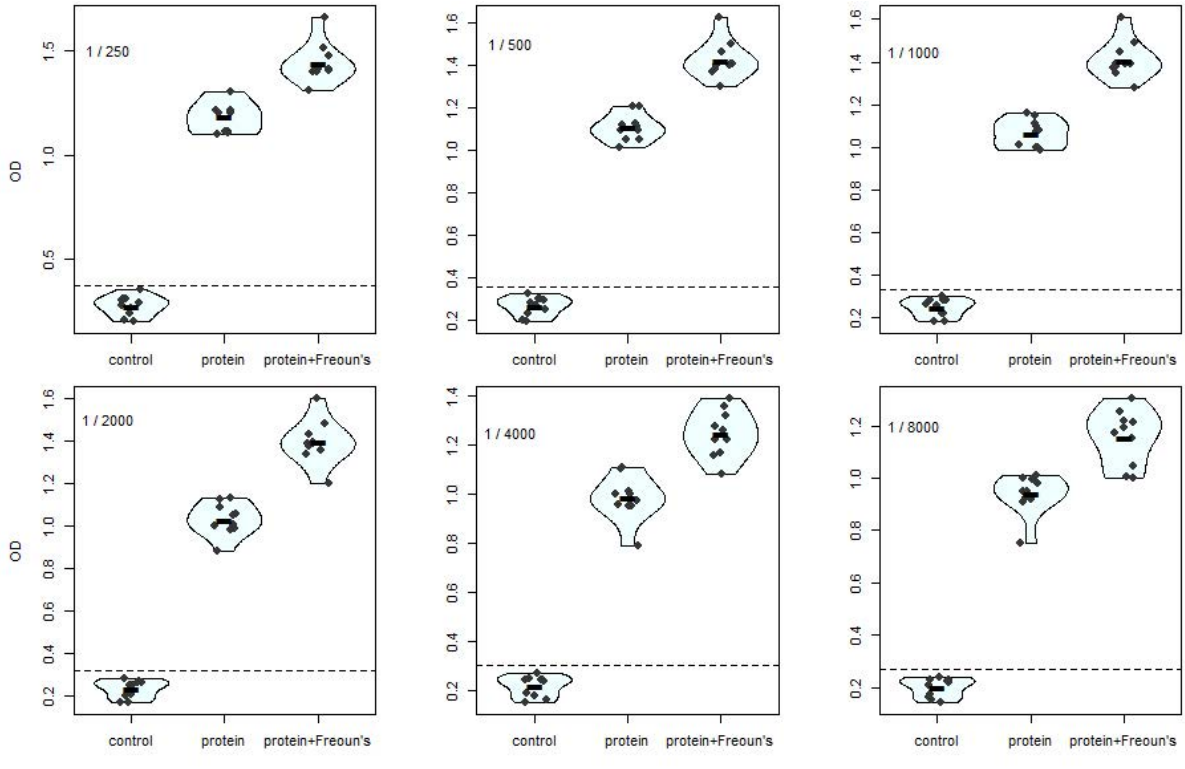

Fig. 6. Violin plot of non/immune sera of mice in days 38. Sera dilution 1/250 to 1/8000 indicate different values of data in three groups: negative control (non-immunized) and protein injected mice with/out Freund's adjuvant. 1/8000 diluted sera from healthy control group yielded ODs between 0.146 and 0.241 (mean, 0.200; SD, 0.036), resulting in a cutoff value of 0.308 (indicated by the broken dashed line). Results show the significant difference between injected groups and negative control groups ($p < 0.05$); there was no significant difference between OD results of injected group with/out adjuvant ($p > 0.05$). Control (non-immunized negative control), protein (injected group without adjuvant) and protein + F (injected group by protein and adjuvant).

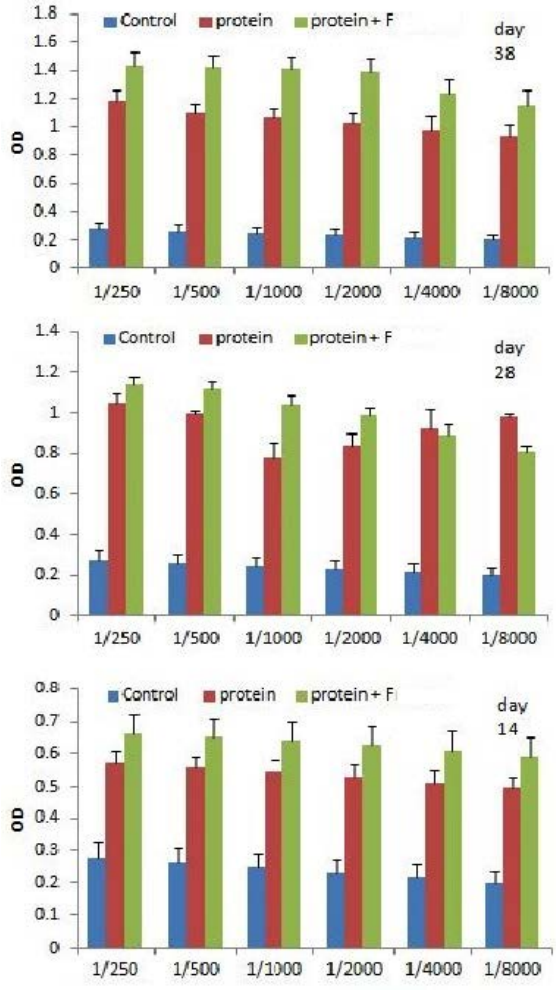

Fig. 7. Sera samples from control and injected group by recombinant protein with/out adjuvant yielded days 14, 28 and 38. ODs mean and SD of each group are showed in detail. High levels of antibody titers in injected mouse in compare with control groups indicate the proper immune-stimulation of recombinant protein ($P < 0.05$); there was no significant difference between OD results of injected group with/out adjuvant ($p > 0.05$). The cutoff value for the assay was calculated as the mean specific OD plus 3standard deviation (SD) for 10 sera from non-immunized mice (control group). Control (non-immunized negative control), protein (injected group without adjuvant) and protein + F (injected group by protein and adjuvant).

immunodeterminant epitopes has been described by other researchers (11-19). Although the immunity validation of TF, Omp31, and Bp26was well defined, the immunogenicity property of these three components combination was not described previously. Because of known proper immune stimulation of these antigenic determinants individually, we constructed a recombinant subunit chimera to increase the immune response against *Brucella* spp. As we described in this study, the *in silico* prediction of chimera indicated that B/T-cell epitopes from each protein caused immune stimulation.

In this study, we evaluated the immunogenicity properties of a chimeric protein in a murine model at days 0, 14, 28, and 38. High level of antibody titers in injected mice in comparison with control groups indicates the proper immune-stimulation by the recombinant protein, as measured by ELISA ($P < 0.05$). Our results demonstrated that the recombinant protein is able to induce vigorous immunoglobulin G response in comparison to control groups ($P < 0.05$). In situation 48–74 residues of Omp31, there is a highly conserved hydrophilic loop regarding as protective epitope (11). Also, antigenic determinants of TF and Bp26 (residues$^{87\text{-}111}$) can induce immune response (14, 16, 18-19). These specifications of TF, Bp26 and Omp31 considerate the potential of these peptides as good component for a subunit vaccine design, as showed in our results. Evaluation of immune responses to Bp26 and Omp31 epitopes in the attenuated *Brucella melitensis* vaccine showed the efficacy of this component in immunity against *Brucella* (29). In other study the protective effect and immune responses against Omp31 and Bp26 was evaluated in mice challenged with *Brucella* (30). It has been shown that *Brucella melitensis* Rev.1 vaccine single and double deletion mutants of the bp26 and omp31 affect the protective efficacy against brucellosis (16). However, in many previous studies, the role of TF, Bp26 and Omp31 immunodominants in immune responses were showed (11, 14, 18-19, 31-32). Results of this report indicate that the antigenic determinants of this recombinant protein could induce antibody titers in injected mice in contrast with the control groups, as measured by ELISA; the OD values of 1/250 to 1/8000 dilution of the mice immune sera indicate that the present protein has been able to stimulate immune system in terms of antibody production. Therefore, the sera of immunized mice reacted with recombinant chimeric protein molecule. In addition, *in silico* data showed induction of both B- and T-cell mediated immune responses, which is important for the design of a protective vaccine. However, it is an ongoing project and further studies focusing on enhancing the efficacy of TF, Bp26, and Omp31 recom-

binant subunit based vaccine using different adjuvant or vaccine strategies are underway. Estimation of the *in silico* and *in vitro* data accuracy and reliability were necessary; and *in vitro* experiments showed the accurate *in silico* designing of recombinant protein in physicochemical parameters, over expression, stability, and immune response prediction (antibody reactivity) in mice. Although different bioinformatics databases can result in different prediction by various software, they do not affect the design of a new recombinant vaccine enormously, as we report in this and previous study.

CONCLUSION

Previous studies showed that multivalent recombinant vaccines can elicit a vigorous stimulation in immune response and better protection efficacy in compare with the pertinent univalent vaccines (4, 6, 8). Our results indicate that this chimeric protein could be a potential immunogenic candidate for development of new subunit vaccines against *Brucella.* Moreover, future studies focusing on enhancing protective activity of Th1/2 response of this recombinant protein are underway.

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REFERENCES

1. Seleem MN, Boyle SM, Sriranganthan N. Brucellosis: A reemerging zoonosis. *Vet Microbiol* 2010; 140:392-398.
2. Pappas G. The changing *Brucella* ecology: novel reservoirs, new threats. *Int J Antimicrob Agents* 2010; 36:8-11.
3. Fugier E, Pappas G, Gorvel JP. 2007. Virulence factors in brucellosis: implications for aetiopathogenesis and treatment. *Exp Rev Mol Med* 2007; 9:1-10.
4. He Y. 2012. Analyses of *Brucella* pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics. *Front Cell Infect Microbiol* 2012; 2:1-17.
5. Nicoletti P. Brucellosis: past, present and future. *Prilozi* 2010; 31:21–32.
6. Perkins SD, Smither SJ, Atkins HS. Towards a *Brucella* vaccine for humans. *FEMS Microbiol Rev* 2010; 34:379-394.
7. Costa Oliveira S, Costa Macedo G, Augusto de Almeida L, Souza de Oliveira F, Oñate A, Cassataro J, et al. Recent advances in understanding immunity against brucellosis: application for vaccine development. *Open Vet Sci J* 2010; 4:102-108.
8. Dorneles EM, Sriranganathan N, Lage AP. Recent advances in *Brucella abortus* vaccines. *Vet Res* 2015; 46:76.
9. He Y, Xiang Z. Bioinformatics analysis of *Brucella* vaccines and vaccine targets using VIOLIN. *Immunome Res* 2010; 6 (Suppl. 1), S5.
10. Avila-Calderón ED, Lopez-Merino A, Sriranganathan N, Boyle SM, Contreras-Rodríguez A. A history of the development of *Brucella* vaccines. *Biomed Res Int 2013;2013-743509.*
11. Cassataro J, Estein SM, Pasquevich KA, Velikovsky CA, Barrera S, Bowden R, et al. Vaccination with the recombinant *Brucella* outer membrane protein 31 or a derived 27-amino-acid synthetic peptide elicits a CD4+ T helper 1 response that protects against *Brucella melitensis* infection. *Infect Immun* 2005; 73:8079-8088.
12. Cassataro J, Pasquevich K, Bruno L, Wallach JC, Fossati CA, Baldi PC, et al. Antibody reactivity to Omp31 from *Brucella melitensis* in human and animal infections by smooth and rough *Brucella*. *Clin Diagn Lab Immunol* 2004; 11:111-114.
13. Cassataro J, Pasquevich KA, Estein SM, Laplagne DA, Zwerdling A, Barrera s, et al. A DNA vaccine coding for the chimera BLSOmp31 induced a better degree of protection against *B. ovis* and a similar degree of protection against *B. melitensis* than Rev.1 vaccination. *Vaccine* 2007; 25:5958–5967.
14. Qiu J, Wang W, Wu J, Zhang H, Wang Y, Qiao J, et al. Characterization of periplasmic protein BP26 epitopes of *Brucella melitensis* reacting with murine monoclonal and sheep antibodies. *PloS One* 2012; 7: e34246.
15. Seco-Mediavilla P, Verger JM, Grayon M, Cloeckaert A, Marin CM, Zygmunt MS, et al. Epitope mapping of the *Brucella melitensis* BP26 immunogenic protein: usefulness for diagnosis of sheep brucellosis. *Clin Diagn Lab Immunol* 2003; 10:647-651.
16. Cloeckaert A, Baucheron S, Vizcaino N, Zygmunt MS. Use of recombinant BP26 protein in serological diagnosis of *Brucella melitensis* infection in sheep. *Clin*

Diagn Lab Immunol 2001; 8:772-775.
17. Debbarh HS, Zygmunt MS, Dubray G, Cloeckaert A. Competitive enzyme-linked immunosorbent assay using monoclonal antibodies to the *Brucella melitensis* BP26 protein to evaluate antibody responses in infected and *B. melitensis* Rev.1 vaccinated sheep. *Vet Microbiol* 1996; 53:325-337.
18. Yang X, Walters N, Robison A, Trunkle T, Pascual DW. Nasal immunization with recombinant *Brucella melitensis* bp26 and trigger factor with cholera toxin reduces *B. melitensis* colonization. *Vaccine* 2007; 25:2261-2268.
19. Deuerling E, Patzelt H, Vorderwulbecke S, Rauch T, Kramer G, Schaffitzel E, et al. Trigger factor and DnaK possess overlapping substrate pools and binding specificities. *Mol Microbiol* 2003; 47:1317-1328.
20. Ghasemi A, Ranjbar R, Amani J. *In silico* analysis of chimeric TF, Omp31 and BP26 fragment of *Brucella melitensis* for development of a multi subunit vaccine candidate. *Iran J Basic Med Sci* 2014; 17:172-180.
21. Yang X, Skyberg JA, Cao L, Clapp B, Thornburg T, Pascual DW. Progress in *Brucella* vaccine development. *Front Bio* 2013; 8:60-77.
22. He Y. Analyses of *Brucella* pathogenesis, hostimmunity, and vaccine targets using systems biology and bioinformatics. *Front Cell Infect Microbiol* 2012; 2:2.
23. Olsen S. Recent developments in livestock and wildlife brucellosis vaccination. *Revue Scientifique et Technique (International Office of Epizootics)* 2013; 32:207-217.
24. Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. *Vet Microbiol* 2002; 90:479-496.
25. Skendros P, Boura P. Immunity to brucellosis. *Rev Sci Tech* 2013; 32:137-147.
26. Oliveira SC, Macedo GC, Augusto de Almeida L, Souza de Oliveira F, Oñate A, Cassataro J, et al. Recent Advances in Understanding Immunity Against Brucellosis: Application for Vaccine Development. *The Open Vet Sci J* 2010; 4:102-108.
27. Baldwin CL, Goenka R. Host immune responses to the intracellular bacterium *Brucella:* does the bacterium instruct the host to facilitate chronic infection? *Crit Rev Immunol* 2006; 26:407-442.
28. Moreno-Lafont MC, López-Santiago R, Zumarán-Cuéllar E, Paredes-Cervantes V, López-Merino A, Estrada-Aguilera A, et al. Antigen-specific activation and proliferation of CD4+ and CD8+ T lymphocytes from brucellosis patients. *Trans roy Soc trop Med Hyg* 2002; 96:340-347.
29. Wang W, Wu J, Qiao J, Weng Y, Zhang H, Liao Q, et al. Evaluation of humoral and cellular immune responses to BP26 and OMP31 epitopes in the attenuated *Brucella melitensis* vaccinated sheep. *Vaccine* 2014; 32:825-833.
30. Gupta V, Radhakrishnan G, Harms J, Splitter G. 2012. Invasive *Escherichia coli* vaccines expressing *Brucella melitensis* outer membrane proteins 31 or 16 or periplasmic protein BP26 confer protection in mice challenged with *B. melitensis. Vaccine* 2012; 30:4017-4022.
31. Díaz AG, Clausse M, Paolicchi FA, Fiorentino MA, Ghersi G, Zylberman V, et al. Immune response and serum bactericidal activity against *Brucella ovis* elicited using a short immunization schedule with the polymeric antigen BLSOmp31 in rams. *Vet Immunol Immunopathol* 2013; 154:36-41.
32. Estein SM, Fiorentino MA, Paolicchi FA, Clausse M, Manazza J, Cassataro J, et al. The polymeric antigen BLSOmp31 confers protection against *Brucella ovis* infection in rams. *Vaccine* 2009; 27:6704-6711.