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Protective Immunity of a Novel Multi-Epitope Vaccine Encoding OMP31, TF, BLS, SOD, BP26, and L9 Against Brucella spp. Infection

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ABSTRACT

Background*: Brucella* is a type of bacteria that causes a disease known as brucellosis in both humans and animals. Many different vaccine formulations are available for this disease; however, vaccines based on epitopes have shown to be effective, especially in combating this pathogen. In the present study, we designed a multi-epitope vaccine against brucellosis using a chimeric protein that combines segments from various *Brucella* proteins known to contain both B- and T-cell epitopes.

Methods: In this study, a vaccine candidate was developed using multiple epitopes derived from various proteins, including OMP31, TF, BLS, SOD, BP26, and L9. These epitopes were selected based on their high density of both B-cell and T-cell epitopes. The construct of the vaccine candidate was inserted into a pEGFP-N1 vector and introduced into HEK-293T cells. Subsequently, the vaccine was tested on different groups of mice; some received the expressed protein in *E. coli*, while others received the DNA vaccine candidate. An ELISA assay was employed to evaluate the humoral immune response.

Results: Both the MEB protein (Pro/Pro) and pCI-MEB plasmid/MEB protein (DNA/Pro) groups showed a specific humoral response. The anti-DNA vaccine antibody titer did not rise as high as that of the protein groups; however, the observed protection indicated the efficiency of the DNA vaccine in activating the immune system.

Conclusion: While the chimeric DNA vaccine candidate induced a weaker humoral response, it remained effective in protecting against virulent strains of *B. abortus* and *B. melitensis* in the challenge route.
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Keywords: Brucellosis, DNA vaccines, Humoral immunity, Subunit vaccines

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List of Abbreviations:
B. abortus: *Brucella abortus*; **CFU:** colony-forming units; **DAB:** 3,3'-Diaminobenzidine; **ELISA:** enzyme-linked immunosorbent assay; **ELISA:** Enzyme-linked immunosorbent assay; **IEDB:** Immune Epitope Database; **MEB:** multi-epitopes of *Brucella spp.*; **NS:** normal saline; **PBST:** Phosphate Buffered Saline-Tween; **3D:** three dimensional; **TMB:** 3,3´,5,5´-tetramethylbenzidine

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INTRODUCTION

Brucella is a genus of Gram-negative bacteria that can infect both humans and animals, leading to a disease known as brucellosis. This disease is caused by a facultative intracellular pathogen and classified as a zoonotic disease, i.e. it can be transmitted from animals to humans[1]. Each year, brucellosis affects over 500,000 individuals, making it a major public health concern[2]. In humans, the disease is recognized as a potentially hazardous condition, while in animals, it causes significant economic losses due to abortion and reduced milk production[3].

Brucellosis is an infectious disease caused by various species of *Brucella*, including *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*[4]. *B. pinnipedialis*, *B. ceti*, and *B. microti* are other *Brucella* species that have also been identified[5]. Among all these species, *B. melitensis* and *B. abortus* are considered the most pathogenic to humans[6]. *Ba. abortus* primarily affects bovines, while *B. ovis* and *B. melitensis* are responsible for causing brucellosis in sheep[5]. Live attenuated strains of *Brucella*, including *B. abortus* strain S19, *B. abortus* strain RB51, and *B. melitensis* strain Rev.1, are currently used to protect animals against brucellosis[7]. However, these vaccines have limitations and are not effective in eliminating the disease. Moreover, they can cause abortion in pregnant animals and may pose risks to humans[8,9]. Therefore, it is crucial to develop an efficient vaccine to completely eradicate brucellosis[3].

In modern biotechnology, epitope-based vaccines are considered more effective against *Brucella spp.* compared to other vaccine types. These vaccines can induce both cellular and humoral immunity. The cellular immune system plays a crucial role in eliminating intracellular pathogens, while specific antibodies against *Brucella* antigens can neutralize the pathogen[10]. Epitope-based vaccines offer several advantages as they are uncomplicated to be constructed, can target key antigenic segments, have enhanced biosafety due to their simple chemical composition, and are able to control the activation of the immune response[11]. One study has identified several potential protective antigens in *Brucella*[12]. To develop epitope-based diagnostic tools and vaccines, it is essential to identify suitable epitopes for therapeutic and preventive interventions, as well as diagnostic purposes[13]. Given the time-consuming and costly nature of experimental screening of large sets of peptides, in silico strategies utilizing $CD4^+$ T-cell epitope mapping on protein antigens, can play a crucial role in developing epitope-based vaccines[11]. These antigens have ability to induce both cellular and humoral immune responses, and their epitopes can be applied in the production of multi-epitope vaccines targeting *Brucella*. Multi-epitope vaccines include fragments of various antigens with a high density of B- and T-cell epitopes, stimulating both arms of the immune system for enhanced protection against *Brucella* infection[14-20].

Our goal was to develop a multi-epitope vaccine against brucellosis using a chimeric protein that combines segments from various *Brucella* proteins known to contain both B- and T-cell epitopes, namely OMP31, TF, BLS, SOD, BP26, and L9. This approach could offer several advantages, including increased immunogenicity, simplified design, enhanced biosafety, and improved control over the activation of immune response. We selected these specific antigens based on the previous studies that identified them as potential protective antigens against brucellosis. Herein, by combining these elements into a single chimeric protein, we aimed to further enhance their protective potential.

MATERIALS AND METHODS

Experimental animals

BALB/c mice were purchased from Razi Vaccine and Research Institute, Tehran, Iran, and were housed under the care of staff with access to water and food ad libitum. Mice were kept in accordance with the standard guidelines of the National Institutes of Health in the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, Revised 1978).

Bacterial strains

E. coli ATCC 25922 was used as a host for plasmid propagation and cloning procedure. Tryptic soy agar sourced from Razi Vaccine and Research Institute was used to cultivate the virulent strains of *B. melitensis*16M ATCC 11649 and *B. abortus* 544 ATCC 21749.

Sequence availability and homologies

The sequences of the *OMP31*, *TF*, *BLS*, *SOD*, *BP26*, and *L9* genes were obtained from UniProtKB (www.uniprot.org). To ensure that all the selected sequences were preserved, they were submitted to the NCBI database for blasting.

Design of chimeric construction and codon optimization

In this study, we employed hydrophobic linkers to combine epitopes from *OMP31*, *TF*, *BLS*, *SOD*, *BP26*, and *L9* in order to create a novel antigenic sequence. The mRNA of the chimeric gene was assessed using Mfold software, and the chimeric codons were optimized for optimal expression in a eukaryotic host using Gene

Designer software[21,22]. The immunogenicity and allergenicity of the entire antigen were predicted using VaxiJen and AllerTOP servers[23,24].

Prediction of secondary and tertiary structures

Secondary structure of the chimeric protein was predicted using the GOR4 server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html)[25,26]. The third and final structures were found using SWISS-MODEL software[27]. To assess the stability and reliability of the structure, we employed the Ramachandran plot via the PROCHECK server and the ProSA-web server[28,29].

Prediction of mRNA structure

The evaluation of chimeric gene mRNA was performed by the Mfold web server (http://www.unafold.org/)[30,31].

Immunoinformatic studies

Several features were identified using methods from the IEDB (http://tools.iedb.org/main/bcell/) to predict the location of B-cell epitopes[32]. Additionally, the ABCpred server (http://www.imtech.res.in/raghava/abcpred/ABC_submission.html) was employed for predicting B-cell epitopes[33]. The ElliPro server was used to predict the spatial epitopes of B cell (http://tools.iedb.org/ellipro/)[34]. Peptide components associated with MHC classes I and II were also predicted using SYFPEITHI software (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm)[35].

Synthesis of the chimeric gene and subcloning in mammalian expression plasmids pCI and pEGFP-N1

The chimeric gene was produced by Biomatic Company (Canada) and inserted into the pUC57 cloning vector. The pCI vector was selected to express the MEB (multi-epitopes of *Brucella spp.*) protein in mouse models. The pEGFP-N1 vector was used to confirm the MEB protein expression in vitro via a GFP tag. To prepare for cloning, the synthetic gene construct (MEB) was digested using *Eco*RI and *Sal*I restriction enzymes (Fermentas, USA) for the pCI vector and *Eco*RI and *Bam*HI for the pEGFP-N1 vector. The digested gene was ligated into vectors at 10 °C overnight using T4 DNA ligase enzyme (Fermentas). The ligation product was then transformed into *E. coli* DH5α competent cells and cultured on LB agar containing 100 µg/ml of ampicillin and 50 µg/ml of kanamycin for the pCI and pEGFP-N1 vectors, respectively. Colonies were analyzed using *Eco*RI and *Sal*I for pCI vector and *Eco*RI and *Bam*HI for the pEGFP-N1 vector. Positive clones were then sequenced. Plasmid purification was performed based on the large-scale method[36]. The concentration and purity of the DNA plasmids were determined by a nanodrop at 260 nm and 260/280 nm, respectively.

Expression in eukaryotic cell line

The HEK-293T cell line, which was obtained from the National Cell Bank of Pasteur Institute of Iran, was cultured in RPMI 1640 media. The plates were incubated in a 5% CO_2 incubator at 37 °C overnight. For transfection, lipofectamine 2000 (Invitrogen, USA) was used, and each well of six-well tissue culture plates received 2.5 ml of complete culture medium containing 5×10^4 cells. Afterwards, the plates were incubated in a 5% CO_2 incubator at 37 °C overnight. On the following day, 3 µg of DNA was mixed with 250 µl of RPMI medium, and 10 µl of lipofectamine was mixed with 250 µl of RPMI medium in separate tubes. The two mixtures were then combined and incubated at room temperature for 20 minutes. The mixture was added to six-well plates containing the attached cells, and the plates were again incubated at 37 °C. After six hours, the supernatant was replaced with fresh complete RPMI medium, and the plates were incubated at 37 °C for 72 h. Finally, the expression of MEB protein was analyzed.

Expression in *E. coli* BL21 (DE3)

The MEB construct was subcloned into the pET28a vector using *Eco*RI and *Bam*HI restriction enzymes for expression in *E. coli* BL21 (DE3). The recombinant MEB clones were grown in LB broth medium containing 50 µg/ml of kanamycin in a shaking incubator at 37 °C. Cells were induced with 1 mM of β-D-1-thiogalactopyranoside (IPTG) and harvested after 4 hours by centrifugation at 5600 ×g 10 minutes. Following centrifugation to collect the cells, the pellet was suspended in a phosphate buffer containing 100 mM of NaH_2PO_4 and 10 mM of Tris-HCl, and then lyzed by ultrasonication. The MEB protein was purified through a Ni-NTA column (Qiagen, Netherlands) following the manufacturer's instructions. After equilibrating the column with the binding buffer, the sample was loaded onto the column. Non-specifically bound proteins were removed by washing the column with increasing concentrations of imidazole (20, 40, and 100 mM). The MEB protein attached to the column was eluted using 250 mM of imidazole and subsequently refolded by dialysis. The purified MEB protein with a molecular weight of 47 kDa was dissolved in normal saline for future use. The purity of the MEB recombinant protein was assessed using the SDS-PAGE method, while its identity was confirmed through a Western blot analysis.

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Western blot analysis

The protein produced from the *E. coli* BL21 (DE3) host was separated by electrophoresis on 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Merck, USA) with a transfer buffer. After washing, the membrane was blocked to prevent non-specific binding. A mouse horse radish peroxidase-conjugated anti-His-tag IgG (1:10000 in PBST) was added to the membrane, which was incubated for two hours. The membrane was then washed with PBST and treated with DAB. The reaction was finally stopped using distilled water.

Immunization of mice

Six-week-old female mice (20-24 g; Razi Vaccine and Research Institute) were divided into five groups of 10 mice and immunized with four booster doses on days 0, 14, 28, and 42. To assess the immunogenicity of the GFP tag in the pEGFP-N1 vector, an additional group of five mice was included for immunization route. Immunization was carried out with subcutaneous administration of 10 µg of MEB protein in 100 µl of NS combined with Freund's adjuvant (Sigma Aldrich, USA). Following this, three booster doses were administered. To compare the antigenicity of the inserted GFP tag, the mice groups received intramuscular injections of either the pCI vector or pEGFP-N1 vector containing the MEB construct (100 µg of the vector in 100 µl NS, with 50 µl of the solution injected into each quadriceps muscle). The mice group primed with DNA/Pro received an intramuscular injection of 100 µg of MEB-synthetic gene pCI plasmid in 100 µl of NS, followed by subcutaneous boosting with 10 µg of MEB protein in 100 µl of NS combined with Freund's adjuvants for three additional doses. Control groups consisted of the mice treated with NS or the pCI plasmid. Blood samples were collected from various groups of mice at 20, 34, and 48 days after the initial immunization. The sera were then stored at -20 °C for subsequent ELISA.

Indirect ELISA assay

To determine the antibody titer specific to MEB protein, an indirect ELISA method was conducted. First, a purified recombinant synthetic protein obtained from a bacterial expression system was diluted to 3 µg/ml in carbonate buffer with a pH of 9.6. The diluted protein was then used to coat the wells of polystyrene plate, with a volume of 100 µl per well. The plates were stored at 4 °C overnight and subsequently washed four times with washing buffer (PBST). After washing, the wells were blocked with 5% skim milk in PBST at 37 °C for 1 hour. Following this step, the wells were incubated with serial dilutions of the sera at 37 °C for 2 hours. After washing phase, an appropriate dilution of anti-mouse IgG HRP conjugate (1/10000) obtained from Abcam, UK was added to the plates at a volume of 100 µl per well. Following a two-hour incubation at 37 °C, the plates were washed four times with a washing buffer, and then 100 µl of a solution containing TMB from Abcam, was added to each well. After 20 minutes of incubation at room temperature, the reaction was stopped by adding 100 µl of 3N sulfuric acid to each well. Finally, the absorbance of each well was measured at 450 nm.

Challenge approach

Mice were subjected to a challenge through the stimulation of spleen cells as described previously[37]. Each group of mice that was immunized with the pCI-MEB plasmid and MEB protein, was divided into two subgroups of five mice each. Thirty days after the last booster, the mice were challenged by an intraperitoneal injection of 100 µl of PBS containing 2×10^5 CFU of *B. abortus* 544 or *B. melitensis* 16M. Thirty days post bacterial challenge, the mice were sacrificed via cervical dislocation. Their spleens were removed aseptically and weighed. Each spleen was homogenized in a stomacher bag, and then serially diluted. The samples were plated on tryptic soy agar and incubated for three days in 10% CO_2 at 37 °C. The number of CFU of bacteria per spleen was counted, and the results were represented as the mean log CFU ± SD per group. The protection unit was determined by subtracting the mean log CFU of the immunized groups from the control group, which represents the level of protection provided by the vaccine.

Statistical analysis

All presented data from the ELISA assay are the mean values of three readings. Normal data were analyzed using a one-sample t-test and post hoc t-test comparisons. For the statistical analysis, GraphPad Prism software, version 6, and IBM SPSS software, version 22 were used. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Design and construction of chimeric gene

The structure of the MEB gene with linker sites is illustrated in **Figure S1**. Linkers containing HMHMHM and GGGGS repeats were used to separate the gene fragments. These linkers are form an alpha helix to provide optimal flexibility and space between the gene fragments. A histidine tag was added at the end of the gene to facilitate the purification of the recombinant protein. The Kozak sequence, which is important for

efficient initiation of translation, was also added at the beginning of the gene to enhance protein expression. A schematic diagram of the chimeric structure (MEB), with linker sites and the placement of the histidine tag and Kozak sequence, can be seen in **Figure S1**. The VaxiJen database score of 0.8528 suggests that the MEB protein is likely to be an effective antigen. Additionally, predictions from the AllerTOP server exhibit that the MEB protein is unlikely to be an allergen.

Prediction of the second and third structures of the protein

The MEB protein was analyzed for its secondary structure composition, which consists of 28.32% alpha helix, 49.49% random coil, and 22.19% extended strand (Fig. 1). The secondary structure of the epitopes was also evaluated. To generate 3D model of the MEB protein, we made several predictions using the SWISS-MODEL software. The Z-score of QMEAN for the MEB protein model was -0.27, which falls within the normal range of -6 to +2, indicating a high-quality model. As shown in the results obtained from analyzing the tertiary structure of the protein, the domains are effectively separated by the linkers used in the protein design.

Stability and predictability of the structure

The Ramachandran plot is a tool used for evaluating the quality of protein structures. In this case, the plot generated by the PROCHECK server confirms the stability of the MEB protein (Fig. 2). Additionally, the ProSA server provides a Z-score, which assesses the overall quality of the protein model; higher negative scores indicate better quality. In this condition, the Z-score of -2.58 suggests that the MEB protein model is of good quality. Altogether, these results provide evidence that the MEB protein structure is both reliable and stable.

Codon optimization

Codon bias is a crucial factor in the design of DNA vaccines, as it significantly influences their effectiveness in the host. Using rare codons can result in a slower translation rate due to the limited availability of the corresponding tRNAs, which increases the likelihood of ribosome pausing and can affect protein folding. Additionally, rare codons can lead to higher frequency of misincorporation errors, and further reducing the efficiency of protein expression. Therefore, optimizing codon usage to incorporate more frequently used codons can increase overall expression level and stability of the protein, ultimately improving the efficacy of the DNA vaccine. The codon adaptation index ranges from 0 to 1, with a value above 0.8 indicating high expression level of the gene in the host. We optimized the sequence by utilizing eukaryotic codon usage preferences and optimizing GC content and unfavorable spikes to improve mRNA half-life.

Prediction of mRNA structure and its stability

The Mfold software predicted the optimal structure for the genetic construct, yielding a ΔG value of -444.2 kcal/mol. No stable secondary structures, such as stem-loop or hairpin, were observed in the 5' mRNA region, as shown in Figure 3. Furthermore, we assessed the energy level of the first 10 nucleotides of the optimized mRNA, which are crucial for initiating translation by the ribosome. The ΔG of these 10 nucleotides was -8 kcal/mol, suggesting efficient translation.

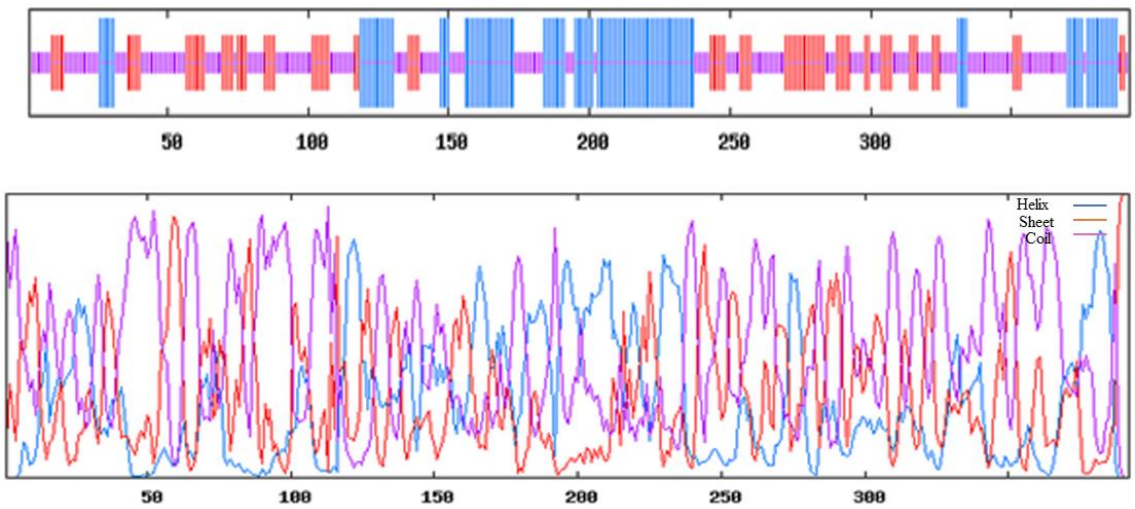

Fig. 1. The GOR4 secondary structure prediction of the MEB protein to find alpha helix, beta sheet, and turn or random coli.

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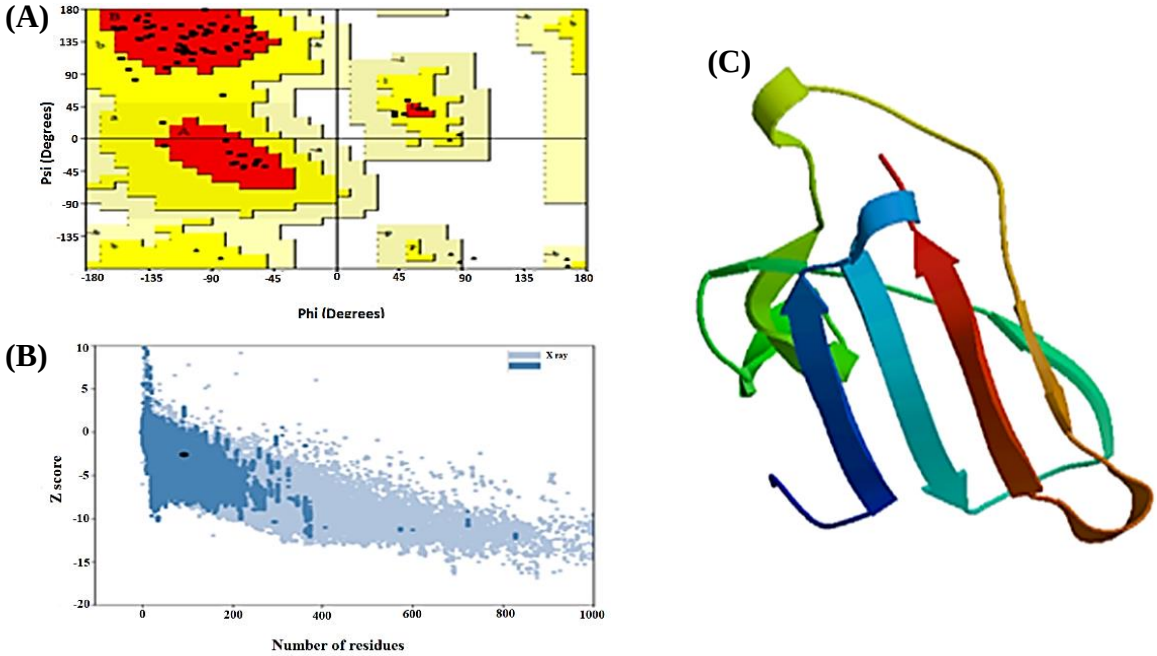

Fig. 2. (A) Ramachandran plot for the MEB protein. The number of residues in the most favored region was 61 (87.1%) and 285 (69.2%). The number of residues in the additional allowed region was 12.9%; (B) 3D structures of the MEB protein; (C) validation of the MEB 3D structure by ProSA-web server.

Results of the immunoinformatic analysis

Predicting the continuous and discontinuous B-cell epitopes

To predict B-cell epitopes of the recombinant protein, we analyzed flexibility, antigenicity, and availability of epitopes on the protein surface. The results are presented in **Table S1**. To predict the B-cell epitopes, the IEDB and ABCpred software were utilized (Fig. 3). The ABCpred software identified 39 linear epitopes, each containing 16 amino acids that could potentially interact with B cells in the recombinant protein sequence. Several epitopes with the highest score are listed in Table 1. Additionally, **Table S2** presents the result of determining discontinuous B-cell epitopes based on the 3D-structural epitopes identified using ElliPro software.

T-cell epitopes and MHC binding peptide affinity

Based on the data presented in Table 2, the IEDB software identified the peptide sequences with high binding scores to MHC Class I, to predict T cell epitopes and MHC Class I binding peptides of the MEB protein.

Expression of the MEB gene in the eukaryotic system

To confirm the proper expression of the constructed DNA vaccine in mammalian cells, the DNA vaccine was cloned into a pEGFP-N1 vector and then transformed into HEK-293T cells. The efficiency of the vaccine was confirmed using fluorescence microscopy, as depicted in Figure 4.

Expression of the MEB gene in the prokaryotic system

After inducing *E. coli* BL21(DE3) with 1 mM IPTG, the MEB protein was expressed. SDS-PAGE analysis of the cell lysate from the induced cells showed the expected recombinant protein with a size of around 47 kDa **(Fig. S2)**. The identity of the purified MEB protein was confirmed by Western blotting analysis. The concentration of the purified protein was estimated to be 300 µg/ml using the Bradford assay. These results are presented in **Figure S3**.

Selected epitopes stimulate immune system

The antibody titers were assessed using an indirect ELISA method. The results indicated a significant increase in the antibody titer against the MEB protein in the prime group after three booster doses, especially for the MEB protein, confirming the immunogenicity of the selected epitopes as vaccine candidates. The antibody levels in DNA vaccine groups also increased after the third boosters compared to the control group. Although the anti-DNA vaccine antibody level did not rise as high as that of the protein groups, the observed protection indicates the efficiency of the DNA vaccine in activating the immune system. All data are presented in Figure 5.

Animal challenges with *B. melitensis* and *B. abortus*

To demonstrate the efficiency of the vaccine, we challenged immunized mice with the pCI MEB

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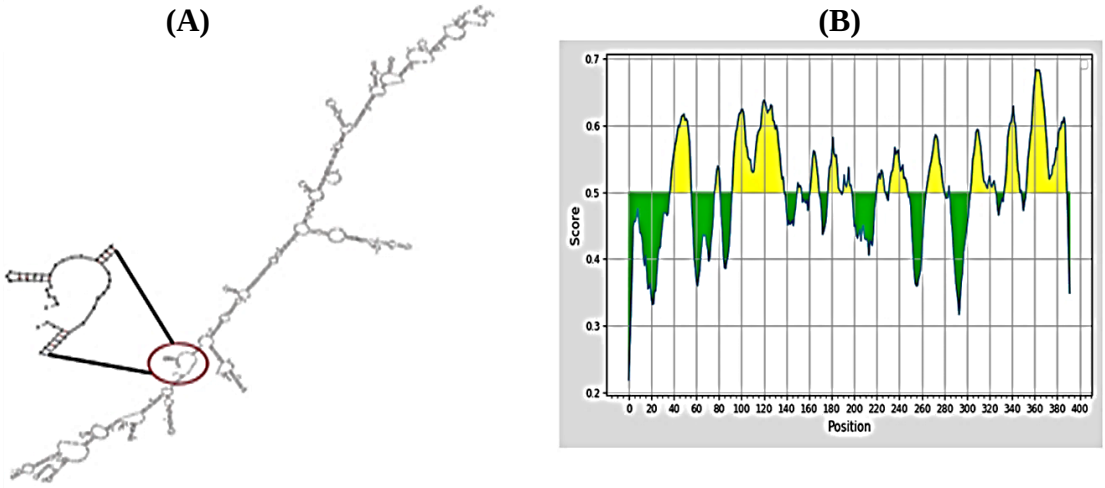

Fig. 3. (A) Analysis of mRNA stability and start codon in the structure; (B) Physiochemical properties of amino acids based on predictive server for linear B-cell epitope. Green regions under threshold color denote unfavorable regions related to the properties of interest. Yellow colors above the threshold share higher scores. The horizontal red line is the threshold. The circle indicates the starting and final nucleotide of mRNA for the attachment of the polymerase enzyme.

gene and MEB protein, along with *B. abortus* and *B. melitensis.* The level of infection was evaluated by determining the CFU of bacteria in spleen. The results, in Table 3 indicated that the groups of immunized mice were significantly protected against *B. abortus* and *B. melitensis* compared to the control group that received either NS or pCI vector. The protection unit presented here refers to the efficiency of the prime group in activating immune system against both strains of *Brucella*, while the efficacy decreased with the DNA vaccine and MEB protein, respectively. Conversely, the data showed that the DNA vaccine could effectively stimulate the immune system against *B. melitensis*, compared to *B. Abortus*. There was no decrease in the CFU in the animals that received pCI vector compared to the control group. Therefore, immunization with the pCI MEB gene provided a higher level of protection against *Brucella* infection compared to MEB protein.

Table 1. Prediction of linear B-cell epitopes in MEB protein based on different parameters using ABCpred software

Sequence	Start position	Score	Sequence	Start position	Score
QGGGGSPTGPGKEVGT	45	0.94	MRLGFTPTERLMVYGT	280	0.81
KGGGGSDGYARNFLLP	363	0.93	NMEKLTPGYHGFHVHE	75	0.80
YIGINAGYAGGKFKHP	255	0.93	GIEDRDLQTGGINIQP	336	0.80
FSGIEQHMHMRLGFTP	271	0.92	RAPIFEEKVVDHLLAN	202	0.80
TVIRGETYHFDIVSNE	2	0.92	HFDIVSNESCRALTDL	10	0.80
ENPSCAPGEKDGKIVP	90	0.91	VHMHMHMFTSSAMAAD	220	0.79
VVISEAPGGLHFKVNM	61	0.90	MHMHMAEKVLDGKADF	122	0.77
ADIIVAEPAPVAVDTF	234	0.90	QPIYVYPDDKNNLKGG	350	0.76
DGKIVPALAAGGHYDP	100	0.90	TSSAMAADIIVAEPAP	228	0.76
LSAYDDGESFSAGNSK	307	0.89	EKVLDGKADFVFSLNY	128	0.76
EKEIYDFLRRTPDAVA	184	0.88	FLLPQGKALRANEANK	375	0.74
PGYHGFHVHENPSCAP	81	0.87	KTKAGGGGGSMKKAGI	322	0.74
GGSMKKAGIEDRDLQT	329	0.86	DLSVEESIAIGNGILT	24	0.74
GGHYDPGNTHHHMHMH	110	0.86	FSAGNSKTKAGGGGGS	316	0.73
TGGINIQPIYVYPDDK	344	0.85	VLPAIEVKDFSKIAVT	145	0.71
DQVRRYPGQEKEIYDF	175	0.85	AGGKFKHPFSGIEQHM	263	0.68
MVYGTGGLAYGKVKTS	291	0.84	HLLANINVHMHMHMFT	213	0.67
REVVDIHMHMRAVYDQ	161	0.84	VFSLNYEVLPAIEVKD	138	0.62
TGPGKEVGTVVISEAP	52	0.83	FLRRTPDAVANLRAPI	190	0.56
TVENEEQGGGGSPTGP	39	0.83			

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Table 2. T-cell epitopes and MHC class I binding peptides in MEB protein predicted by IEDB software

Allele	Seq-num	Start	End	Length	Peptide	Score	Percentile rank
HLA-B*44:03	1	64	72	9	SEAPGGLHF	0.989393	0.01
HLA-A*68:01	1	162	171	10	EVVDIHMHMR	0.987295	0.01
HLA-B*44:02	1	64	72	9	SEAPGGLHF	0.982541	0.01
HLA-B*35:01	1	285	293	9	TPTERLMVY	0.980651	0.01
HLA-A*26:01	1	162	170	9	EVVDIHMHM	0.97996	0.01
HLA-A*26:01	1	247	255	9	DTFSWTGGY	0.969945	0.01
HLA-B*35:01	1	180	188	9	YPGQEKEIY	0.958717	0.02
HLA-A*68:01	1	314	322	9	ESFSAGNSK	0.953382	0.03
HLA-B*15:01	1	302	310	9	KVKTSLSAY	0.94591	0.01
HLA-B*35:01	1	308	316	9	SAYDDGESF	0.941698	0.02
HLA-A*30:02	1	172	180	9	AVYDQVRRY	0.93507	0.01
HLA-A*68:01	1	144	152	9	EVLPAIEVK	0.931915	0.05
HLA-A*33:01	1	153	161	9	DFSKIAVTR	0.926565	0.01
HLA-A*68:02	1	162	170	9	EVVDIHMHM	0.913018	0.02
HLA-B*40:01	1	183	191	9	QEKEIYDFL	0.911219	0.06
HLA-B*40:01	1	64	72	9	SEAPGGLHF	0.906375	0.06
HLA-A*02:06	1	137	145	9	FVFSLNYEV	0.903858	0.04
HLA-B*40:01	1	206	214	9	FEEKVVDHL	0.894356	0.06
HLA-A*68:02	1	137	145	9	FVFSLNYEV	0.888187	0.02
HLA-B*07:02	1	50	58	9	SPTGPGKEV	0.879542	0.05
HLA-A*26:01	1	172	180	9	AVYDQVRRY	0.869395	0.03
HLA-B*53:01	1	240	249	10	EPAPVAVDTF	0.866308	0.03
HLA-B*15:01	1	291	300	10	MVYGTGGLAY	0.864729	0.03
HLA-A*30:02	1	302	310	9	KVKTSLSAY	0.860933	0.01
HLA-B*15:01	1	172	180	9	AVYDQVRRY	0.851855	0.04
HLA-A*02:01	1	137	145	9	FVFSLNYEV	0.84511	0.06
HLA-B*35:01	1	284	293	10	FTPTERLMVY	0.837414	0.06
HLA-A*68:01	1	65	73	9	EAPGGLHFK	0.829585	0.19
HLA-A*03:01	1	172	180	9	AVYDQVRRY	0.826607	0.06
HLA-B*35:01	1	269	278	10	HPFSGIEQHM	0.824662	0.07
HLA-A*33:01	1	162	171	10	EVVDIHMHMR	0.82199	0.02

Seq-num: sequence number

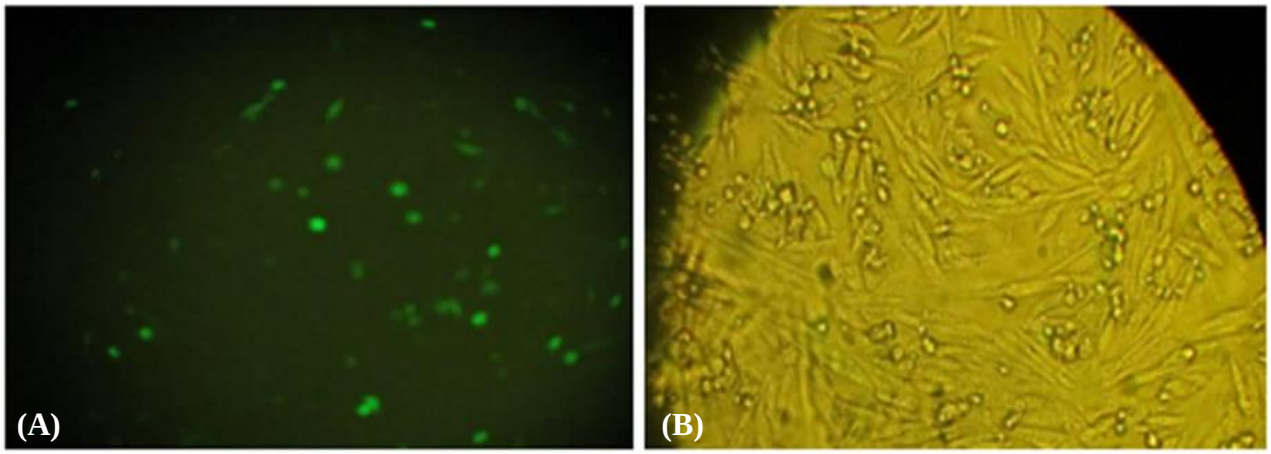

Fig. 4. Fluorescence microscopy results. (A) MEB protein-GFP expressing cells, (B) HEK-293T (negative control).

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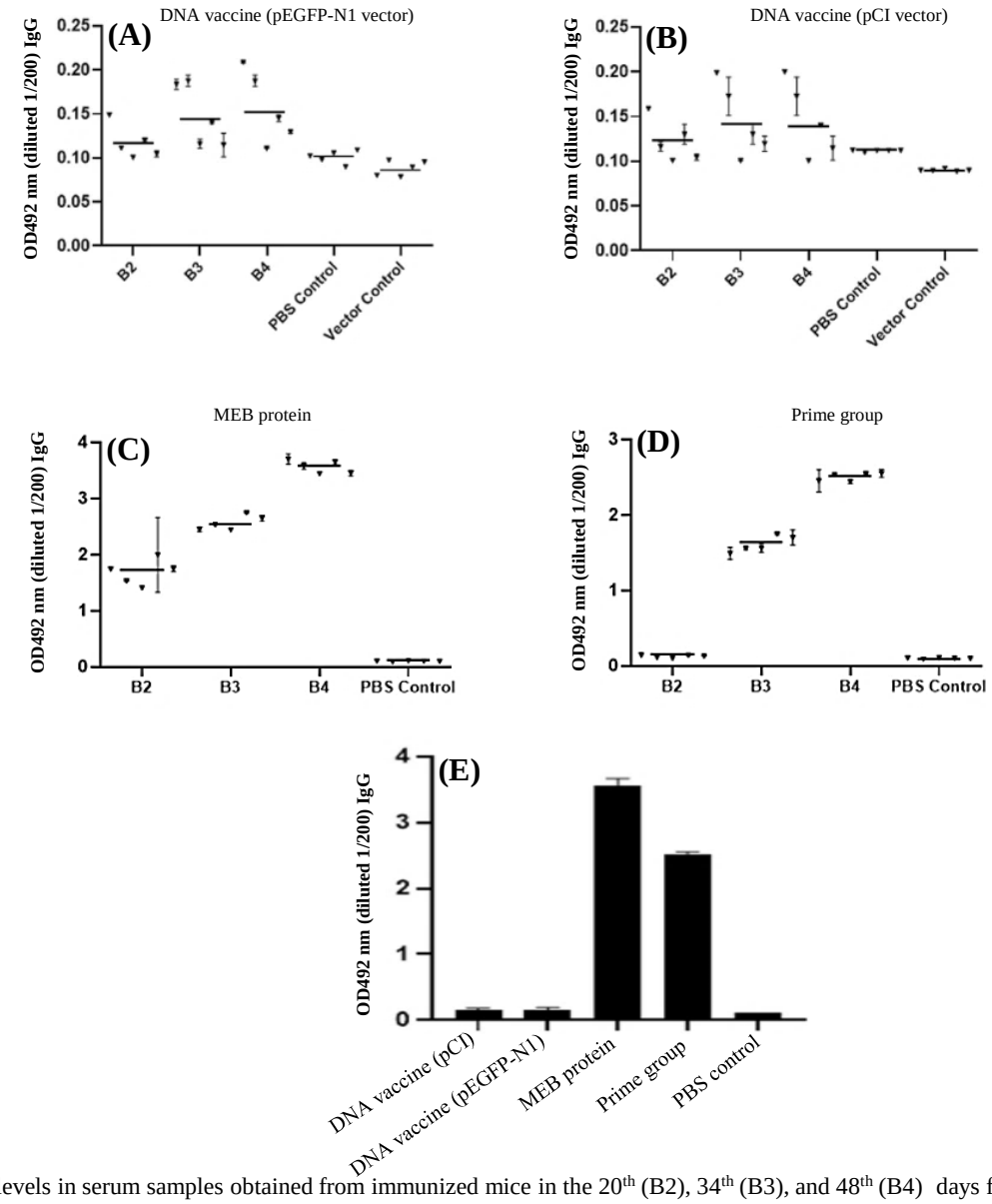

Fig. 5. IgG levels in serum samples obtained from immunized mice in the 20th (B2), 34th (B3), and 48th (B4) days following the first immunization, after 1/200 dilution in PBS. The IgG titers of sera collected from the mice immunized intramuscularly with pEGFP-N1 DNA vaccine (A) or pCI DNA vaccine (B); IgG titers from sera obtained from the mice immunized subcutaneously with MEB protein plus Freund's adjuvant (C) and sera from the prime group of the mice that received intramuscular administration of the pCI DNA vaccine, followed by a subcutaneous boost with MEB protein (D); The immune responses of four groups were evaluated on the 48th day after the first immunization. Each column represents the mean antibody titer of an independent group of immunized mice (E). The horizontal bars indicate the median results from each data set.

DISCUSSION

Brucellosis is a significant infectious disease that affects both humans and domestic animals, resulting in significant economic burden worldwide[8,38]. Given the limitations of the live attenuated *Brucella* vaccine, it is crucial to develop more effective gene-specific subunit vaccines to prevent this disease[39]. While various *Brucella* antigens have been shown to induce an immune response, no studies have investigated the combined effect of these antigens as a DNA vaccine. Previous research has demonstrated the efficacy of individual antigens, such as TF and bp26, as subunit vaccines[20], BLS as a purified recombinant protein, the BLS-based and L9-based DNA vaccines in stimulating both cellular and humoral immune responses[16,18].

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Table 3. The protection against challenges with *B. melitensis* and *B. Abortus* in pCI MEB gene and MEB protein in immunized mice

Mice group	Bacterial	Log_{10} CFU of *Brucella* at spleen	Protection units
	B. Abortus	3.60 ± 0.21	1.50
	B. melitensis	3.54 ± 0.23	1.63
	B. Abortus	3.82 ± 0.21	1.28
	B. melitensis	3.87 ± 0.19	1.30
	B. Abortus	3.15 ± 0.23	1.80
	B. melitensis	3.21 ± 0.23	1.80
	B. Abortus	5.10 ± 0.1	-
	B. melitensis	5.17 ± 0.1	-
	B. Abortus	4.9 ± 0.1	0.20
	B. melitensis	5.0 ± 0.1	0.17

Five groups with five mice in each.

Therefore, in this study, we selected effective epitopes from TF, OMP31, BP26 from *Brucella melitensis*, L9, and SOD from *Brucella abortus*, and BLS from *Brucella ovis*, all characterized by a high density of B- and T-cell epitopes. These epitopes were linked using GGGGS and HMHMHM repeats to ensure their specific display in the final structure of the MEB protein without interference[14-20].

The mRNA was analyzed using bioinformatics and Mfold software, which revealed a thermodynamically stable model with a long-term codon. The Kozak sequence, 5'GCCACC3', was found to play a significant role in enhancing the efficiency of the translation process[31,40]. According to the GOR database[26] the predicted model of MEB protein consists of 28.32% alpha-helix, 49.49% random coil, and 22.19% extended strand. The I-TASSER server[41,42] was used to predict the 3D structure of the MEB protein, and a TM score greater than 0.5 confirmed the accuracy of the topology of protein. To assess the overall quality of the MEB protein model, the Z-score was calculated. The Z-score measures the deviation of the total energy distribution of the 3D model from that of a random conformation. The high reliability of the MEB protein model was further confirmed by the C-score. The results of the Ramachandran plot showed that only a small percentage (12.4%) of residues was located in the outlier region, which may be due to the presence of the MEB junction. B-cell epitopes play a critical role in the development of peptide vaccines. To predict B-cell epitopes, ABCpred was used, which is based on machine learning algorithms. Higher scores for peptides indicate a greater probability of B-cell epitope prediction, as previously demonstrated in other investigations[33,43].

The IEDB was utilized to predict important factors, such as hydrophilicity, flexibility, surface accessibility, and beta-turn formation. The SYFPEITHI database[35] was employed to predict T-cell epitopes. The MEB gene was then subcloned into the pCI and pEGFP-N1 vectors to create a DNA vaccine. To confirm the expression of the MEB protein, the pEGFP-N1 vector containing the synthetic MEB gene was transformed into the HEK-293 T cell line. Successful expression of the MEB protein was confirmed by the expression of GFP. The MEB gene was subsequently subcloned into the pET28a vector for being expressed in bacteria as a recombinant protein. While cellular immunity is often regarded as the primary mechanism for protection and elimination of intracellular bacteria, the antibody titer also plays a crucial role in neutralizing the pathogen before it enters the cell.

In this study, mice were immunized in different vaccination groups to investigate their immune response. Although immunization with the pCI MEB DNA vaccine did not result in a high IgG antibody titer, it still created significant protection in the mice challenged with *B. melitensis* 16 M and *B. abortus* 544 when compared to the control group. A study performed by Cassataro et al.[15] evaluated the immunogenicity and protective efficacy of the *B. melitensis Omp31* gene cloned in the pCI plasmid. While mice vaccinated with pCI *Omp31* did induce cytotoxic $CD8^+$ and $CD4^+$ responses, the observed humoral response was weak. Another study by Onate et al.[17] evaluated the effectiveness of the CuZn SOD DNA vaccine. They found that intramuscular injection of Pacyna-SOD into BALB/c mice induced a robust T-cell proliferative response and increased level of IFN-γ. Our ELISA results showed no significant difference in the antibody titer of the mice immunized with MEB DNA vaccine using either the pCI or pEGFP-N1 vectors, indicating that the GFP expression in the pEGFP-N1 vector did not enhance the immunogenicity of the vaccine. Since the DNA vector is designed for intracellular expression,

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only antigen fragments will be presented on MHC I, preventing B cells from detecting the antigen and producing specific antibodies against this multi-epitope antigen. While ELISA test of sera obtained from the immunized mice can identify B-cell epitopes by detecting antibodies against the recombinant protein, it is not possible to confirm the presence of T-cell epitopes, which may be more effective in reducing *Brucella* infection rates. Therefore, only the immune response against the entire MEB construct could be evaluated through the ELISA assay, while the activity of both humoral and cellular immunity were observed in the challenge rout confirming the efficiency of the designed DNA vaccine.

CONCLUSION

DNA vaccines represent a valuable strategy for long-term antigen expression and immune stimulation. However, the humoral immune response may be limited since the expressed antigens are presented to T cells on MHCI. To address this limitation, recombinant antigens could be presented to enhance the humoral immune response. Furthermore, considering the role of various immunogens from different species may provide broad-spectrum protection against brucellosis. Additional studies are required to improve the efficacy of MEB construct immunization and gain a better understanding of the underlying protection mechanisms.

DECLARATIONS

Acknowledgments

No artificial intelligence services were used for the preparation of this manuscript.

Ethics approval

All experiments described in this study were conducted in compliance with the standard guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No.8023, Revised 1978) to ensure ethical treatment of animal subjects. This includes measures such as proper housing, feeding, and care of animals, as well as minimizing the pain and discomfort of animals used in the study. All methodology was conducted in accordance with national and international standards for the care and use of laboratory animals, with approval from the Ethics Committee at Shahed University (IR.SHAHED.REC.1400.102).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

SSS: investigation, project administration, writing–review; RRA: investigation, writing–original draft preparation, and editing, data analysis; ShN: supervision, conceptualization; AF: investigation, writing–review; SLMG: conceptualization, supervision, writing–review, and editing.

Data availability

All relevant data can be found within the manuscript or in supplementary file.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version contains supplementary material.

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