Evaluation of the designed multi-epitope protein of *Brucella melitensis* in guinea pigs

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A R T I C L E I N F O	A B S T R A C T
	Objective(s): One of the causes of human and animal zoonotic infections is *Brucella melitensis*, which is transmitted to humans through dairy products. It seems for prevention of human infection we might protect the livestock by an efficient protein as a vaccine candidate. For this purpose, the use of immunogenic proteins of bacteria is able to create immunity the same as the traditional vaccines. ***Materials and Methods:*** In this study, by finding the immunogenic antigens of this bacterium by 2-dimensional gel electrophoresis and MALDI-TOF methods and also the proteins reported in other studies, we found the epitopes of the bacterial antigenic determinants *in silico.* Nineteen peptides of T and B epitopes were selected. They were ligated with linkers and after gene synthesis, the designed polypeptide was expressed in *Escherichia coli* BL21. The purified recombinant MEL protein mixed with chitin was injected subcutaneously into three 300 g male guinea pigs three times. Also, PBS control and Rev.1 commercial vaccine groups were considered. ***Results:*** The results show that MEL polypeptide is equal to the Rev.1 vaccine in stimulating secretion of IFNγ and IL2 and specific IgG. High levels of IL-2 emphasize the activation of the cellular immunity, and in particular comparison of PI in guinea pig's spleen cells treated with recombinant MEL protein on days 0 and 5 show that it has significant proliferation compared with PBS unstimulated cells. ***Conclusion:*** This recombinant protein could be a subunit protein with sufficient efficiency in stimulating the humoral and cellular-mediated immune system against *B. melitansis.*

Introduction

Brucellosis is the most common bacterial disease of humans and animals worldwide, with over 500000 infections in humans annually (1, 2). Brucellosis causes high economic loss in livestock by abortion and is known as a life-threatening multisystem disease in humans (3-5). It is an endemic infection in many parts of the world, including the Middle East, Africa, Latin America, Central Asia, and many regions of the Mediterranean basin; and Iran is an endemic region for brucellosis (6, 7). Therefore, this infection is an enormous challenge for health. *Brucella melitensis* is the main cause of brucellosis in sheep, goats, and humans (8). The dairy products of infected animals may contain large numbers of viable organisms (9, 10). The live vaccine B. melitensis strain Rev.1 is used worldwide for prevention of brucellosis in small ruminants (11, 12). The potential risks to veterinarians have always been raised, and confirmation of Rev.1 injection in humans with high-dose experimental inoculation has been demonstrated in volunteers (13). Therefore, a subunit vaccine that is protective against *B. melitensis* is necessary. A number of studies with computational approaches have predicted

epitopes of antigens that are effective in stimulating the immune system and have used these findings in an experimental study aimed at obtaining an epitope-based vaccine (14-17). An immunogenic multitope protein containing antigenic epitopes from several dominant parts of the bacterial structure may provide protective immunity against brucellosis (18). Establishing an effective immune response against Brucella infection requires cell-mediated responses, particularly Th1, which is associated with the production of interferon-gamma, and humoral response, which produces specific antibodies (19-21). In this study, we employed a reverse vaccinology approach and bioinformatics analysis to find protective complex candidates for induction of both cellular and humoral immunity in sheep and goat brucellosis.

Materials and Methods

Antigenic protein identification

Choice of anti-sera and bacterial strain

To analyze the *B. melitensis* immunoreactive protein profile, a total of 10 sheep sera samples naturally infected with *B. melitensis* (contains antibody) and ten

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non-infected, and seven *Mycobacterium* infected sheep (as the negative controls) sera were collected from the Meysam slaughterhouse (Tehran, Iran) for this study. The characterization of negative or positive status of serum samples was performed by using the confirmation serological methods including, Rosebengal, Tube wright, and Coombs wright (22). We created a serum pool of each group of samples. The bacterial strain for *B. melitensis* used in this study was taken from the culture collection of the Razi vaccine and serum research institute (Karaj, Iran).

Extraction of structural proteins

To isolate the whole-cell structural proteins, the strain was cultured for 48 hr in Tryptic Soy Broth (TSB) at 37 °C with shaking (23, 24) at the Razi Vaccine and Serum Research Institute (Karaj, Iran). Briefly, the culture was harvested by centrifugation and washed twice with phosphate buffer saline. The cells were reconstituted in 80% ethanol and centrifuged. Subsequently, the cell pellet was suspended in lysis buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 40 mM Tris base, and 0.002% Bromophenol Blue), sonicated on ice for 1 min (duty cycle: 1.5, amplitude: 90%, UP100H), centrifuged at 12,000g for 10 min at 4 °C and the supernatant was collected and kept at -20 °C.

Two-dimensional polyacrylamide gel electrophoresis (2DE) and Western blotting

Seven-centimeter immobilized pH gradient (IPG) strips with nonlinear range of pH 3-10 (Bio-Rad) were used to perform isoelectric focusing (IEF) and processed as described previously (25). For the Immune blotting procedure, 25 µl of total protein lysate was separated using a precast preparative 15% SDS–PAGE and blotted onto a nitrocellulose membrane (Sigma–Aldrich) by semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) and processed as described previously. We used infected sheep sera (1:500 diluted) as the primary antibody source and the goat anti-sheep total IgG HRP-conjugated as a secondary antibody for *B. melitensis* protein antigens detection. The visualization of signals was performed with substrate buffer (Diaminobanzoic acid), 10 mL PBS, and 30 µl hydrogen peroxidase.

Protein identification by mass spectroscopy and mascot analysis

The protein bands which were reacted with infected sera immunoglobulin were excised from the SDS–PAGE gels and destained, and protein extraction was proceeded by the manufacture process described previously (25). Then, the isolated peptides were purified using 25 µl of acetonitrile, mixed, and dried using a vacuum centrifuge. The protein sample preparation was proceeded according to standard techniques. Subsequently, a database exploration for protein characterization was carried out using MS/MS ion search (MASCOT, www.matrixscience.com) against all entries of NCBInr. Protein identification is valid when more than 2 peptides match and MOWSE scores are significant (*P*<0.05).

Construction design and immuneinformatics

Amino acid sequence retrieval, epitope predicting, and plasmid designing

Identified proteins with significant MOWSE scores were considered for the antigens of *B. melitensis*. To assess the primary protein structure of each antigen the ProtParam tool was used to obtain the molecular weight, isoelectric point (PI), grand average of hydropathicity (GRAVY), amino acid composition, and other physicochemical features. The T cell epitopes from all of the obtained antigens (considering MHC I&II) were predicted, using the online prediction server Immune Epitope Database (IEDB) (https://www.iedb. org/). Online servers like ABCpred (https://www.bio. tools/) were applied to predict the linear B-cell epitopes. The IEDB server predicts the peptides based on Chou and Fasman beta-turn, Karplus and Schulz flexibility, Emini surface accessibility, Kolaskar and Tongaonkar antigenicity, and Parker hydrophilicity. The selected epitopes are fused using glycine-serin amino acid-rich linkers in such a way that the best poly-epitope antigen (named MEL) is obtained, which expresses all of the epitopes at the surface. For computation of several physicochemical parameters of the MEL protein, we used ProtParam at (http://expasy.org/tools/protparam.html). After validation of the construct's confidants, the poly-tope protein coding sequence was synthesized chemically by GeneCreate Biological Engineering Co (China).

In silico evaluation of multi-epitope protein

The VaxiJen v.2.0 server at (http://www.jenner.ac.uk/ VaxiJen) was used for prediction of the immunogenicity of the MEL protein. The accuracy of this server based on the type of study varies from 70% to 89%. The prediction of protein allergenicity was performed via the AlgPred webserver at (http://www.imtech.res. in/raghava/algpred/). The accuracy of the hybrid prediction approach is near 85% with a threshold of 0.4. I-Tasser generates reliable protein models without close homologs in the Protein Data Bank (PDB). *P*-value and RMSD indicate the relative global quality and absolute local model quality, respectively.

Cloning, expression, purification, and confirmation

The computational approved sequence was synthesized into the *pET22b* expression vector (GeneCust, Luxembourg S.A) using restriction enzymes (*SacI* & *HindIII*) and named *pETmel22b*. The transformed BL21 (DE3) (Invitrogen, Germany) strain of *E. coli* was considered for protein expression. Protein concentration was measured using the Bradford assay. Then, the bacterial lysate was electrophoresed onto a 12% Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) beside the protein marker (Fermentas, Lithuania). The expression of recombinant protein was compared with that of the control samples' *E. coli* BL21without plasmid. The recombinant multi-epitope protein was purified under native conditions on His-Tag resin (Invitrogen, Germany) according to the manufacturer's guidelines. The purified MEL protein and the controls were separated by the SDS-PAGE method using 12% and transferred onto a nitrocellulose membrane (Sigma-Aldrich, USA) by blotting BioRad system. To assay, the antigenicity of the purified multi-epitope peptide, the membrane, after blocking with a solution containing 3% skim milk (Fluca), was incubated for 100 min at 25 °C with the sheep's 1:200 dilutions of sera, obtained from 20 sheep in Razi Institute, which was previously confirmed by a serology test. The membrane was washed and incubated with goat anti-sheep IgG Alkaline

phosphatase conjugate (1:5000) (Abcam, UK) as the secondary antibody for two hours at 37 °C. Following the washing stage, the immune complex was visualized by incubating the membrane with a solution containing NBT-BCIP for 15 min at 37 °C. The reaction was stopped using distilled water.

Preparation of chitin microparticles as an adjuvant in immunogenic complex

The commercial powder of the chitin microparticles was suspended in distilled water, sonicated, and filtered using a 40-μm filter (BD Falcon, Mexico) (26). The pellet of the microparticles after centrifugation (2800g for 10 min) was oven-dried at 50 °C. Particle size and distribution were analyzed using a laser particle size analyzer. Before applying it as an adjuvant in the immunogenic complex, we checked it for the presence of LPS using the Limulus Amebocyte Lysate kit (Cambrex, USA).

In vivo evaluation of multi-epitope protein immunogenicity
Animals

We performed the study on 9 guinea pigs (*Cavia porcellus*) weighing from 300 g in the majority of the experiments, obtained from the Medzist company, and kept under isolation as a routine. They were graded into 3 groups called PBS group, Rev.1 vaccine, and MEL protein. We have followed all ethical principles of research on laboratory animals under the National Institutes of Health guide for the Care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978) (Ethics Code: IR.SBMU.REC.1396.126).

Immunization of guinea pigs

The animals were immunized by injection subcutaneously. The injection solution was 200 μl containing 10 mg MEL protein and chitin microparticles (100 mg/mL) for the MEL group; 200 μl PBS and 200 μl Rev.1 vaccine were used for the two other groups.

Samples collection and primary cell culture

Before sacrification of the animals, the blood of each experimental group was collected from the cardiac puncture to perform the antibody ELISA (27, 28) (Enzyme-Linked Immune Sorbent Assay) and also for bactericidal antibody responses test. After sacrifice and cutting off guinea pigs' lower jaws the spleens were dissected, the lymphocytes were isolated by Ficol 70(Sigma), and then cultured in RPMI (Bio sera) (with penicillin 100 u/ml and streptomycin 100 μg/ml) at 37 °C, 5% Co_2, and 80% humidity at 6×10^6 density of cells for proliferation assay. Proliferation stimulation by adding injected components was performed 24 hr after cell culture. Liver tissues were kept in formalin until slide staining for examination of any pathological changes of the protein.

Measurement of specific IgG against MEL protein in guinea pig's sera by ELISA

For evaluation of IgG production stimulation in guinea pig's sera, after an immunogenic complex injection, different concentrations of purified protein in 1x PBS were coated on a crystal-grade polystyrene 96-well microtiter plate (SPL Life Sciences) (29). The volume of 100 μl of 1/200 diluted serum samples in 1x PBS was added to each well. The plate was left at room temperature for 2 hr . The plate was washed three times, and 100 μl of HRP-conjugated rabbit anti-guinea pig IgG (1:10000 dilutions in 1x PBS) was added to each well. After 1 hr and re-washing the plate, 100 μl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and after 15 min at room temperature, the reaction was stopped by 50 μl of 2N H_2SO_4. The Optical Density (OD) of each well was assayed by ELISA reader at wavelength 450 nm with reference wavelength 630 nm. The method was applied for all of the serum samples in 1:200 dilutions and with 1 μg/ml of purified protein. All of the 25 anti-*Brucella* IgG negative samples were considered to set a cutoff value using mean ± 2SD.

Measurement of IL2, IL5, IL4, IL10, TNFα, and IFNγ cytokine genes expression by real-time q-PCR

Total RNA was extracted from spleens by using a Gene All Kit(Korea) according to the manufacturer's guidelines. RNA was then treated with 5 U of DNase I (Fermentas, Lithuania). RNAs were measured using a Biophotometer (Eppendorf, Germany). To synthesize cDNA, 2 μl of oligo (dT) primer, 2 μl of 2.5 mM stock of dNTPs (Invitrogen), and 16 μl of RNA were incubated at 65 °C for 5 min and then put on ice for 3 min. The following reagents were added: 4 μl of 5× buffer (Fermentas) and 0.5 μl of 200 unit/μl Revert Aid™ (Fermentas) followed by keeping at 42 °C for 50 min. Table 1 lists the six guinea pig cytokines and Gapdh as housekeeping genes (GenBank).

Real-time RT-PCR was carried out with a Bioneer Master mix (Korea) using 1 μl of the cDNA in a volume of 20 μl with the specific primers mix. Quantitative PCR was carried out in the LightCycler 480 for 45 cycles using the following program: denaturation at 95 °C for 10 sec, annealing at 60 °C for 10 sec, and extension at 72 °C for 15 sec. Melting curve analysis was achieved after

Table 1. Primers used for cytokine gene expression assay by real-time qPCR

| Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
IL4	tgacggtcattctcttctgcctc	aggagagtgtgttgaggtgctg
IL-2	gttatgctttctcagagcaaccc	gctaaatttagcacctgctccac
IL12	acctccctagggcctcaccag	ggccttgtgaagttcactgttc
IL5	ggaagctctggcaacactattc	tgcttcactctccgtcgctcc
IL10	gccagccaaggcacgaacacc	accctgccaaaggcagctcgg
TNFα	tgcactttgggtgatcggcc	agccaccggcttgtcattatcg
IFNγ	atgttggctcttcagttctg	catctgagttatctgcattc
Gapdh	cgagacaagatggtgaaggtc	cattgatggctacaatatccact
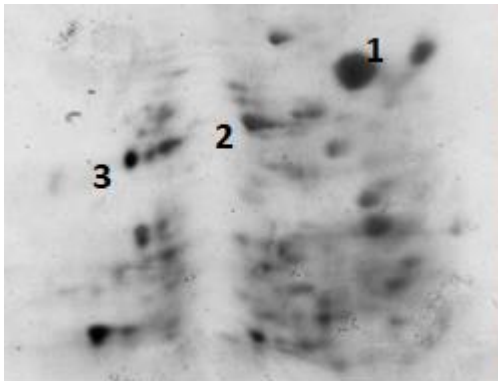
Figure 1. 2D gel electrophoresis of *Brucella melitensis* lysate. The spots number 1, 2, and 3 were positive in interaction with infected sheep pooled serum
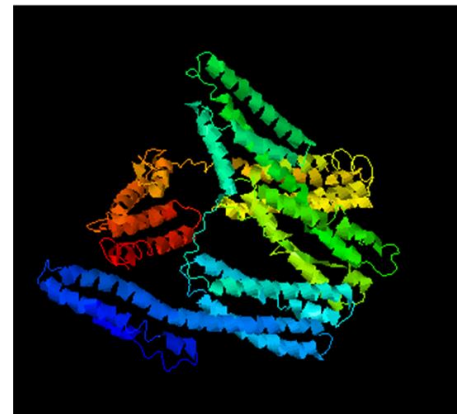

Figure 2. Global model of MEL protein (6bfiA) by I-TASSER

the amplification phase. Cytokine amplification was confirmed by 2% agarose gel electrophoresis.

Lymphocytes proliferation assay by flow cytometer

The proliferation character of the three groups was carried out by adding the MEL, PBS, and Rev.1 vaccines separately 24 hr after cell culture. Following 6 day incubation of cells at 37 °C in a 5% CO_2 humidified incubator, they were harvested and the proliferation rate was assessed via measuring fluorescent label carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies, USA) uptake. The assay described in this section is used to track proliferating cells due to the CFSE intracellular fluorescent label (30). A FACS Aria II flow cytometer (BD Biosciences, San Diego, CA, USA) was used for the flow cytometry technique, Which has two power lasers: 488 nm and 635 nm. The data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Protein toxicity assay on guinea pigs' liver tissue by microscopic evaluation

The histology of liver samples of guinea pigs was prepared with paraffin and cut. After Hematoxylin and Eosin staining, they were examined by a specialist.

Statistical analysis

All analyses were performed in three versions. Values were assumed as means±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) and then multiple comparison tests in Turkey's. *P*-values less than 0.05 were considered to be statistically significant. REST® 2009 Software (Qiagen, Hilden, Germany) was used to analyze RT-qPCR data.

Results

Immunoreactive proteins in Brucella melitensis cells

Western blotting of bacterial strains with confirmed naturally infected animal sera showed that there are several antigenic proteins in *B. melitensis* cells that

induce the humoral immune system in sheep. Figure 1 demonstrates the analysis of *B. melitensis* proteome by 2DE and we selected 3 sharp spots that were positive in interaction with infected sheep pooled serum for MALDI TOF MS spectroscopy.

Proteomics analysis of B. melitensis

The protein bands which reacted with infected sera were prepared for analysis by MALDI TOF MS/MS. Protein identification is valid when more than 2 peptides match and MOWSE scores are significant (*P*<0.05) (Table 2). Table 3 shows isolated proteins of *B. melitensis* that referred to from other articles.

Selected epitopes for construct design

Using servers that were described, the highest scored T-cell and B-cell epitopes for *B. melitensis* immunogen proteins were selected and sorted in Table 4.

Predicted structure and physicochemical properties

The 3D structure of the MEL protein was predicted by the I-TASSER server (C516795 project) (Figure 2). The best template resulting for the homology modeling was **6bfiA** with a normalized Z-score 1.35 which means a good alignment and shows the high quality of the model. The tertiary model quality score and limited errors were analyzed using the pGenTHREADER server, which predicted the C-score to be 1.13, and computed TM-Score 0.57±0.14 and accounted RMSD 10.6±4.6 which represents a model of correct global topology.

ProtParam server administered basic physicochemical parameters of the protein. The number of amino acids was considered 431 with a molecular weight of 45116.47 Da. The pI value was 6.30, and the computed half-life of the MEL protein was 30 hr (mammalian reticulocytes, *in vitro*), >20 hr (yeast, *in vivo*), and >10 hr (*Escherichia coli in vivo*). The instability index was computed to be 38.29 which classified it as a stable protein. The GRAVY index score was 0.161 which shows

Table 2. *Brucella melitensis* identified proteins by MALDI TOF MS/MS

Number	Protein	MW (kD)	Location
1	ABC transporter ATP-binding Protein	100	Bacterial pellet
2	Chaperonin 60	70	Media
3	Aldehyde Dehydrogenase	40	Media

Table 3. *Brucella melitensis* identified proteins by other studies (2DE)

Selected proteins	Reference
25 kDa outer membrane protein-omp25	31
BvrR (*Brucella* virulence-related Regulatory protein)	32
BvrS (*Brucella* virulence-related Sensory protein)	
BtpA&BtpB (*Brucella* TIR domain-containing protein A&B), TcpB (TIR domain-containing protein from *Brucella)*	33
TIRAP9 (TIR domain-containing adaptor protein)	34
p-type DNA transfer protein VirB5	35
Type IV secretion system protein virB10	36
Type IV secretion system protein VirB11	37
Type IV secretion system protein virB3	38
D-erythritol 1-phosphate dehydrogenase	39
Erythritol kinase	40
D-erythrulose 1-phosphate 3-epimerase	41
L-erythrulose 1-phosphate isomerase& D-erythrulose 4-phosphate isomerase	42

the hydrophobic nature of MEL by Kyte-Doolittle and Hopp Woods formula. VaxiJen overall prediction of the protective antigen for MEL represented that it is a probable antigen with a score of .0.5772.

The MEL recombinant protein production and confirmation by Western blotting

The MEL fragment was cloned into the pET22b expression vector (Figure 2) and then expressed in *E. coli* and purified. Western blotting analysis of MEL recombinant protein revealed with infected sheep serum and His Tag monoclonal antibody showed that the protein has expressed after induction and purified successfully.

Specific IgG detection in guinea pigs' sera

To determine whether recombinant *B. melitensis* MEL protein elicited antibody responses in the guinea pig, we obtained sera from five guinea pigs experimentally injected with recombinant *B. melitensis* MEL protein, sera from five guinea pigs injected with the whole *B. melitensis* antigen (Rev.1 vaccine), and sera from five guinea pigs injected with PBS as the negative control. The levels of IgG specific for recombinant MEL protein and Rev1 vaccine with a mean A450 of 1.405± 0.068 and 1.075± 0.144, respectively, did not differ between guinea

pigs. In contrast, for two groups of animals infected with *B. melitensis* antigen, the levels of IgG-specific increased significantly above those measured in guinea pigs infected with PBS with a mean A450 of 0.258±0.056 (*P*<0.0001). These results suggest that recombinant MEL protein is capable of responding to guinea pigs like the *B. melitensis* Rev1 vaccine (Figure 3).
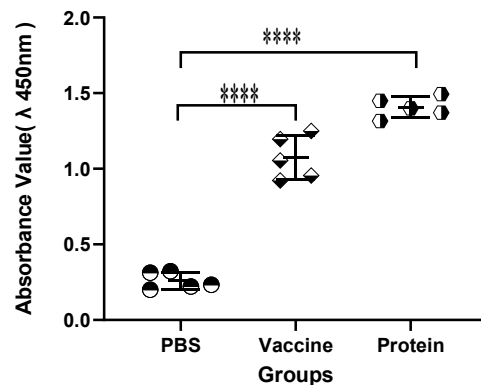

Figure 3. Rate of absorption in the sera of guinea pigs treated with MEL, Rev.1 vaccine and PBS

Table 4. Epitope mapping outcome of *Brucella melitensis* immunogen proteins

B-cell Epitopes*	T-cell MHCI binding Epitopes*
KSLVIVSAALLPFSATA	VPAPVEVAPQYSWAG
EPVTLTVTEFLILHSLAQRPGVVKS	EASATQTI ALVDDDRNILTSVSIALESEGY
ERMAVFRVFGVVSAVMVILSLFLASTIANPLR	ERRARRQRSVFLRRYLSPLRKFLGQY
SEDKEAFVQDLVAALRDLGAKIFYDAYTLKVGDSLR	KQSLSSMRTTASATMEAEEEYDFFISHASEDKEAFVQDLV
RRGQVRIEYEFIPVQPFLTVADFD	ELAELTRLPTIFAYEAACKLDP
SEDKEAFVQDLVAALRDLGAKIFYDAYTLKVGDSL	K MSELERATRDGAAIGKKRAD
SEDKEAFVQDLVAALRDLGAKIFYDAYTLKVGDSL	AHKAQQAISSAKSLSTQKSKMSELERATRDGAAIGKK
SEDKEAFVQDLVAALRDLGAKIFYDAYTLKVGDSL	ALTVTSTAHAQLPVTDAGSI
GRTRKVLLFLFVVGFIVVLLLLLVFHMRG	MTQENIPVQPGTLDGERGLP
LPYEIIRRLLYLVVDVVVHVHNGVHDG	MMSNRSDFIVPDEAAVKRAA
RPAMLFGVPVIPLVIVGGSIVLLSVWISMFILPIVPIVLVMRQI	MTTAPQESNARSAGYRG

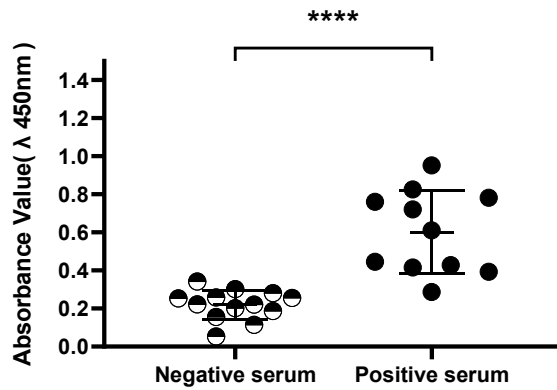* These are the highest scored T-cell and B-cell epitopes

Figure 4. Absorption of MEL protein in healthy sheep sera and sheep with *Brucella melitensis*

Figure 6. Analysis of the splenocytes proliferation rate (%) after treatment with antigen (Flowjo V7.6.1). Cells in culture with MEL exhibited significant proliferation after 5 days compared with the PBS group. The difference was also significant in the MEL group compared with the PBS group ($P<0.001$). Significance was flagged with star(s); ** $P \leq 0.01$ and **** $P \leq 0.0001$

MEL protein and sheep serum IgG antibody complex-forming

Thirteen positive and twelve negative serum samples from sheep for antibodies to the whole *B. melitensis* antigen were examined by ELISA for existence of specific antibody (IgG) for recombinant *B. melitensis* protein. The results are shown in Figure 4 that levels of IgG against the recombinant protein (MEL) were significantly higher in positive sera with a mean A450 of 1.375± 0.145 compared with negative sera with a mean A450 of 0.328± 0.120 ($P<0.0001$) (Figure 4).

Cytokine genes expression evaluation by real-time RT PCR

As shown in Figure 5, Real-time RT-PCR analysis of immunized guinea pigs' spleen lymphocytes in 3 groups showed that the expression levels of IL-2 and INFγ increased in guinea pigs immunized with MEL protein significantly. While a decrease in TNFα, IL-4, IL-12, IL-10, and IL-5 was observed in this group compared with the controls (Rev.1 and PBS).

Lymphocyte proliferation assay

To assess the lymphocyte proliferation activity, the CFSE-labeled cells were cultured with purified protein following five days of culture, the proliferation rate of the cells was assessed via measuring CFSE uptake.

Splenocyte proliferation was assessed by tracking the decrease in CFSE fluorescence in proliferating cells (CD3-gated). The percentage of the proliferation rate
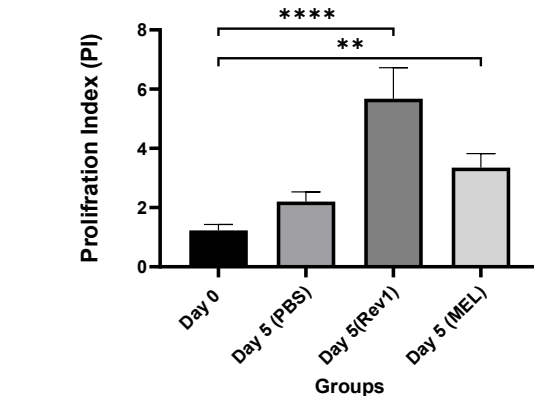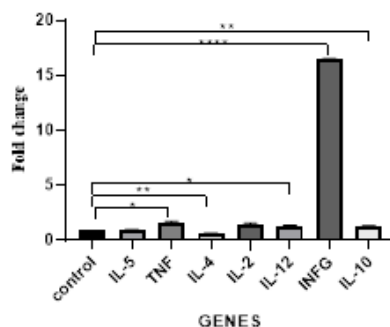
was compared between the groups as demonstrated in Figure 6. The results showed that the cells cultured with the MEL antigen group exhibited significant proliferation compared with PBS unstimulated cells.

Microscopically evaluation

Histopathological evaluation revealed that in all cases of experimented and sham groups mild portal and/or parenchymal hepatitis (mononuclear and polymorph nuclear cells), vacuolar degeneration, cell swelling, and individual necrotic cells were seen that were not related to the treatment (Figure7).

Discussion

Iran is one of the endemic regions for brucellosis with a noticeable incidence of brucellosis among human and domestic animal populations. According to the annual report of the Iranian Centers for Disease Control, the most important causes of human brucellosis are *B. melitensis* and *B. abortus* (43, 44). *B. melitensis* Rev.1, an attenuated smooth strain was able to control *B. melitensis* infection and is currently used as the only vaccine for the prophylaxis of caprine brucellosis (45-47). However, major problems like the ability of this strain to cause brucellosis in humans (48) and creation of resistance to streptomycin used to treat brucellosis, have prevented
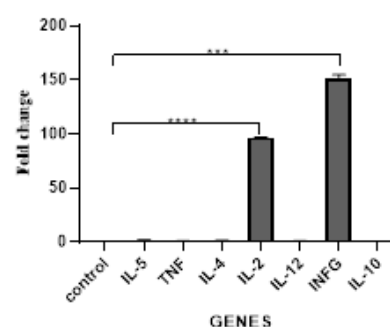

Figure 5. Expression levels of Cytokines. Cytokine RNA transcriptions were compared between MEL protein (right), Rev.1 conventional vaccine (left), and PBS control groups

its use for human vaccination (49). Therefore, a subunit vaccine that is protective against *B. melitensis* is in demand. Many studies similar to our research have reported that a multitope vaccine containing protective epitopes from several immunodominant proteins may exert protective immunity against brucellosis (50). Thus, we employed a reverse vaccinology approach to identify immune-reactive proteins of bacteria and access a potential vaccine candidate for goat brucellosis. Bacterial cell structural and secreted antigens could be the most critical selections for designing vaccines since they might participate in the initial interaction with the host cells. In the production of good and ideal vaccines against brucellosis important functions of the immune system must be strengthened and activated.

Each of the selected peptides from the proteins obtained from this study and the results of previous research are able to stimulate the immune system as peptide vaccines. By connecting them and making a recombinant protein (MEL) and forming a proper folding, we were able to stimulate the guinea pig's immune system even more effectively than the commercial *Brucella* vaccine, which is a weakened bacterium and can return to the active form. One of the properties of small phagocytosable chitin particles is that they activate alveolar macrophages, leading to the expression of cytokines such as IL-12, tumor necrosis factor-α (TNFα) and IL-18, and then NK cells producing INF-γ (51). Therefore, the use of chitin as a safe adjuvant has contributed to the immunogenicity of the recombinant MEL protein. There has been much evidence demonstrating that the gamma interferon (IFN- γ)-mediated T helper 1 (Th1) immune response is vital and important for the control of *Brucella* infection (19, 52-54). After ruminant vaccination, IFNγ production by Th1 is reported (55). The results from this study indicated that approximately high expression levels of Th1 cytokines (IFN-γ and IL-2) were observed in guinea pigs immunized with Rev-1 vaccine and MEL recombinant protein. High levels of IL-2 emphasize the activation of cellular immunity and in particular, Th1 lymphocytes which are aimed at the elimination of pathogens. In this study guinea pigs immunized with MEL protein or Rev.1 vaccine produced significantly higher specific IgG levels compared with the PBS group. The profile of the antibody response is a reaction to the T helper cell type, all of these results indicate the induced Th1 response against MEL protein. A comparison of PI in guinea pig spleen cells treated with recombinant protein (MEL) on days 0 and 5 suggested that MEL protein has significant proliferation compared with PBS unstimulated cells.

Our results suggest that this recombinant protein could be a subunit protein together with chitin with sufficient efficiency in stimulating the humoral and cellular-mediated immune system against *B. melitansis* compared with common live attenuated *B. melitensis* Rev.1 vaccines.

Conclusion

The MEL recombinant protein containing determinant peptides of *B. melitensis* with chitin could be a potential subunit immunogenic complex against *B. melitensis* with magnificent ability in inducing both types of humoral

and cellular-mediated immunity without any toxicity compared with common live attenuated *B. melitensis* Rev.1 vaccine.

Conflicts of Interest

The authors declare no conflicts of interest in this work.

References

1. Chang C, Beutler BD, Ulanja MB, Uche C, Zdrnja M. Brucellosis presenting with febrile pancytopenia: an atypical presentation of a common disease and review of Brucellosis. Case Rep Infect Dis 2021; 2021:2067570.
2. Wubishet Z, Sadik K, Abdala B, Mokonin B, Getachew T, Getachew K. Small ruminant brucellosis and awareness of pastoralists community about zoonotic importance of the disease in Yabello districts of Borena Zone Oromia Regional State, Southern Ethiopia. Curr Trends Biomed Eng Biosci 2018; 12:5-10.
3. Pappas G. The changing *Brucella* ecology: Novel reservoirs, new threats. Int J Antimicrob Agents 2010; 36: S8- S11.
4. Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: A re-emerging zoonosis. Vet Microbiol 2010; 140: 392-398.
5. Sauret JM, Vilissova N. Human brucellosis. J Am Board Fam Pract 2002;15:401-406.
6. Golshani M, Buozari S. A review of brucellosis in Iran: Epidemiology, risk factors, diagnosis, control, and prevention. Iran Biomed J 2017;21:349-359.
7. Foster JT, Walker MF, Rannals DB, Hussain HM, Drees PK, Tiller VR, *et al.* African lineage *Brucella melitensis* isolates from Omani livestock. Front Microbiol 2017; 8:2702.
8. Samadi A, Ababneh M, Giadinis ND, Lafi SQ. Ovine and caprine brucellosis (*Brucella melitensis*) in aborted animals in Jordanian sheep and goat flocks. Vet Med Int 2010; 2010: 458695.
9. Yumuk Z, O'Callaghan D. Brucellosis in Turkey-an overview. Inter J Infect Dis 2012;16: e228-e235.
10. Akbarmehr J. The prevalence of *Brucella abortus* and *Brucella melitensis* in local cheese produced in Sarab city, Iran and its public health implication. Afr J Microbiol Res 2011; 5:1500-1503.
11. Ponsart C, Riou M, Locatelli Y, Jacques I, Fadeau A, Jay M, *et al. Brucella melitensis* Rev. 1 vaccination generates a higher shedding risk of the vaccine strain in Alpine ibex (Capra ibex) compared to the domestic goat (Capra hircus). Vet Res 2019; 50:1-3.
12. Alton GG, Elberg SS. Rev. 01 *Brucella melitensis* vaccine. A review of ten years of study. Vet Bull 1967; 371: 893-900.
13. Ollé-Goig JE, Canela-Soler J. An outbreak of *Brucella melitensis* infection by airborne transmission among laboratory workers. Am J Public Health 1987; 77:335-338.
14. Sekhavati MH, Heravi RM, Tahmoorespur M, Yousefi S, Abbassi-Daloii T, Akbari R. Cloning, molecular analysis and

epitopics prediction of a new chaperone GroEL *Brucella melitensis* antigen. Iran J Basic Med Sci 2015; 18:499-505.

15. Soria-Guerra RE, Nieto-Gomez R, Govea-Alonso DO, Rosales-Mendoza S. An overview of bioinformatics tools for epitope prediction: implications on vaccine development. J Biomed Inform 2015; 53:405-414.

16. Simon GG, Hu Y, Khan AM, Zhou J, Salmon J, Chikhlikar PR, *et al*. Dendritic cell mediated delivery of plasmid DNA encoding LAMP/HIV-1 Gag fusion immunogen enhances T cell epitope responses in HLA DR4 transgenic mice. PLoS One 2010; 5: e8574.

17. Michel-Todó L, Reche PA, Bigey P, Pinazo MJ, Gascón J, Alonso-Padilla J. *In silico* design of an epitope-based vaccine ensemble for Chagas disease. Front Immunol 2020; 10:3124-3156.

18. Sadeghi Z, Fasihi-Ramandi M, Bouzari S. Evaluation of immunogenicity of novel multi-epitope subunit vaccines in combination with poly I: C against *Brucella melitensis* and *Brucella abortus* infection. Inter Immunopharmacol 2019;75:105829.

19. Vitry MA, De Trez C, Goriely S, Dumoutier L, Akira S, Ryffel B, *et al*. Crucial role of gamma interferon-producing CD4+ Th1 cells but dispensable function of CD8+ T cell, B cell, Th2, and Th17 responses in the control of *Brucella melitensis* infection in mice. Infect Immun 2012;80:4271-4280.

20. 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;2013:743509-743527.

21. Svetić A, Jian YC, Lu P, Finkeiman FD, Gause WC. *Brucella abortus* induces a novel cytokine gene expression pattern characterized by elevated IL-10 and IFN-γ in CD4+ T cells. Inter Immunol 1993;5:877-883.

22. Gómez MC, Nieto JA, Rosa C, Geijo P, Escribano MA, Munoz A, *et al*. Evaluation of seven tests for diagnosis of human brucellosis in an area where the disease is endemic. Clin Vaccine Immunol 2008;15:1031-1033.

23. Wahl KL, Wunschel SC, Jarman KH, Valentine NB, Petersen CE, Kingsley MT, *et al*. Analysis of microbial mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 2002;74:6191-6199.

24. Valentine N, Wunschel S, Wunschel D, Petersen C, Wahl K. Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. Appl Environ Microbiol 2005;71:58-64.

25. Dadfarma N, Nowroozi J, Kazemi B, Bandehpour M. Identification of the effects of acid-resistant Lactobacillus casei metallopeptidase gene under colon-specific promoter on the colorectal and breast cancer cell lines. Iran J Basic Med Sci 2021; 24: 506-513.

26. No HK, Meyers SP. Preparation and characterization of chitin and chitosan-a review. J Aquat Food Prod Technol 1995;4:27-52.

27. Birck MM, Tveden-Nyborg P, Lindblad MM, Lykkesfeldt J. Non-terminal blood sampling techniques in guinea pigs. J Vis Exp 2014;11:e51982.

28. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother 2010;1:87-93.

29. Khodabakhsh T, Arabi A, Pakzad P, Gheflat S, Bahreinipour A, Bandehpour M. A New ELISA Kit based on antigenic epitopes for diagnosing of *Brucella abortus*. Microbiol Biotechnol Lett 2019; 47:158–163.

30. Sharifnia Z, Bandehpour M, Hamishehkar H, Mosaffa N, Kazemi B, Zarghami N. *In-vitro* transcribed mRNA delivery using PLGA/PEI nanoparticles into human monocyte-derived dendritic cells, Iran J Pharm Res 2019;18:1659-1675.

31. Yousefi S, Tahmoorespur M, Sekhavati MH. Cloning, expression and molecular analysis of Iranian *Brucella melitensis* Omp25 gene for designing a subunit vaccine. Res Pharm Sci 2016;11:412-418.

32. López-Goñi I, Guzmán-Verri C, Manterola L, Sola-Landa A, Moriyón I, Moreno E. Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. Vet Microbiol 2002;90:329-339.

33. Coronas-Serna JM, Louche A, Rodríguez-Escudero M, Roussin M, Imbert PR, Rodríguez-Escudero I, *et al*. The TIR-domain containing effectors BtpA and BtpB from *Brucella abortus* impact NAD metabolism. PLoS Pathog 2020;16:e1007979.

34. Horng T, Barton GM, Medzhitov R. TIRAP: An adapter molecule in the Toll signaling pathway. Nat Immunol 2001;2:835-841.

35. Christie PJ, Whitaker N, González-Rivera C. Mechanism and structure of the bacterial type IV secretion systems. Biochim Biophys Acta 2014;1843:1578-1591.

36. Giraldo AM, Mary C, Sivanesan D, Baron C. VirB6 and VirB10 from the *Brucella* type IV secretion system interact via the N-terminal periplasmic domain of VirB6. FEBS Lett 2015;589:1883-1889.

37. Atmakuri K, Cascales E, Christie PJ. Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. Mol Microbiol 2004;54:1199-1211.

38. Fronzes R, Christie PJ, Waksman G. The structural biology of type IV secretion systems. Nat Rev Microbiol 2009;7:703-714.

39. Sangari FJ, Agüero J, García-Lobo JM. The genes for erythritol catabolism are organized as an inducible operon in *Brucella abortus*. The GenBank accession number for the sequence reported in this paper is U57100. Microbiol 2000;146:487-495.

40. Lillo AM, Tetzlaff CN, Sangari FJ, Cane DE. Functional expression and characterization of EryA, the erythritol kinase of *Brucella abortus*, and enzymatic synthesis of L-erythritol-4-phosphate. Bioorg Med Chem Lett 2003;13:737-739.

41.Chain PS, Comerci DJ, Tolmasky ME, Larimer FW, Malfatti SA, Vergez LM, *et al*. Whole-genome analyses of speciation events in pathogenic *Brucella*. Infect Immun 2005;73:8353-8361.

42. DelVecchio VG, Kapatral V, Redkar RJ, Patra G, Mujer C, Los T, *et al*. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc Natl Acad Sci 2002;99:443-448.

43. Esmaeili H. Brucellosis in Islamic republic of Iran. J Med Bacteriol 2014;3:47-57.

44. Dastjerdi MZ, Nobari RF, Ramazanpour J. Epidemiological features of human brucellosis in central Iran, 2006–2011. Public Health 2012;126:1058-1062.

45. Marin CM, Barberan M, De Bagués MJ, Blasco JM. Comparison of subcutaneous and conjunctival routes of Rev 1 vaccination for the prophylaxis of *Brucella ovis* infection in rams. Res Vet Sci 1990;48: 209-215.

46. Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. Vet Microbiol 2002;90:479-496.

47. Ko J, Splitter GA. Molecular host-pathogen interaction in brucellosis: Current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev 2003;16:65-78.

48. Blasco JM, Diaz R. *Brucella melitensis* Rev-1 vaccine as a cause of human brucellosis. Lancet 1993; 342: 805.

49. de Bagués MJ, Elzer PH, Blasco JM, Marin CM, Gamazo C, Winter AJ. Protective immunity to *Brucella ovis* in BALB/c mice

following recovery from primary infection or immunization with subcellular vaccines. Infect Immun 1994;62:632-638.

50. Vishnu US, Sankarasubramanian J, Gunasekaran P, Rajendhran J. Novel vaccine candidates against *Brucella melitensis* identified through reverse vaccinology approach. OMICS 2015;19:722-729.

51. Li X, Min M, Du N, Gu Y, Hode T, Naylor M, *et al*. Chitin, chitosan, and glycated chitosan regulate immune responses: the novel adjuvants for cancer vaccine. Clin Dev Immunol 2013; 2013: 387023.

52. Baldwin CL, Parent M. Fundamentals of host immune response against *Brucella abortus*: what the mouse model has revealed about control of infection. Vet Microbiol 2002;90:367-382.

53. Dornand J, Gross A, Lafont V, Liautard J, Oliaro J, Liautard JP. The innate immune response against *Brucella* in humans. Vet Microbiol 2002;90:383-394.

54. Alizadeh H, Dezfulian M, Rahnema M, Fallah J, Esmaeili D. Protection of BALB/c mice against pathogenic *Brucella abortus* and *Brucella melitensis* by vaccination with recombinant Omp16. Iran J Basic Med Sci 2019;22:1302-1307.

55. Olsen SC. Recent developments in livestock and wildlife brucellosis vaccination. Rev Sci Tech 2013;32:207-217.