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Original Research Article

Induction of protective immune responses in mice by double DNA vaccine encoding of *Brucella melitensis* Omp31 and *Escherichia coli* Eae genes

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Abstract

Purpose: To investigate the efficacy of a double DNA vaccine encoding of *Brucella melitensis* omp31 gene and of *Escherichia coli* eae gene in inducing protective immune response in a mouse model.

Methods: After performing PCR assays and cloning both the eae and omp31 genes, the generated DNA vaccines were intramuscularly injected into BALB/c mice. Purified proteins and transformed cell lysates were identified by western blot and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by enzyme-linked immunosorbent assay (ELISA).

Results: pcDNA3-omp31-eae provokes a significant, high level of protection in BALB/c mice against challenges with *E. coli* and *B. melitensis*. Titers ranging from 90:160 to 2,570:5,110 were detected 60 days after vaccination. Notably, rOmp31 and rEae proteins significantly induced the production of IFN- γ in cells from pcDNA3-omp31-eae-immunized mice ($p < 0.05$ in both groups) and only low levels of IL-10 were found in all groups. The lymphocytes from mice immunized with pcDNA3-omp31-eae had an apparent T cell proliferative response to rOmp31 and rEae proteins ($p < 0.01$) with a stimulation index of 14.90.

Conclusion: The novel pcDNA3-omp31-eae construct can be used as a DNA vaccine to prevent *E. coli* and *B. melitensis* infections in future studies.

Keywords: Multiple DNA vaccine, Omp31 gene, *Brucella melitensis*, Eae gene, *Escherichia coli*, Protective immune response

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INTRODUCTION

DNA vaccines can induce both cellular and humoral immune responses, but have demonstrated only limited success in animals because of their low immunogenicity [1]. Nonetheless, DNA vaccines have several advantages over traditional protein-based vaccines, including induction of longer lasting immunity, ease of development, and lower production costs [2].

One of the most widespread zoonoses, caused by the pathogenic bacterium *Brucella*, is brucellosis, a disease that affects humans and livestock. *Brucella melitensis* is an intracellular, facultative, Gram-negative bacterium that infects humans and a wide range of animals [3,4]. *B. melitensis* has high pathogenicity in humans and causes a large proportion of human cases [5,6]. Human brucellosis is treatable with antibiotics, but the course of antibiotic treatment must be

long because *Brucella* infections are intracellular [7]. *Brucella* species are therefore considered potential civilian, military, and agricultural bioterrorism agents [7]. In livestock, brucellosis can cause great economic losses, as infection can lead to stillbirths, abortions, and loss of fertility in animals [8,9].

B. melitensis causes miscarriage in cows, goats, and sheep, and is considered to be the most pathogenic *Brucella* sp. for humans [10]. Recombinant Omp31 (rOmp31) vaccination elicits a CD4 Th1 response that provides partial protection against *B. melitensis* infections [11]. High concentrations of the plasmid containing the *omp31* gene are needed to generate an efficient response, probably because of the low *in vivo* transfection efficiency for this type of plasmid vector [3].

Infections with *Escherichia coli* are commonly associated with diarrhea accompanied by bloody hemorrhagic colitis and abdominal cramps, but more serious complications such as thrombotic thrombocytopenic purpura, hemolytic-uremic syndrome, hemorrhagic colitis (HC), and in some cases, death, can also occur [12-14]. Pathogenic strains of *E. coli* are classified as either extra-intestinal (ExPEC), causing a variety of infections in both animals and humans, or intestinal pathogens causing diarrhea [15]. The chromosomal gene *eae*, which encodes intimin, is part of a pathogenicity island termed the locus of enterocyte effacement (LEE) [13]. *Eae* is the first gene to be associated with attaching and effacing (AE) activity, and is essential for intimate bacterial attachment to eukaryotic host cells [14]. The *eae* gene is one of the genes currently used for the molecular diagnosis of enteropathogenic *E. coli* (EPEC) [16]. EPEC produces characteristic AE lesions on intestinal epithelial cells. The AE phenotype is encoded by a cluster of genes, including the *eae* gene located on the LEE [17]. The ability to cause AE lesions in enterocytes is the major virulence factor of *E. coli* in intestinal infections in humans and animals [18].

The *E. coli* AE gene (*eae*) encodes a 94-kDa outer membrane protein, intimin, which is important but not sufficient for the production of AE lesions [18,19]. Inoculation with plasmid DNAs expressing foreign proteins stimulates both cellular and humoral immune responses that protect against bacteria, parasites, viruses, and tumors [20], and is a reasonably powerful and novel technique of immunization [21]. The aim of the present study is to investigate the immunogenicity and protective capacity of a DNA

vaccine encoding the *eae* and *omp31* proteins of *E. coli* and *B. melitensis*, respectively.

EXPERIMENTAL

Bacterial strains and DNA extraction

B. melitensis and *E. coli* were obtained from the Microbiology Research Center of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran. The *B. melitensis* and *E. coli* strains were grown on *Brucella* agar and Luria-Bertani (LB) agar, respectively. Genomic DNA was extracted using a Qiagen DNA extraction kit (Qiagen, Ltd., Crawley, UK). Quality was assessed according to manufacturer's instructions by electrophoresing the extracted DNA on a 1 % agarose gel and measuring the optical density at 260 nm (OD 260) d [22,23].

PCR amplification

Specific oligonucleotide primers for the *omp31* gene (*omp31*-F: 5'-ACCGGATCCACCACCATGA AATCCGTAATTTTG-3' and *omp31*-R: 5'-TGCTC GAGGAACTTGTAGTTCAGACCGAC-3') and the *eae* gene (*eae*-F: 5'-ACACTCGAGCAAACCAA GGCCAGCATTAC-3' and *eae*-R: 5'-CAGTCTA GATTATTCTACACAAACCGCAT-3') were designed and used in their respective amplification reactions. To amplify the *omp31* and *eae* genes, the 25- μ L total volume PCR mixtures consisted of 2.5 μ L 10 \times PCR buffer, 1 μ M each of the appropriate primers, 2 mM MgCl₂, 200 μ M dNTPs, 1 μ g template cDNA, and 1 unit pfu DNA polymerase (Roche Applied Science, Penzberg, Germany). For each gene, the 30-cycle amplification was performed in a thermal cycler system (Palm Cycler Gradient, Sydney, Australia) with their respective amplification programs are shown in Table 1.

Table 1: Amplification program for *omp31* gene

Step	Temperature (°C)	Time (min)	Cycle
<i>B. melitensis</i>			
Initial denaturation	94	5	1
Seg 1: Denaturation	94	1	30
Seg 2: Annealing	61	1	
Seg 3: Extension	72	1	
Final extension	72	5	1
<i>E. coli</i>			
Initial denaturation	94	5	1
Seg 1: Denaturation	94	1	30
Seg 2: Annealing	58	1	
Seg 3: Extension	72	1	
Final extension	72	5	1

Cloning of bacterial genes

Electrophoresis was subsequently performed on a 1 % agarose gel in 1× TBE buffer, followed by staining with ethidium bromide and visualization on a UV transilluminator to verify the PCR product sizes. Bands corresponding to the *omp31* and *eae* genes were cut from the gel and the DNA fragments were purified using a gel extraction kit (Bioneer, Cheongwon, Korea) according to the manufacturer's instructions. TOPO-*omp31* and TOPO-*eae* plasmids were cloned using the TA technique, as follows. *Omp31* and *eae* DNA fragments were ligated to the T-vector using the TOPO T/A cloning kit (pCR8/GW/TOPO, Invitrogen, Carlsbad, California, United States) according to the manufacturer's instructions, to obtain TOPO-*omp31* and TOPO-*eae*. *Bam*HI/*Xho*I and *Xho*I/*Xba*I restriction analysis and PCR techniques were used to confirm insertion. The recombinant plasmids (TOPO-*omp31* and TOPO-*eae*) were sequenced with specific primers using the Sanger method (Macrogen, Seoul, Korea), then linearized using *Bam*HI/*Xho*I. The *omp31* and *eae* genes were released from TOPO-*omp31* and TOPO-*eae*, respectively, using *Bam*HI/*Xho*I and *Xho*I/*Xba*I and inserted into the pcDNA3 plasmid. The pcDNA3-*omp31-eae* plasmid constructs were verified by restriction digestion and sequencing.

Preparation of recombinant *omp31* and *eae* proteins

To prepare recombinant *Omp31* (rOmp31) and *Eae* (rEae) proteins, the *omp31* and *eae* genes were excised from the pcDNA3-*omp31-eae* plasmid by *Bam*HI/*Xho*I and *Xho*I/*Xba*I digestion and then inserted into the same restriction sites of the prokaryotic expression vector pET32a(+) (Novagen, Wisconsin, United States). The recombinant plasmids were transformed into *E. coli* BL21 (DE3) (Novagen). After culturing, the positive clones were selected. According to the manufacturer's protocol, the recombinant proteins were expressed in successfully transformed bacteria by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in LB medium and purified on a Ni²⁺-HiTrap chelating 5-mL pre-packed column (Amersham Pharmacia Biotech, Piscataway, United States) using imidazole as the elution reagent.

SDS-PAGE analysis

The purified proteins and transformed cell lysates were identified by western blot and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified proteins were stored

at -70 °C until use for *in vitro* stimulation of splenocytes or for enzyme-linked immunosorbent assays (ELISAs).

In vivo analysis

Mice were anesthetized by inhalation of 3 % halothane in 1 L/min fresh gas flow composed of an equal N₂O:O₂ mixture and maintenance in 1–1.5 % halothane. The tibialis anterior muscle was then injected with 50 μg of plasmid DNA (pcDNA3-*omp31-eae*, 100 μg DNA/mouse) in 50 μL of phosphate buffered saline (PBS) using a 28-gauge needle insulin syringe. Mice were vaccinated at weeks 0, 2, and 4 with pcDNA3-*omp31-eae*. As negative controls, separate groups of mice were immunized with pcDNA3, with PBS only, or with plasmid lacking either the *omp31* or *eae* inserts. As a positive control, another group of mice was vaccinated intraperitoneally with 2 × 10⁸ CFU of *B. melitensis* strain Rev 1 and *E. coli* in 0.2 mL PBS.

Analysis of IgG isotypes

The presence of serum immunoglobulin G (IgG) isotypes specific for rOmp31 and rEae was determined by indirect ELISA. pPurified rOmp31 and rEae (3 μg mL⁻¹ in carbonate buffer, pH 9.6) were added to standard polystyrene 96-well plates for ELISA (Nunc-Immuno plates with MaxiSorp, Nunk, Roskilde, Denmark), incubated overnight at 4 °C, then washed four times in wash buffer (Tris-buffered saline, pH 7.4, with 0.05 % Tween 20). The plates were blocked with 5 % gelatin in Tris-buffered saline at 37 °C for 2 h, then incubated for 3 h at 24 °C and washed four times with serially diluted sera. Isotype-specific rabbit anti-mouse horseradish peroxidase conjugates were added (ICN Biomedicals, Inc., Aurora, OH, USA) (50 μL well⁻¹) at appropriate dilutions. Plates were washed four times and 100 μL of substrate solution (200 μmol of o-phenylenediamine and 0.04 % H₂O₂) was added to each well after 30 min, followed by incubation at 24 °C. The enzyme reaction was stopped after 20 min incubation at room temperature by the addition of 100 μL 0.18 M sulfuric acid, and the absorbance at 492 nm was measured.

Mice were sacrificed two weeks after the last immunization and spleens were removed under aseptic conditions. After preparation of single-cell suspensions from the spleens, red blood cells were lysed with ACK solution (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.3). Splenocytes were cultured in 5 % CO₂ at 37 °C at a concentration of 4 × 10⁵ cells well⁻¹ in RPMI

1640 medium supplemented with 2 mM L-glutamine and 10 % heat-inactivated fetal calf serum (Sigma, St. Louis, United States) in a 96-well flat-bottom plate in the presence of 0.08 µg purified rOmp31 and rEae protein or no additives (unstimulated control). Cells were cultured for 3 days and pulsed for 8 h with 0.4 µCi of 3H-thymidine (50 Ci mmol⁻¹; Amersham Pharmacia Biotech) per well. Incorporated radioactivity was measured in a liquid scintillation counter.

Cytokine detection and vaccine evaluation

For cytokine detection, spleen cell culture supernatants were tested for the presence of cytokines by antigen-capture ELISA using OptEIA Set Mouse IFN-γ, IL-4, and IL-10 (BD Biosciences, San Diego, CA, USA) and collected after 48 h of antigen stimulation.

Five weeks after vaccination, six mice from each group were challenged by intraperitoneal injection of 10⁴ CFU of *B. melitensis* 16M or *E. coli*. After two weeks, the infected mice were sacrificed. Dilutions of spleen homogenate were plated to determine the number of *E. coli* and *B. melitensis* CFUs per spleen. This assay was performed in triplicate. Log₁₀ units of protection were obtained by subtracting the mean log₁₀ CFU for the experimental group from the mean log₁₀ CFU of the corresponding control group.

Ethical consideration

This work was approved by the ethical committees of the biotechnology research center, Islamic Azad University, Shahrekord, Iran and Research Adjutancy of the Islamic Azad University of Shahrekord (ref no. 2037550IAU). The laboratory animal studies followed international ethical and scientific guidelines on animal testing and research [24].

RESULTS

Amplified *eae* and *omp31* genes

The PCR amplified products for the *B. melitensis omp31* and *E. coli eae* genes were respectively 720 bp and 862 bp in length, as shown in Figure 1.

B. melitensis omp31 and *E. coli eae* genes were successfully cloned into TOPO vector. In addition, positive control colonies were obtained; matrix analyses of the cultured colonies for the *omp31* and *eae* genes are shown in Figures 2 and 3, respectively.

Omp31 gene fragment, which has a *Bam*HI/*Xho*I restriction site, and the *eae* gene fragment, which has a *Xho*I/*Xba*I restriction site, were inserted in the polyclonal site (PCS) of pCDNA3. The result of pCDNA3-*omp31-eae* restriction using *Xho*I/*Xba*I enzymes is shown in Figure 4.



Figure 1: Agarose gel electrophoresis of PCR-amplified *omp31* and *eae* gene products. Lane M: 100-bp DNA marker, lane 1: negative control, lane 2: amplified *eae* gene fragment, lane 3: amplified *omp31* gene fragment

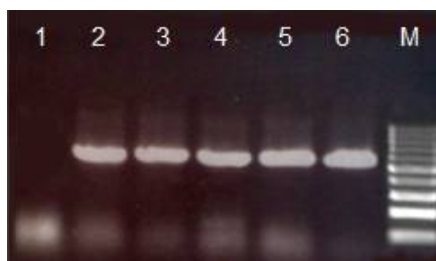


Figure 2: Detection of the *B. melitensis omp31* gene in the cultured colony matrix by PCR. Lane M: 100-bp DNA marker, Lane 1: negative matrix sample, Lane 2: matrix 1, Lane 3: matrix 2, Lane 4: matrix 3, Lane 5: matrix 4, Lane 6: matrix 5

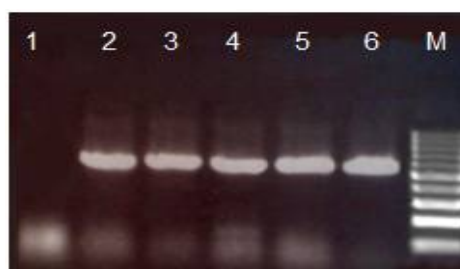


Figure 3: Detection of the *E. coli eae* gene in the cultured colony matrix by PCR. Lane M: 100-bp DNA marker, Lane 1: negative matrix sample, Lane 2: matrix 1, Lane 3: matrix 2, Lane 4: matrix 3, Lane 5: matrix 4, Lane 6: matrix 5

Antibodies and immunological responses

Immunization with pCDNA3-*omp31-eae* led to a rapid IgG response within two weeks after vaccination. Titers ranged from 90:160 to 2,570:5,110; the peak titers were detected 60 days after vaccination. IgG1 and IgG2a antibody analyses revealed that anti-Omp31 and anti-Eae



Figure 4: PcDNA3-*omp31-eae* restriction analysis. Lane M: 1-kb DNA marker, Lane 1: 5428-bp pcDNA3 fragment and 862-bp *eae* fragment (correct clone), Lanes 2–3: 5428-bp pcDNA3 fragment without the *eae* gene

antibodies were found in pcDNA3-*omp31-eae* immunized mice, but not those given pcDNA3. We determined the proliferation responses and cytokine profiles of spleen cells from mice injected with pcDNA3-*omp31-eae*, pcDNA3, and PBS to examine cell-mediated immunity (CMI). Lymphocytes from mice immunized with pcDNA3-*omp31-eae* had an apparent T cell proliferative response to rOmp31 and rEae proteins ($p < 0.01$) with a stimulation index of 14.90.

Cytokine profiles using supernatants of spleen cell cultures revealed higher levels of IFN- γ in pcDNA3-*omp31-eae*-immunized animals compared to the negative control groups. The rOmp31 and rEae proteins significantly induced the production of IFN- γ in cells from pcDNA3-*omp31-eae*-immunized mice ($p < 0.05$ in both groups), and only low levels of IL-10 were found in all groups. In addition, no IL-4 was detected in culture supernatants of splenocytes that were stimulated with specific antigens.

Protection tests were accomplished by challenging control and vaccinated mice with *B. melitensis* and *E. coli* and evaluating infection levels by determining CFU counts in the spleen. The results shown in Table 2 indicate that immunization with pcDNA3-*omp31-eae* resulted in a significantly higher degree of protection in comparison with the unimmunized control groups.

Table 2: Comparison of stimulation by PBS, pcDNA3, and pcDNA3-*omp31-eae* in splenocyte cultures from all groups of mice

Test group	Cytokine			Antibody	
	IL-10	IL-4	IFN- γ	IgG2a	IgG1
PBS	-	-	-	-	-
PcDNA3	-	-	-	-	-
PcDNA3- <i>omp31-eae</i>	+	-	+++	+++	++

DISCUSSION

DNA vaccines consisting of genes cloned into a eukaryotic plasmid are a novel and promising approach that can induce both humoral and cellular immune responses [25]. Control of *Escherichia* and *Brucella* has markedly reduced the incidence of human infection, but these diseases still represent an important cause of morbidity worldwide. To design a new generation of vaccines, more information on the antigenic makeup of *Brucella* and *Escherichia* spp. must be obtained to identify immunodominant proteins and epitopes. A human vaccine would be valuable for individuals who may be occupationally exposed to *Brucella* and *Escherichia* spp.

Numerous attempts have been made to control human disease by vaccination with attenuated *Brucella* strains [21]. However, practical acceptance of these preparations has been very limited, mainly because of the unacceptable local inflammatory responses they elicit, combined with their limited protection. Obviously, there is a need for a better vaccine for brucellosis eradication [21]. Mice immunized with *omp31* were significantly protected against *B. ovis* and *B. melitensis* infections [3].

Eae gene cloning results of the present study were similarly successful as those of for expression of the STX2 gene of *E. coli* O157:H7 in pUC118 and pUC119 vectors [12] and of Hongyan *et al* [26]. Thus, our DNA construct can be used as a DNA vaccine against *E. coli* in future studies.

The development of a safe and effective vaccine against *B. melitensis* and *E. coli* has proven to be complicated. Indeed, numerous intracellular and cell surface components have been assessed as protective antigens against these two bacterial infections, although only low or intermediate levels of protection have been achieved with these isolated proteins [9,12]. The *B. melitensis omp31* and *E. coli eae* genes are promising candidates for use as a subunit vaccine against brucellosis and *E. coli* infection. In the case of *B. melitensis* and *E. coli*, cellular immune responses characterized by the production of IFN- γ are critical to conferring protection [27].

In summary, this study demonstrates that DNA vaccines can be effective against *E. coli* and *B. melitensis* infections. In this study, we also assessed the capacity of pcDNA3-*omp31-eae* to elicit an immune response and protective immunity in BALB/c mice. Since the levels of protection achieved by a multiple DNA vaccine

encoding the *omp31* gene of *B. melitensis* and the *eae* gene of *E. coli* are more than sufficient, this double DNA vaccine could be a useful candidate for the development of DNA vaccines against *E. coli* and *B. melitensis* infections.

Limitations of the study

DNA vaccines are limited to protein immunogens, that is, they can't be used to confer immunity to non-protein based antigens such as bacterial polysaccharide. Induced antibody production against DNA may occur in the host organism. The achieved immune response in mice was satisfactory, but to obtain immunization in larger animals, high-level DNA doses are needed. In addition, insertion of foreign DNA into the host genome in order to obtain immune response may affect the control of cell growth.

CONCLUSION

The DNA construct that was produced in this work can be further developed as a DNA vaccine against *E. coli* and *B. melitensis*. In addition, further studies are required to determine the functions of various T cell types in the protection induced by pCDNA3-*omp31-eae*.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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