

Construction of an Expression Plasmid (Vector) Encoding *Brucella melitensis* Outer Membrane Protein, a Candidate for DNA Vaccine

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Abstract

Background: DNA vaccination with plasmid encoding bacterial, viral, and parasitic immunogens has been shown to be an attractive method to induce efficient immune responses. Bacteria of the genus *Brucella* are facultative intracellular pathogens for which new and efficient vaccines are needed.

Methods: To evaluate the use of a DNA immunization strategy for protection against brucellosis, a plasmid containing the DNA encoding the *Brucella melitensis* (*B. melitensis*) 31 kDa outer membrane protein, as a potent immunogenic target, was constructed.

Results: The constructed plasmid, pcDNA3.1+omp31, was injected intramuscularly into mice and the expression of omp31 RNA was assessed by RT-PCR.

The integrity of the pcDNA3.1+omp31 construct was confirmed with restriction analysis and sequencing. Omp31 mRNA expression was verified by RT-PCR.

Conclusion: Our results indicate that the pcDNA3.1+omp31 eukaryotic expression vector expresses omp31 mRNA and could be useful as a vaccine candidate.

Keywords: *Brucella melitensis*, DNA Vaccine, Omp31, PcDNA3.1

Introduction

Brucellosis, also known as Malta fever and undulant fever, is one of the most important common diseases between human and animals (1). Different species of *Brucella* are responsible for this disease. *Brucellas* are facultative, gram-negative, intracellular bacteria that can infect a wide range of animals, including humans. *Brucella abortus* (*B. abortus*) infects cows, *Brucella melitensis* (*B. melitensis*) infects sheep and goats, *Brucella swiss* (*B. swiss*) infects pigs, and *Brucella canis* (*B. canis*) infects dogs, and all these species can cause disease in humans as well (2-3). Infections can cause abortion, milk reduction, infertility, and financial loss; therefore, this bacteria has both important economical and clinical aspects (4).

Brucellosis has been long known as one of the zoonotic diseases that causes financial and clinical problems, especially in Mediterranean and middle-eastern countries (5-6).

As with infections caused by other intracellular pathogenic bacteria, brucellosis resistance is due to cellular immune responses and activation of T lymphocytes and macrophages (7-8). Production of IFN γ via CD4⁺ Th1 cells, in which IFN γ strengthens macrophage activation against *Brucella* and activation of CD8⁺ T cells, is an important aspect of this response that can aid in lysis of *Brucella*-infected cells ((9, 10).

Rev1, which can cause immunity in goat, is one of the most common live attenuated vaccines against brucellosis. But as with

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Brucella vaccines, abortion, the possibility of human infection, and diagnosis interference of infected vs. vaccinated animals are disadvantages of the Rev1 vaccine (9). High efficiency, safety, and the ability to provide long-lasting immunity after a single immunization are the criteria for an ideal vaccine, and DNA vaccines can meet all these criteria. Because of the ability of DNA vaccines to induce prolonged cellular immune responses, these vaccines can be good candidates for intracellular bacteria such as *Brucella*. In addition, DNA vaccines can cause constitutive antigen expression, which can induce and strengthen memory immune responses (10-13). Expression of different *Brucella* antigens, which can induce immune responses, is another important point in DNA vaccine production. The ability of *Brucella* outer membrane proteins, such as *omp31*, to induce an immune response has been verified (14-16).

Because of the properties and advantages of DNA vaccines, and because of the necessity for a new and an efficient vaccine against *Brucella*, production of a nucleic acid vaccine against *B. melitensis* was our main goal in this project.

Materials and Methods

Construction of pcDNA3.1+omp31 Plasmid

The *omp31* gene was PCR amplified from the *B. melitensis* Rev1 strain using specific primers and *Pfu Taq* polymerase. Primers were designed according to the deposited sequences of *B. melitensis* in the GenBank database and synthesized by Genfanavaran, Macrogene, Seoul, South Korea. The primers sequences were as follows: The forward primer (Fomp31):

5'CTAGAATTCGTAATGAAGTCCGTAATTTTGGCGTCCAT3' with an *EcoR* I restriction site; and the reverse primer (Romp31):

5'TATTGGAGCTCGAGTCAGAACTTGTA GTTCAGACCGACGC3' with an *Xho* I restriction site. Cycling profile for amplification was one starting cycle at 94 °C for 3 min, followed by 5 cycles at 94 °C for

45 sec, 58 °C for 45 sec, and 72 °C for 45 sec. The PCR was continued for 30 cycles at 94 °C for 45 sec, 61 °C for 45 sec, and 72 °C for 45 sec. The final primer extension was performed at 72 °C for 10 min. PCR products were electrophoresed on a 1% (w/v) agarose gel, the corresponding band was cut from the gels, and the PCR product was purified from the gel using a DNA Extraction Kit (Fermentase, Lithuania). Restriction enzyme digestion was performed on the purified PCR product using *EcoR* I and *Xho* I. The pcDNA3.1+plasmid was amplified in *Escherichia coli* (*E. coli*) TOP10 cells. The amplified plasmid was purified by the alkaline lysis method and subjected to digestion using *Xho* I and *EcoR* I. The products were separated on a 1% (w/v) agarose gel and the fragments were purified from the gel with a DNA extraction kit (Fermentase, Lithuania). The purity and concentration of the DNA fragments were determined by gel electrophoresis and spectrophotometry. The pcDNA3.1+Vector and *omp* DNA fragments were ligated at a ratio of approximately 3:1 by T4 DNA ligase at 20 °C for 30 min. Competent *E. coli* TOP10 cells were transformed with 5 µl of ligation reaction mixture and plated on LB agar containing 50 µg/ml ampicillin. The colonies were then transferred to LB medium and incubated overnight at 37 °C with shaking to obtain a saturated culture. Recombinant plasmid DNA was extracted, purified, and analyzed.

Analysis of the plasmid construct

Restriction enzyme analysis was employed to confirm the presence of the *omp31* fragment in the pcDNA3.1+*omp31* vector. Then selected colonies were sequenced (Genfanavaran, Macrogene, Seoul, Korea). Selected plasmids were purified from transformed *E. coli* TOP10 cells by the alkaline method. The purified plasmids were dissolved in sterile, endotoxin-free PBS, pH 7.2, and stored at -20 °C. The integrity of the DNA plasmids was checked by agarose gel electrophoresis after digestion with appropriate

restriction enzymes. The DNA concentration was determined by measuring the optical density at 260 nm.

To examine the capability of *in vivo* expression, one mouse was injected intramuscularly with 100 µg of the pcDNA3.1+omp31 construct. Total RNA was isolated from the injected muscle three days after injection using a Tri Pure Kit (Roche) and used for cDNA synthesis followed by RT-PCR with specific *omp31* primers. α -actin primers were used as an internal control.

Results

Analysis of pcDNA3.1+omp31 DNA

The *omp31* fragment was used as the target gene to construct the pcDNA3.1+omp31 vector after ligation of the *omp31* gene in the *EcoR* I and *Xho* I restriction sites of the eukaryotic expression vector, pcDNA3.1+ (Fig. 1).

Restriction enzyme analysis and DNA sequencing verified the integrity of the construct (Fig. 2). The sequence of *omp31* in this vector has been deposited in the GenBank database (Accession No. GQ403950).

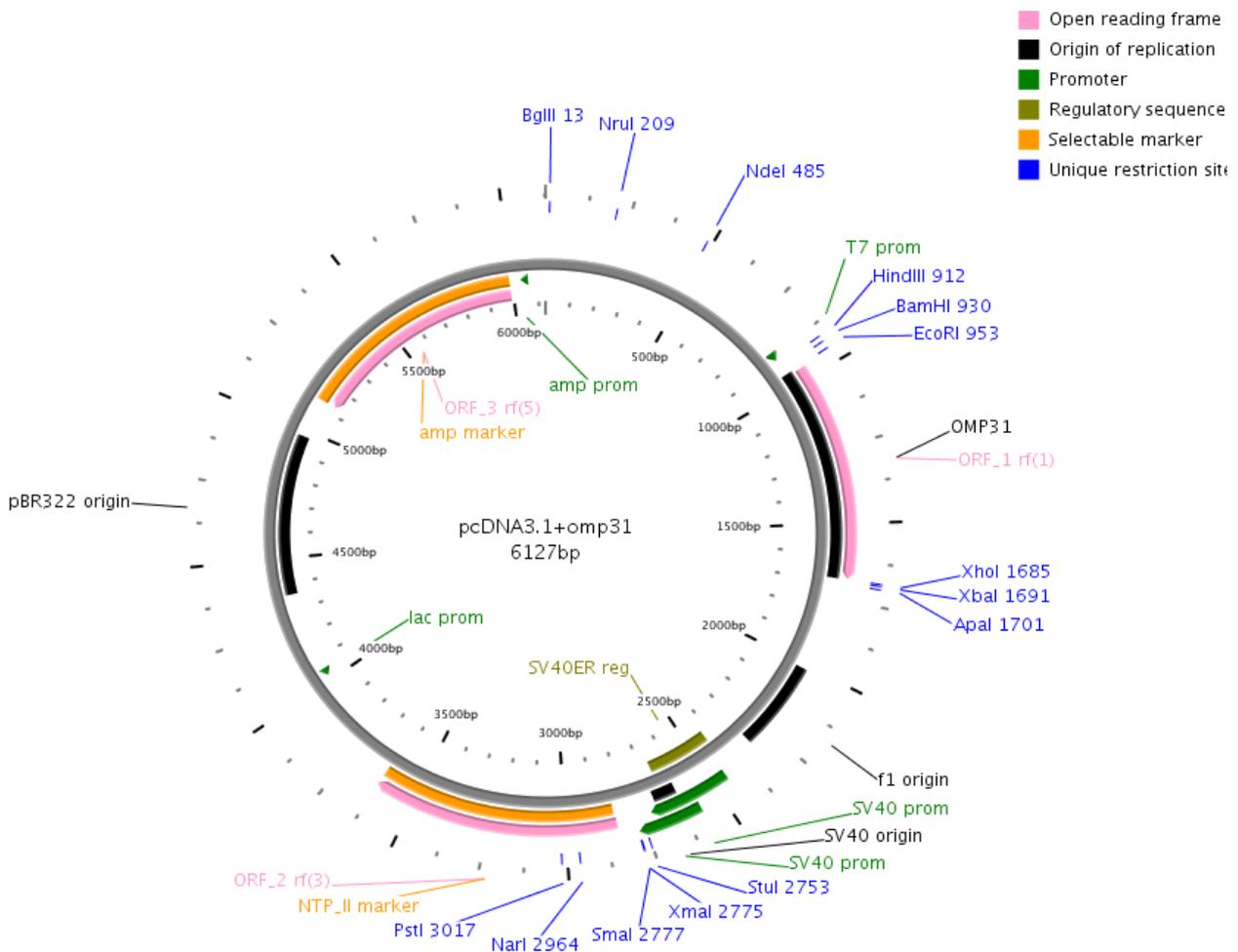


Fig. 1. pcDNA3.1+omp31 vector. The *omp31* gene was cloned into the multiple cloning site (between *EcoR* I and *Xho* I) in pcDNA3.1+.

Analysis of pcDNA3.1+omp31 RNA

To confirm that the pcDNA3.1+omp31 construct can direct expression of *omp31* in eukaryotic cells one mouse was injected intramuscularly with 100 µg of the pcDNA3.1+omp31 construct. The presence of bands of about 750 base pairs (bps) on

electrophoresis gel following RT-PCR of RNA extracts demonstrated that recombinant *omp31* mRNA is transcribed in the muscle (Fig. 3).

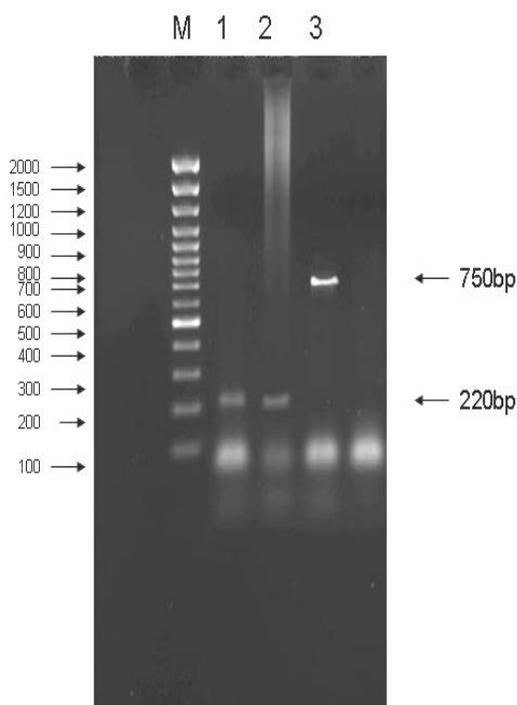


Fig. 2. Agarose gel electrophoresis of the pcDNA3.1+omp31 plasmid restriction digest. Lane M, DNA size marker; lane 1, plasmid digested with *EcoR* I and *Xho* I.

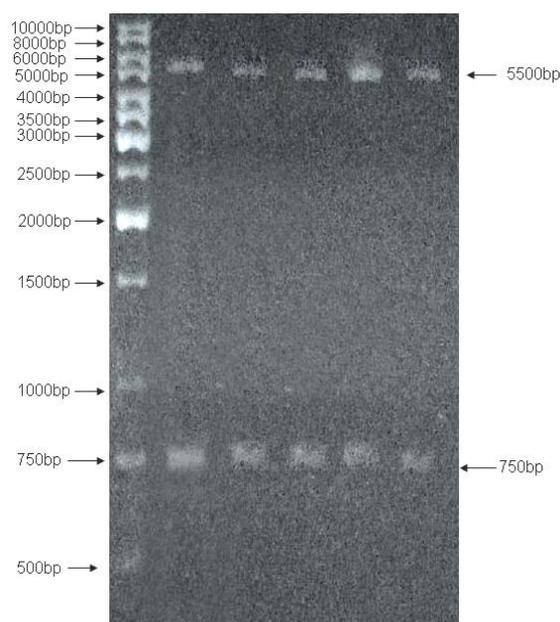


Fig. 3. Analysis of pcDNA3.1+omp31 mRNA by RT-PCR. Lane M, DNA size marker; lanes 1 and 2, RT-PCR product of immunized mouse myocyte RNA using β -actin primers as a control for cDNA synthesis; lane 3, RT-PCR product of immunized mouse myocyte RNA using Fomp31 and Romp31 primers.

Discussion

In this study, we constructed a plasmid encoding the omp31 gene of *B. melitensis*, a potent immunogenic target, as a candidate DNA vaccine for brucellosis. First, the omp31 gene fragment was amplified from

total extracted DNA of *B. melitensis*. Then the purified omp31 gene was cloned into the eukaryotic expression vector pcDNA3.1+. Both enzyme digestion and sequencing confirmed the construction of recombinant plasmid pcDNA3.1+ omp31. Competent *E. coli* TOP10 cells were transformed with plasmid pcDNA3.1+ omp31, and expression of omp31 RNA in mouse muscle was verified by RT-PCR.

These results suggest that the pcDNA3.1+omp31 plasmid may be used as a DNA vaccine against brucellosis. DNA vaccines provide a valuable technology for rapid development of safe and efficient vaccines needed for emerging infectious diseases.

In a few studies DNA vaccines have been determined to induce immune responses in animals. Eukaryotic expression vectors pCIBFR and pCIP39, encoding BFR and P39 antigens, respectively, were injected. Both vectors elicited T-cell-proliferative responses, induced strong gamma interferon production and strong, long-lived memory immune responses that persisted at least three months after the final vaccination (17).

The immunogenicity and protective efficacy of a DNA vaccine encoding the GroEL heat-shock gene from *B. abortus*, tested in BALB/c mice, showed a significant level of protection (18). Lumazine synthase (19), Cu, Zn superoxide dismutase (20), and superoxide dismutase (SOD) of *B. abortus* (21) have been tested and elicited different levels of protection in animals.

The outer membrane proteins (Omps) of *Brucella* spp. have been identified as the best potential immunogenic candidates for making vaccines. The immunogenicity and protective efficacy of the *B. melitensis* Omp31 gene cloned in the pCI plasmid (pCIOmp31) in BALB/c mice have been evaluated against *B. ovis* and *B. melitensis* infections. The pCIOmp31-induced cytotoxic protection responses were mediated predominantly by CD8+ T cells, although CD4+ T cells were also identified (22).

Further studies are necessary to determine whether this form of vaccination is effective against brucellosis. In the future, the efficacy of this vaccine in immune response induction will be investigated by our team.

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