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Full Length Research Paper

Recombinant *Brucella abortus* outer membrane protein 19 (rOmp19) significantly stimulates splenic lymphocytes of immunized BALB/c mice

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Several live vaccines have been introduced so far to protect brucellosis but they have not been adopted broadly or their use has been discontinued for a variety of reasons. Immunity against facultative intracellular bacterial pathogen depends on acquired cell mediated immunity, distinguished by the activation of T-lymphocytes and subsequent activation of macrophages for increased killing of such intracellular pathogens. Three minor Outer membrane proteins (Omp19, Omp16.5 and Omp10) are expressed in all six *Brucella* species and all of their biovars. We investigated the capacity of *Brucella abortus*, outer membrane protein 19 (Omp19) to elicit splenic lymphocytes stimulation response. We assessed the use of this protein as vaccine against *Brucella* in BALB/c mice. *B. abortus Omp19* was cloned in pET28a(+) vector and Competent E. coli BL21(DE3) was transformed with the corresponding constructs, and rOmp19 was successfully expressed after induction with 1 mM IPTG. The expressed protein was purified using nickel affinity chromatography and confirmed by western blot analysis using polyclonal rabbit antibodies. BALB/c mice immunization with rOmp19 conferred significant protection against B. abortus infection. The potential usefulness of this antigen of *B. abortus* would be of great importance in eradication of brucellosis in future.

Key words: *Brucella abortus*, recombinant outer membrane protein 19 (rOmp19), Lymphocyte proliferation.

INTRODUCTION

Brucellosis is an important zoonosis with worldwide distribution. Facultative intracellular bacteria cause abortion, infertility and leading to important economic losses in animals (Emslie and Nel, 2002). The main pathogenic species for domestic animals are, *Brucella abortus*, responsible for bovine brucellosis; *Brucella melitensis*, the major etiologic cause of small ruminant brucellosis; and *Brucella suis* is responsible for swine brucellosis (Matthew and Axel, 2002) Brucellosis is also a human disease which distinguished by fever, chills, malaise and a chronic course that requires six weeks of therapy with at least two antibiotics for treatment (Buzgan et al., 2009). Human brucellosis has also been attributed to some marine mammal strains recently. Control and eradication of brucellosis in domestic animals have important public health and economic implications. Vaccination is the most important method of control of animal brucellosis (Davis and Elzer, 2002). Thus, prevention of human brucellosis depends predominantly on the control of the disease in animals. Currently, *B. abortus S19* or *B. abortus RB51* is used to immunize cattle, whereas the *B. melitensis Rev 1* strain is used to immunize goats and sheep (Anne and Vale, 2002). No other vaccines are licensed for other animals, and a human brucellosis vaccine does not exist. Generally, the use of live attenuated organisms as vaccines have some problems in terms of safety during vaccine production (Andrew et al., 1973; Erasmus and Bergh, 1985; Schurig et al., 1991). To provide safety, there were no replication vaccines which are easy to reproduce, for quality

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assurance; we worked on the development of a subunit vaccine against *Brucella*. In particular, we aim to develop a recombinant subunit vaccine. Outer membrane proteins (OMPs) are important immune gens in most of the gram negative bacteria (Guillermo and Giambartolomei, 2004). They are particularly effective for development of vaccine candidates and diagnostic kits. Previous studies on OMPs of different *B. abortus* strains have shown 3 main groups including group I proteins having molecular masses between 88-94 kDa, group II between 36 and 38 kDa and group III between 31 and 34 kDa and 25-27 kDa (Cloeckaert et al., 2002), several low molecular weight proteins have been identified as minor Omp in *B. abortus* (Omp19, Omp16.5 and Omp10) (Bowden et al., 2000; Tibor et al., 2002). The 19-kDa Omp is a surface-exposed lipoprotein expressed in all six Brucella species and all their biovars. The sequence information suggests that Omp19 is a lipoprotein (Guillermo and Giambartolomei, 2004). For this reason, we evaluated the immunologic features of rOmp19 to prevent *Brucella* infection. The subunit vaccine was tested in mice model and was able to proliferate splenic lymphocytes of immunized BALB/c mice.

MATERIALS AND METHODS

Plasmid vectors and primers design bacterial strain and cultivation

For this study, *Brucella* agar was utilized to culture *B. abortus S19* routinely. pJET1.2 (Fermentas) and pET28a(+) (Novagen) were applied for cloning and expression vectors, respectively. *Escherichia coli* $DH_5\alpha$ and *E. coli* BL21(DE3) were applied as cloning and expression prokaryotic hosts, respectively. Primers were designed to amplify the whole coding region of Omp19 (ACCESSION: U35742) consisting of FB18F 5'-AAC**GGATCC**ATGGGAATTTCAAAAGCAAGTCTGCTC-3' and FB18R 5'-AAA**AAGCTT**TCAGCCCAACAGCGTCACGGCCTGC containing either restriction sequences of BamHI and HindIII, respectively. The forward and reverse primers included an additional AAC and AAA sequences at the 5´ends respectively, so, the polymerization was performed from the right site. Complete *omp19* ORF was amplified by PrimSTAR® HS DNA polymerase with high proof reading power which synthesized the insert with blunt end.

Cloning of the gene encoding the 18-kDa protein in a pET28a(+) vector

A DNA fragment which encodes for approximately 18 kDa protein from *Brucella abortus S19* was amplified with *PrimSTAR® HS DNA* polymerase, cloned in pJET1.2. pJET1.2 including the gene coding for *B. abortus* Omp19 was double digested with BamHI and HindIII and vertified by sequencing and aligned with reference sequence. To obtain recombinant Omp19 (rOmp19), the corresponding gene was sub cloned in pET28a(+) and cloned.

Expression of the protein

The plasmid DNA of a clone containing the inserted (pET28a(+)-*omp19*) was used to transform *E. coli* BL21(DE3) competent cell. Recombinant clones were grown in LB medium containing kanamycin for 3 h at 37°C to reach an ODo.6. The culture was induced by adding 1 mM IPTG and samples which incubated at 37°C were collected. Crude cells were boiled in sample buffer and subjected to SDS-PAGE in acrylamide 15% gel and followed by Coomassie Brilliant Blue G-250 staining. Cell pellet of two liters of four hour inducted culture collected and was purified according to the universal protocol by QIAGEN manual (QIA*expressionist*™). Briefly, cell pellet of four hour induced BL21 harboring pET28-omp19 were suspended again in a lysis buffer (Buffer A) containing PMSF as antiprotease and sonicated. Cell debris was separated by centrifugation.

Purification, refolding of the protein and immunization

rOmp19 was purified using NI-NTA resin. NI-NTA resin (QIAGEN) were added to the supernatant and shacked for one hour. Resins were gathered on a cartridge and washed subsequently. Recombinant protein was eluted with 250 mM imidazole, and the elution reagent was removed by dialysis against phosphate buffered saline (PBS). After SDS-PAGE in 15% polyacrylamide gel, purified rOmp19 and crude induced BL21 cell lysate, were separated and transferred to PVDF sheet using BioRad transblott system. Sheet was blocked by bovine serum albumin. The protein was probed by polyclonal rabbit antibodies to *B. abortus* outer membrane protein preparations. HRP conjugated anti-rabbit IgG was applied to the sheet as the secondary antibody. Sheets were finally developed with diamino benzidine. This Purified recombinant protein, designated BA18K, reacted in Western blot analysis and probed by 1:3000 dilutions of 10 serum samples from experimentally and brucellosis patients who were treated with HRP-Anti-Human IgG and developed as described above. The experimental test performed in BALB/c mice was subcutaneously vaccinated with 20 µg of recombinant protein combined with ferund's adjuvant. Antigen was administered and primary followed by two boosters with 2 weeks of intervals. Splenic lymphocytes were extracted and cultured. Lymphocytic cultures were stimulated with 10 µg/ml of rOmp19. Lymphocyte proliferation response to the protein antigen was assessed via MTT test and compared to that of nonimmunized mice lymphocytic cultures.

RESULTS

The 18kDa protein of *B. abortus* was expressed in PET28a(+) as inclusion bodies in BL2 1 (DE3) cells. The inclusion bodies were solubilised in 8 M urea and the protein was refolded by dialysis against PBS. The expression system and refolding procedures used resulted in a good yield of the recombinant protein properly folded. SDS-PAGE analysis of induced culture samples compared to noninduced ones and non-transformed *E. coli* BL21(DE3) shows the expression of a protein of about 20 kDa. This was consistent with the expected protein of 17.53 kDa plus pET28a(+) added amino acids. The expression reaches the maximum rate two hours after induction and remains unchanged in the next two hours. 20 µg of the mentioned protein combined with ferund's adjuvant was used to immunize BALB/c mice. Cultured lymphocytes were stimulated with 10 µg/ml of rOmp19 (Figure 1). Lymphocyte proliferation response to the antigen was evaluated by MTT test and measure up to that of nonimmunized mice lymphocytic

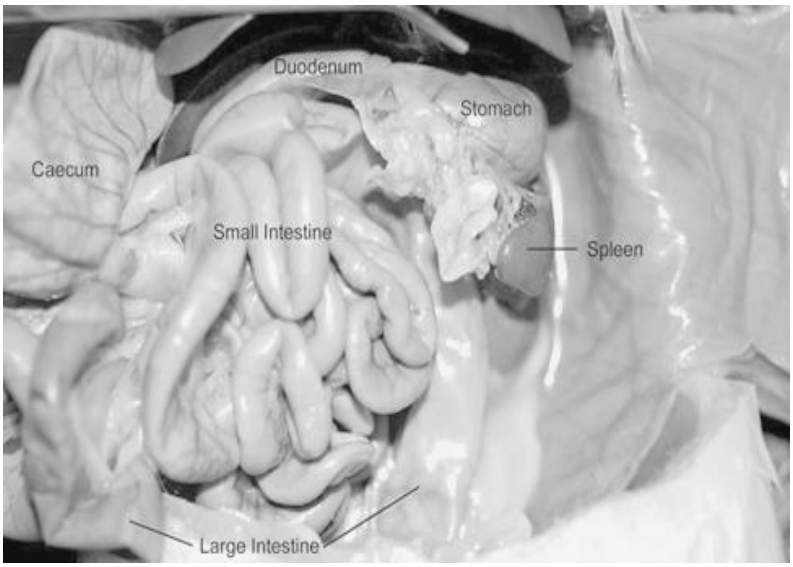

Figure 1. The extracted spleen was cultured. Lymphocytes were stimulated with 10 µg/ml of rOmp19.

cultures. Polyclonal antiserum successfully recognized rOmp19 in crude cell lysate. Purified recombinant Omp19 successfully recognized by rabbit polyclonal anti serum and sera from 10 microbiologically confirmed hospitalized patients. The epitops of rOmp19 afforded a level of protection similar to that conferred by the native component. Proliferation rates of lymphocytes from immunized mice were significantly higher than that of lymphocytes from non immunized ones. One of the major aims of this work was to develop of new vaccine which, being protective, should not interfere with the diagnosis of infections due to smooth Brucellae. The overall result of the present study showed that rOmp19 of *B. abortus* is an appropriate antigen for further investigations in vaccines and serologic diagnosis programs.

DISCUSSION

In order to establish the protective value of the subunit vaccine, a study of immunization and experimental infection with *B. abortus* in mice was performed. The first effective *Brucella* vaccine was based on live *B. abortus* strain 19. This induces suitable protection against *B. abortus*, but at the cost of persistent serological responses (Mark and Steven, 1995). A comparable problem happens with the *B. melitensis Rev.1* strain that is still the most efficient vaccine against caprine and ovine brucellosis (Adone et al., 2008). This vaccines kills cells of virulent strains administered with adjuvant induced considerable protection, but also unacceptable levels of antibodies interfering with diagnostic tests (Axel and Isabelle, 2004). The rifampicin-resistant mutant *B. abortus RB51* strain has been proved safe and effective in the field against bovine brucellosis (Julia and Marisa, 2008), and exhibits negligible interference with diagnostic serology (Sheela and Neelima, 2007). Various studies have examined recombinant proteins as candidate protective antigens, with or without adjuvants (Raúl and Silvia, 2000). We were interested in *B. abortus Omp19* which is expressed in all six *Brucella* species and all their biovars (Guillermo and Giambartolomei, 2004).

Omp19 gene was cloned in pET28a(+) and expressed, in the previous study (Fatemeh and Esmaeil, 2012). The mice were immunized by rOmp19 with ferund's adjuvant. Splenic lymphocytes were extracted and cultured. Cultured lymphocytes were stimulated with 10 µg/ml of rOmp19. Lymphocytic stimulation response to rOmp19 was evaluated by MTT test (Figure 2) and compared to that of nonimmunized mice lymphocytic cultures. Proliferation rates of lymphocytes from rOmp19 immunized mice were significantly higher than lymphocytes from non immunized mice. The use of rOMP19 of *B. abortus* for providing protection against experimental challenge in laboratory mice is reported in Table 1. Recognition of antibody to the recombinant protein in serum samples of infected cases suggests the stimulation of immune response to this protein during brucellosis.

Conclusion

Several brucella proteins have been sequenced and cloned, but only a few have been characterized at the structural and functional level. The ability of the recombinant Omp19 to stimulate lymphocytes implies the potential of the protein to be concerned as an antigen for the serological diagnosis of brucellosis.

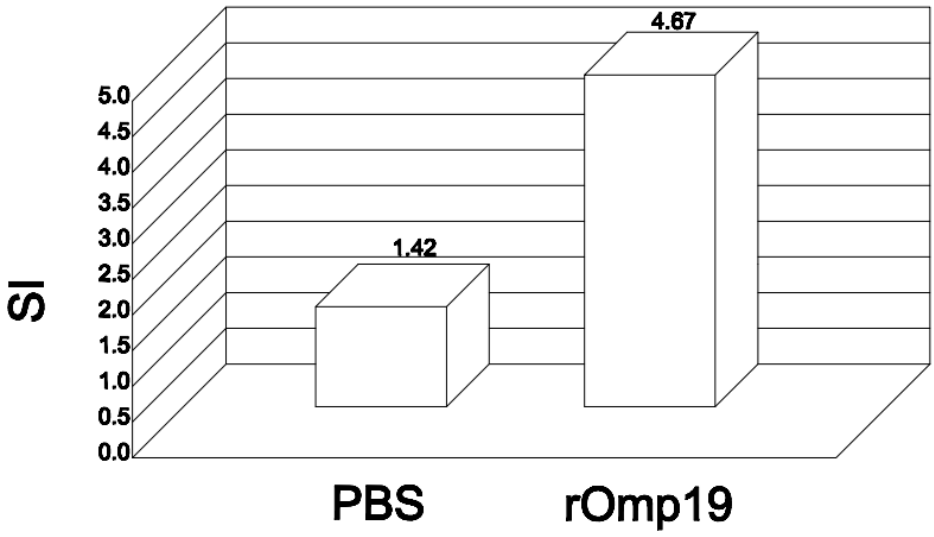

Figure 2. Lymphocytic proliferation response to the antigen was assessed via MTT test.

Table 1. rOMP19 provided reasonable protection in laboratory mice.

Recombinant lipoprotein	Log Unit Protection	Clearance (%)
rOmp19	1.35	26

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