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Recombinant outer membrane protein 25c from *Brucella abortus* induces Th1 and Th2 mediated protection against *Brucella abortus* infection in mouse model

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ABSTRACT

Development of a safe and efficacious vaccine for brucellosis is a long standing challenge for scientists. Recognizing potential antigens towards developing vaccine candidate is crucial. Omp25c, a porin protein of *Brucella*, is a paralog of two previously identified promising vaccine candidates namely, Omp25 and Omp31, with notable sequence identity. Also, Omp25c is conserved in all major *Brucella* species. This highlights the possibility of employing this protein in multivalent subunit vaccine based approach of *Brucella* management. In this study, we were interested in examining the immunogenicity and protective efficacy of Omp25c against *Brucella* infections. Recombinant unlipidated form of this antigen (rOmp25c) produced, upon intraperitoneal immunization in BALB/c mice along with Freund's adjuvant, was confirmed to be highly immunogenic; leading to high IgG antibody titers during the study duration. The IgG2a/IgG2b ratio of anti-rOmp25c antibodies revealed elicitation of Th2 based humoral immunity. Lymphocyte proliferation study divulged induction of specific memory response and secretion of both Th1-type (IFN-γ, GM-CSF and TNF-α) and Th2-type cytokine (IL-5) from restimulated splenocytes of rOmp25c immunized mice. CD4 T-cell subpopulation was comparatively increased than total B cell subpopulation in case of immunized mice, indicating the induction of strong cell-mediated (Th1 biased) immunity than humoral (Th2) immunity. The collective Th1 plus Th2 immune response specific to rOmp25c could be the reason for protection against *Brucella* challenge observed in mice groups that was comparable with S19 vaccine strain. Thus, the study encourages rOmp25c as a potent candidate vaccine against brucellosis.

1. Introduction

Brucella species are Gram-negative, aerobic, non-motile and non-spore-forming coccobacilli that cause brucellosis among cattle and humans (Guilloteau et al., 1999). Brucellosis results in abortion and sterility in case of cattle infection. Among susceptible humans, infection results in chronic illness known as undulant fever (Corbel, 1997). As successful pathogens, they have adopted efficient strategies to evade and interpose with innate immune detection and thus establish a chronic infection (Barquero-Calvo et al., 2007). The capability of this bacteria to survive, replicate and persist within phagocytes, including macrophages, dendritic cells (DCs) and placental trophoblasts, makes it a potent pathogen (Salcedo et al., 2008; Archambaud et al., 2010; Copin et al., 2012). To achieve this, the bacterium has inherent strategies of resisting and escaping the killing mechanisms of phagocytic cells and inhibiting the elicitation of host immune response (Splitter et al., 1996). Furthermore, intracellular lifestyle of *Brucella* and acidic environment surrounding the bacteria within the host cell limits the innate and adaptive immune responses (Martirosyan and Gorvel, 2013); in turn helps them to evade from the action of antibiotic therapy (de Figueiredo et al., 2015). As an add-on threat, the aerosolization and high invasiveness features have made this pathogen a biothreat agent (de Figueiredo et al., 2015). Therefore, prevention, rather than cure of brucellosis, is critical.

Various vaccination strategies including attenuation and subunit candidate vaccines have been extensively explored. However, the effort remains same to identify additional protective antigens for conferring long term effective and safe protection against brucellosis (Rep. W. H. O. Meet., W. H. O. document EMC/ZDI/98.14, p. 40–43, World Health Organization, Geneva, Switzerland, 11–12 December 1997; Olsen and Stoffregen, 2005; Yang et al., 2013). In such a case, multiple subunit vaccine or multiple protein species could possibly induce better immunogenicity and effective protection against brucellosis in mice model.

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Outer membrane proteins (Omps) of *Brucella* are reported to be immunogenic in bovine and human and hence are a subject of interest towards better vaccine strategy against brucellosis (Jacques et al., 1992; Cloeckaert et al., 1996; Bowden et al., 1998; Guilloteau et al., 1999; Connolly et al., 2006). So far, Omp25 and Omp31 have been successfully characterized for vaccine potential in animal models (Jubier-Maurin et al., 2001; Goel and Bhatnagar, 2012; Estein et al., 2003). Interestingly, these two proteins have 34% identity and thus belong to group 3 Omps based on molecular masses and amino acid sequence identity (Salhi et al., 2003). Further on, Omp25 is present in all *Brucella* species and Omp31 in all except *B. abortus* (Vizcaino et al., 2001). This highlights the possibility of deducing more promising immunogens from the group for making better vaccine molecules with multivalent antigen approach. In this regard, we sorted for paralogs with higher identity to Omp25 and Omp31 for careful characterization of novel putative Omps. This analysis revealed an autotransporter barrel protein termed Omp25c, with 39 and 37% identity with Omp25 and Omp31 respectively. This antigen was also present in *B. melitensis, B. abortus, B. ovis* and *B. suis* with a very little polymorphism (Salhi et al., 2003).

Having confirmed for notable amino acid sequence identities with promising candidate vaccines, Omp25c merited further investigation with respect to vaccine potential. In this study, our main interest was to evaluate Omp25c for its protective efficacy against *B. abortus* 544 challenge in mouse model. To achieve this, unlipidated form of Omp25c (rOmp25c), devoid of the signal peptide and N-terminal cysteine, was expressed in bacterial heterologous expression host and immunized in BALB/c mice intraperitoneally. The range of immune response evoked by BALB/c mice against this recombinant protein was studied by determining immune system parameters including IgG antibody titer, isotype profile, proliferation index, specific cytokine production, lymphocyte subpopulation enumeration and challenge studies.

2. Materials and methods

2.1. Ethics statement

The project was approved by Defence Food Research Laboratory (DFRL), Defence Research Development Organization with project no. NBC-ST1. All the animal experiments were performed with the approval and written consent of the Institutional Animal Ethics Committee (IAEC) of DFRL. All animal surgeries were performed with highly minimized pain and sufferings as per institutional guidelines.

2.2. Mice

5–6 weeks of inbred specific pathogen-free female BALB/c mice were obtained from Central Animal Facility, Defence Food Research Laboratory (DFRL). Mice were housed in standard cages (n = 10 mice/cage) under pathogen free condition and were provided water and feed *ad libitum*. Animals were acclimatized to housing for a week before initiating the immunization study. After BALB/c mice were injected with *B. abortus* 544, they were kept in Biosafety Level 3 animal facilities. The animals were housed and experiments were carried out as per the standards of Institutional Animal Ethics committee (IAEC) and institutional regulations with permit no SS-10/DRD(DOM)-001.22/DFR (ST).

2.3. Bacterial strains and propagation

Bacterial strains of *B. abortus* (live attenuated vaccine strain S19) and *B. abortus* 544, *B. melitensis* 16 M and *B. suis* 1330 were obtained from Indian Veterinary Research Institute, Izatnagar, Bareilly, India. *E. coli* strains of DH5α and BL21(DE3)pLysS cells (Invitrogen, CA) were used for propagation and expression of plasmids respectively. *Brucella* strains were maintained in Tryptic Soy medium (TSA) whereas *E. coli* strains were grown in LB medium (Himedia, India). Antibiotics were used at the final concentration of 100 μg/ml for ampicillin and 35 μg/ml for chloramphenicol. *Brucella* antibiotics including bacitracin (500 μg/ml) and polymyxin B sulfate (1 μg/ml) (Sigma, India) were used as per manufacturer's instruction.

2.4. Antigen (Ag) production

The unlipidated version of the *Omp25c* gene devoid of the signal peptide and N-terminal cysteine was cloned in pRSET A vector (Invitrogen, CA). Briefly, the reference nucleotide sequence of *Omp25c* (Accession no. AY484546) from *B. abortus* 544 strain was used to design the cloning primers for the PCR amplification of the truncated gene sequence with 5′-terminus *BamH*I and *Hind*III restriction sites. Thus, designed primers Omp25c-F–5′ CGCGGATCCGCTGACGCCGTCATTG AAC-3′ and Omp25c-R-5′ CCCAAGCTTTTAGAACTTGTAAGCGACAC-3′, incorporating *BamH*I and *Hind*III restriction sites (underlined), were commercially synthesized (Sigma, India). Full length *Omp25c* gene was amplified using genomic DNA (gDNA) of *B. abortus* 544 strain isolated by CTAB method (Murray, 1980) as template with *Pfu* DNA polymerase (Fermentas, India). Each 25 μl PCR reaction included 2 μl of genomic DNA (gDNA), 1.25 U *Pfu* polymerase (Sigma, India), 50 μM of each deoxynucleotide triphosphate (Fermentas, India), 1x PCR buffer, 2.5 mM $MgSO_4$ and 0.6 pmol of each oligonucleotide primer. Thermal cycling was carried out in Master cycler-Pro thermal cycler (Eppendorf, Germany). PCR condition was as follows: initial denaturation for 4 min at 94 °C followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C, and extension at 72 °C for 60 s with a final 10 min extension at 72 °C. Approximately 10 μl of each resultant PCR product was visualized by agarose gel electrophoresis. The PCR amplified product was further purified using Machrey-Nagel PCR purification kit (Machrey-Nagel, USA). The resultant purified PCR product and pRSET A vector were digested with above mentioned restriction enzymes (Catalog No. FD0054 and FD0504, Fermentas) and again purified by the same procedure. The restricted PCR product and pRSET A vector were ligated using T4 ligase enzyme (Catalog No. EL0014, Fermentas). The ligation mix was used to transform DH5α competent cells. The resulting plasmid DNA (pRSET A-*Omp25c*) was purified from overnight positive DH5α clones using Nucleospin Plasmid Miniprep kit (Machrey-Nagel, USA) and confirmed by DNA sequencing for proper ligation in frame and presence of N-terminal 6x His-tag.

2.5. Protein expression and rOmp25c production

Competent *E. coli* BL21(DE3)pLysS competent cells were transformed with the pRSET A-*Omp25c* construct, and recombinant Omp25c (rOmp25c) was successfully expressed after 3 h of induction with isopropyl-β-D-thiogalactoside (1 mM). The expressed protein was found to be localized in inclusion bodies. This recombinant protein was purified by urea denaturation method. Briefly, one liter IPTG induced culture pellet was re-suspended in 50 ml of buffer containing, 8 M urea, 0.1 M potassium phosphate buffer and 150 mM NaCl, pH 8.3; subjected to sonication and further incubated for lysis at 37 °C for 1 h under shaking condition. Further the resultant lysate was centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant obtained was kept at room temperature for binding with Ni^{2+}-NTA resin (Qaigen, UK) for 2 h. After collecting the flow through of unbound proteins, the Ni^{2+}-NTA column was washed thrice to remove residual unbound proteins using 8 M urea buffer in 0.1 M potassium phosphate buffer and 150 mM NaCl, pH 6.3. Finally, the rOmp25c bound to Ni^{2+}-NTA with 6x His-tag was eluted in 8 M urea buffer in 0.1 M potassium phosphate buffer and 150 mM NaCl, pH 4.5. Presence of the purified rOmp25c in the eluted fractions of proteins was analyzed by SDS-PAGE followed with Commassie blue staining and then confirmed by Western blotting using anti-His antibody (Sigma, India). Purified protein was further dialyzed against gradient concentrations of urea in 1x phosphate-buffered saline (7 mM