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# Immunization of Mice with Recombinant *Brucella abortus* Organic Hydroperoxide Resistance (Ohr) Protein Protects Against a Virulent *Brucella abortus* 544 Infection

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In this study, the *Brucella abortus ohr* gene coding for an organic hydroperoxide resistance protein (Ohr) was cloned into a maltose fusion protein expression system (pMAL), inserted into *Escherichia coli*, and purified, and its immunogenicity was evaluated by western blot analysis using *Brucella*-positive mouse sera. The purified recombinant Ohr (rOhr) was treated with adjuvant and injected intraperitoneally into BALB/c mice. A protective immune response analysis revealed that rOhr induced a significant increase in both the IgG1 and IgG2a titers, and IgG2a reached a higher level than IgG1 after the second and third immunizations. Additionally, immunization with rOhr induced high production of IFN-γ as well as pro-inflammatory cytokines such as TNF, MCP-1, IL-12p70, and IL-6, but a lesser amount of IL-10, suggesting that rOhr predominantly elicited a cell-mediated immune response. In addition, immunization with rOhr caused a significantly higher degree of protection against a virulent *B. abortus* infection compared with a positive control group consisting of mice immunized with maltose-binding protein. These findings showed that *B. abortus* rOhr was able to induce both humoral and cell-mediated immunity in mice, which suggested that this recombinant protein could be a potential vaccine candidate for animal brucellosis.

**Keywords:** *Brucella abortus*, organic hydroperoxide resistance protein (Ohr), immunization, vaccine

## Introduction

Brucellosis is a re-emerging zoonotic disease responsible for great economic losses in animal production for food, and it represents a significant human health threat with an annual incidence of approximately 500,000 cases worldwide [2, 4, 20]. The etiologic agents are highly contagious to humans; an estimated aerosol dose of 10 to 100 organisms is sufficient to establish an infection [3]. Although horizontal human transmission is possible, most cases of human infection are due to exposure to infected animals or contaminated animal matter, such as uterine secretions or aborted fetuses, and consumption of unpasteurized milk and dairy food products [4, 7]. A human vaccine would be useful to protect farmers, veterinarians, animal care workers, laboratory personnel, and the general population; however, no licensed vaccine is available to prevent brucellosis in humans [2]. The use of the currently available live attenuated vaccines has several drawbacks with respect to safety and is not adequate to eliminate brucellosis in any host species [8,18].

The ability of *Brucella* species to invade host cells is a key step in their pathogenesis, and the ability to evade host immune surveillance favors their survival and proliferation. Therefore, host resistance depends mainly on acquired cell-mediated immunity (CMI). Vaccines that can stimulate

strong CMI responses would effectively control the disease [15]. During the last decades, the focus of many research studies has been on the development of safer and more effective *Brucella* vaccines [2].

On the other hand, subunit vaccines are being considered to be developed as effective vaccines for human brucellosis because it has already been used and is currently available against infections such as meningococcus and influenza. Additionally, different studies have evaluated surface structures and antigens of *Brucella* as immunopotent components, to design an efficient brucellosis subunit vaccine, including L7/L12 ribosomal protein [17], outer membrane proteins Omp16 and Omp19 [20], Omp28 [13], and CobB and AsnC proteins [7]. Consequently, we aim to particularly develop a recombinant subunit vaccine that would elicit sufficient protection in host species. A potential candidate is the organic hydroperoxide resistance (Ohr) protein, which belongs to a family of peroxiredoxins that detoxify organic peroxides (except hydrogen peroxide) and has been identified and characterized in several bacteria [5]. In our previous study, this protein displayed a strong immunoreactivity as analyzed by two-dimensional electrophoresis (2DE) followed by western blot analysis with *Brucella*-infected bovine serum [12]. In the present study, the *B. abortus ohr* gene was cloned and expressed in a maltose fusion protein (pMAL) expression system and expressed and induced in *Escherichia coli*. The purified protein showed high immunogenicity without cross-reaction in immunoblotting analysis. This protein was used as a subunit vaccine against brucellosis in a mouse model, and its protective efficacy was evaluated based on the animals' response to immunization.

## Materials and Methods

### Bacterial Strain and Growth Conditions

A smooth, virulent *B. abortus* 544 biovar 1 strain was kindly provided by the Animal and Plant Quarantine Agency in Korea, and *Escherichia coli* DH5α cells were purchased from Invitrogen (USA). *B. abortus* was routinely cultured overnight in *Brucella* broth (BD Biosciences, USA) at 37°C. Solid medium was made by supplementing *Brucella* broth with 1.5% (w/v) agar (Takara, Japan) when needed. *E. coli* DH5α cells were used for producing the necessary plasmid constructs. *E. coli* cultures were grown at 37°C in LB broth or agar with or without 100 μg/ml of ampicillin (Sigma, USA).

### Cloning, Expression, Induction, and Purification of rOhr

The *B. abortus ohr* gene was amplified by PCR with the following primer pairs: 5′ - AAT TC GAA TTC ATG CCA ATT CTT TAC ACG - 3′ (EcoRI site underlined) and 5′ - AGG C AAG CTT TCA GGC TAC GCT CAG GCG - 3′ (HindIII site underlined). The amplified DNA product was digested with the appropriate restriction enzymes (EcoRI and HindIII; Takara, Japan). The digested product was cloned into a pMAL vector (New England Biolabs, USA) and then transformed into *E. coli* DH5α host cells. Bacteria confirmed to express the fusion plasmid were cultured overnight and transferred into ampicillin-containing LB broth, and the recombinant protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM, with further incubation at 37°C for 4 h. The cells were harvested by centrifugation at 5,000 ×*g* for 10 min and resuspended in column buffer (20 mM Tris HCl, 200 mM NaCl, 1 mM EDTA, 0.1% Triton X100, and 10% glycerol, pH 7.4), and then frozen at −70°C and thawed three times at 4°C. The suspensions were sonicated (Bandelin Electronic, Germany) at 10,000 Hz on ice, centrifuged at 8,000 ×*g* for 20 min to collect the supernatant, and then loaded onto a maltose resin column (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. The purified protein was stored at −70°C.

### SDS-PAGE and Immunoblotting

Lysates of the induced cells and the purified proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis as previously described [6, 13]. Briefly, the purified recombinant proteins were boiled for 5 min in 2× SDS buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8). After electrophoresis, the separated proteins were transferred onto Immobilon-P membranes (Milipore, USA) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol, pH 8.3) for 60 min using a semi-dry electroblot assembly (Bio-Rad, USA). The membranes were blocked with 5% skim milk (Difco, USA) for 30 min at room temperature and subsequently washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and then incubated with *Brucella*-positive or -negative mouse sera (produced from mouse immunized by virulent *B. abortus*, 1:1,000 dilution) in blocking buffer at 4°C overnight. The membranes were washed with 0.05% PBS-T and incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000 dilution; Sigma, USA) in blocking buffer for 1 h at room temperature and finally washed with 0.05% PBS-T. The proteins were detected with ECL solution (Thermo Scientific, USA).

### Protection Experiment in Mice

Fifteen six-week-old female BALB/c mice (Japan SLC, Japan) were randomly allocated to three groups. Each mouse was immunized intraperitoneally with 20 μg of rOhr, 20 μg of maltose-binding protein (MBP) (positive control), or PBS (negative control) in 100 μl of incomplete Freund's adjuvant (IFA) (Sigma, USA) on weeks 0, 2, and 5. Serum samples were obtained from the tail vein of all animals on weeks 5 and 7 after the first immunization. After

2 weeks from the last immunization, mice were intraperitoneally challenged with approximately 5 × $10^4$ CFU of *B. abortus* in 100 µl of PBS.

Two weeks postinfection, all mice were sacrificed, and the spleens were removed, weighed, and homogenized in PBS [13]. The homogenates were serially diluted 10-fold with PBS and plated on *Brucella* agar and then incubated for 3 days at 37°C. The $Log_{10}$ number of CFUs for each spleen sample was calculated. Log protection was calculated as the mean $Log_{10}$ CFU of the PBS group minus the $Log_{10}$ CFU of the experimental group. All of the procedures described were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University (Authorization No. GNU-130125-M0008).

**Immune Response Analysis**

After 5 and 7 weeks of the first immunization, blood samples were collected from the tail vein of all mice. After centrifugation at 4,000 ×*g* for 10 min at 4°C, the supernatants were carefully collected as serum samples. The serum titer of the IgG1 and IgG2a isotypes specific for rOhr was subsequently determined by ELISA according to the manufacturer's instructions (Abcam, USA). The cutoff value was calculated as the mean specific OD plus the standard deviation (SD) for non-immunized mouse sera diluted 1:100. The titer was defined as the highest dilution of serum giving an OD of twice the cutoff value. The levels of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 in the sera were determined by cytometric bead array (BD CBA Mouse Inflammation Kit, USA).

**Statistical Analysis**

The data are expressed as the mean ± SD. Student's *t*-test or one-way ANOVA was used to statistically compare the groups. Results with $p < 0.05$ were considered significantly different.

## Results

**Cloning and Immunoreactivity of rOhr Protein**

The *B. abortus ohr* gene product (423 bp) was amplified by PCR, cloned into a pMAL expression vector, and transformed into competent *E. coli* cells. The successfully transformed cells were grown in ampicillin-containing LB broth, and the expression of target protein was induced with 0.2 mM IPTG. The molecular masses of purified rOhr and MBP were approximately 59.15 kDa and 43.5 kDa on SDS-PAGE, respectively (Fig. 1A). The immunoreactivity of the purified rOhr was evaluated by immunoblotting, showing that purified rOhr strongly reacted with *Brucella*-positive serum but did not react with *Brucella*-negative serum (Fig. 1B).

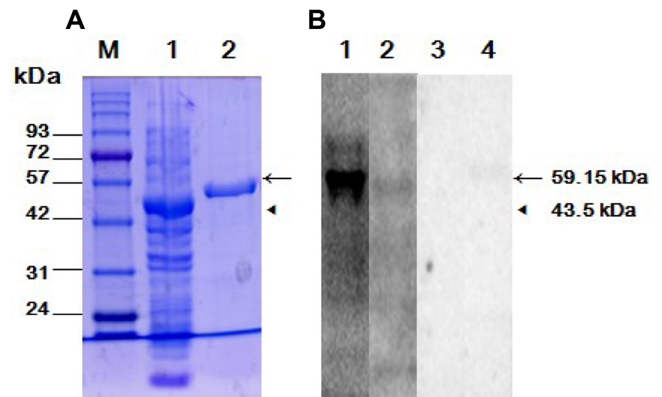


**Fig. 1.** Immunoreactivity of *B. abortus* recombinant Ohr (rOhr). (**A**) Lysate proteins of untransformed cells (lane 1) and purified rOhr (lane 2) separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. (**B**) Immunogenicities of rOhr (lanes 1, 3) and MBP (lanes 2, 4) were determined by western blotting using *Brucella*-positive (lanes 1, 2) or *Brucella*-negative (lanes 3, 4) mouse serum. rOhr (arrows) and MBP (arrowheads) are indicated. M: Marker.

**Immune Response Analysis of rOhr Protein in Mice**

The animal immune response was evaluated from three groups of mice immunized with rOhr, MBP, or PBS with IFA on weeks 0, 2, and 5. Blood samples collected on weeks 5 and 7 after the first immunization were processed, and the sera were analyzed to determine the IgG1 and IgG2a titers using an ELISA kit. Our results showed that at weeks 5 and 7, the IgG1 and IgG2a mean log titers of the rOhr-immunized mice were significantly higher than the MBP-immunized group (Fig. 2). Production of IgG1 and IgG2a increased approximately 1.7-fold and 1.8-fold after the second immunization, respectively. Additionally, elevated IgG1 and IgG2a were observed after the third immunization with approximately 1.75-fold and 2.1-fold increases, respectively. The results indicated that the administration of rOhr induced a strong humoral immune response.

Further information on the type of immune response was determined by measuring the concentration of cytokines from sera collected at weeks 5 and 7 after the first immunization. Immunization of mice with MBP did not significantly induce the production of any cytokines compared with the PBS-immunized group. In contrast, at 5 weeks after the first immunization, rOhr-immunized mice showed a significant increase in the production of IL-10 and TNF, but not much IFN-γ. However, 2 weeks after receiving full immunization, IL-12p70 and IFN-γ were markedly stimulated with concentrations reaching up to 3.485 pg/ml and 5.21 pg/ml, respectively. Similarly, IL-6 and IL-10 were also slightly increased, but changes in TNF and MCP-1 could not be detected (Table 1). Our results suggested that immunization with rOhr greatly induced the production of antigen-presenting cell-related cytokines

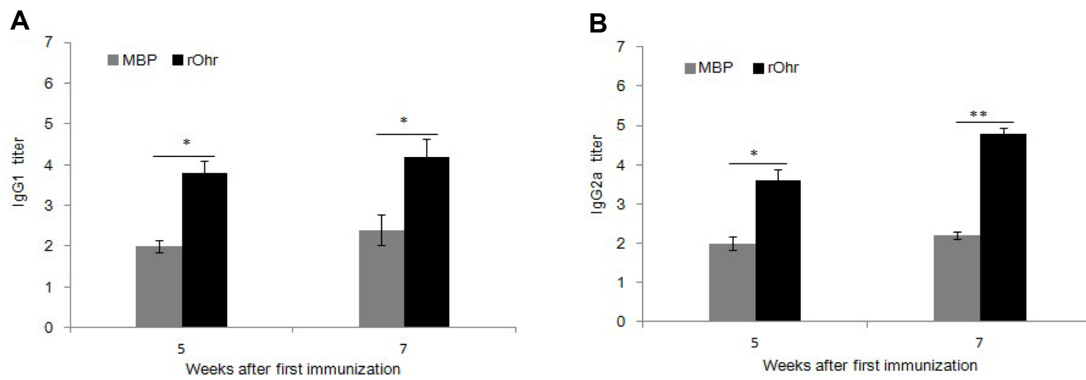


**Fig. 2.** Humoral immune response elicited after immunization with rOhr.
BALB/c mice were immunized with rOhr, MBP, or PBS on weeks 0, 2, and 5. Serum samples were obtained from the tail vein on weeks 5 and 7 after the first immunization. The specific anti-Ohr IgG1 titer (**A**) and IgG2a titer (**B**) were determined by ELISA. Data are presented as the mean ± SD ($n = 5$ per group). Asterisks indicate a significant difference ($p < 0.01$).

**Table 1.** Concentration of cytokines after 5 and 7 weeks in mice vaccinated with rOhr, MBP, or PBS.

| Cytokine | Concentration (pg/ml) of cytokines in mice vaccinated with | | | | | |
|---|---|---|---|---|---|---|
| | PBS | | MBP | | rOhr | |
| | 5* | 7 | 5 | 7 | 5 | 7 |
| IL-12p70 | 0 | 0.82 ± 0.39 | 0 | 0.90 ± 0.30 | 1.83 ± 0.05[a,1] | 3.49 ± 0.99[a,1] |
| TNF | 1.97 ± 1.46 | 2.76 ± 0.81 | 4.28 ± 1.33 | 5.77 ± 1.04[a] | 12.51 ± 2.41[a,1] | 12.59 ± 3.10[a,1] |
| IFN-γ | 0.06 ± 0.08 | 0.75 ± 0.74 | 0 | 0.34 ± 0.28 | 0.36 ± 0.28 | 5.21 ± 0.98[a,1] |
| MCP-1 | 25.23 ± 9.09 | 26.24 ± 8.44 | 30.81 ± 11.82 | 29.28 ± 5.41 | 50.07 ± 3.15[a] | 47.82 ± 1.13[a,1] |
| IL-10 | 0 | 0.12 ± 0.11 | 0 | 0 | 1.49 ± 0.03[a,1] | 2.96 ± 1.35[a,1] |
| IL-6 | 0 | 0 | 0 | 0.05 ± 0.04 | 0.99 ± 0.84[a,1] | 1.49 ± 1.09[a,1] |

*Weeks after first vaccination.

[a]Significantly different from the PBS-immunized group ($p < 0.05$).

[1]Significantly different from the MBP-immunized group ($p < 0.05$).

and chemokines (IL-12p70, IL-6, MCP-1, TNF) as well as the Th1-related cytokine IFN-γ and the Th2-related cytokine IL-10.

### Protection Efficiency of the rOhr Protein

To evaluate the host protection response, BALB/c mice were immunized with purified rOhr, MBP in IFA adjuvant, or PBS and then challenged with *B. abortus* administered by the intraperitoneal route. In this experiment, protection was defined as a significant reduction ($p < 0.01$) in the log units of bacterial load in the spleen of mice treated with the rOhr protein in comparison with the control group treated with PBS. As shown in Fig. 3B, mice immunized with rOhr had a significantly higher degree of protection than mice immunized with MBP or PBS. Moreover, the mean spleen weight of the rOhr protein-vaccinated mice was approximately 1.29-fold lower, which was significant compared with the PBS-treated group (Fig. 3A).

## Discussion

Brucellosis causes great economic loss and significant health risks to humans. Because the incidence of human brucellosis is strongly dependent on the prevalence of the disease in animals, disease prevention in humans also depends predominantly on the control of the disease in animals, and vaccination in conjunction with test and slaughter programs is the most important method of control in animals [7, 8]. There is no currently available licensed vaccine for human brucellosis. Although control and eradication of animal brucellosis have been achieved in some areas owing to intense regional efforts, it remains as an important disease in many parts of the globe [2, 8].

Animal vaccination is a critical factor for the control and

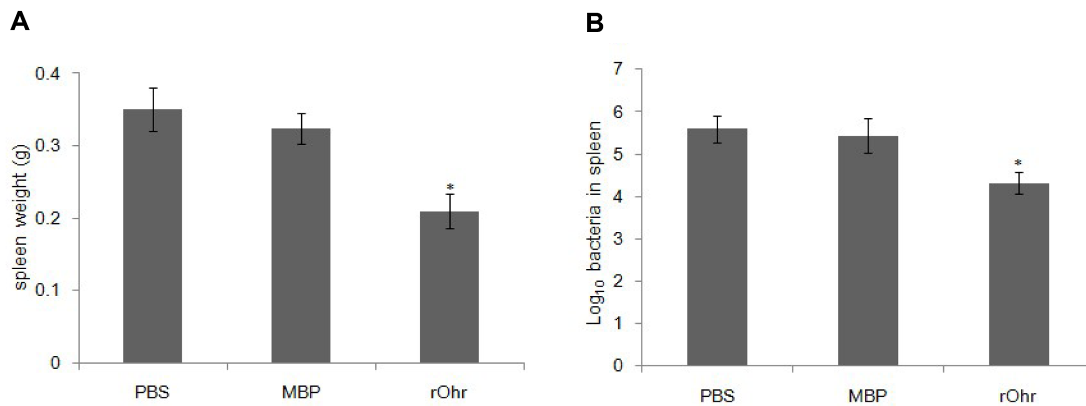


**Fig. 3.** Protection against *B. abortus* 544 in mice vaccinated with rOhr.

Mice were immunized with rOhr, MBP, or PBS. Two weeks post-vaccination, mice were challenged with *B. abortus* 544. Two weeks later, mice were sacrificed, and the weight of spleen (**A**) and bacterial proliferation in spleen (**B**) were determined. Data are presented as the mean ± SD ($n$ = 5 per group). Asterisks indicate a significant difference ($p < 0.01$).

prevention of animal and human brucellosis, but the currently available live attenuated vaccines have several safety-related problems, including reversion to virulence, the potential to cause human infection, abortions in pregnant animals, and shedding in milk. These live vaccines also possess a limited ability to prevent infection, and seroconversion after exposure, and may interfere with disease diagnosis due to a high antibody titer [2, 12, 19]. Therefore, the development of a new, safe, and improved vaccine is a challenge to the scientific community. Much more attention has been focused on screening *Brucella* protective antigens as subunit vaccine candidates because they are completely inert and have a definite composition, controllable production, and high homogeneity as compared with live attenuated vaccines [7]. Furthermore, subunit vaccines can induce an immune response to a single protein, which could improve serological diagnostic methods by allowing the design of diagnostic tools that can differentiate vaccinated animals from naturally infected animals. Accordingly, several protein antigens from *Brucella* have been examined as recombinant protein vaccines in mouse models and have shown a significant protective immune response [2].

Organic hydroperoxide resistance protein is involved in the bacterial response to oxidative stress and has been known to protect *B. abortus* 2308 from organic hydroperoxide stress in in vitro assays, but it is not required for virulence of the wild type in cultured murine macrophages or experimentally infected mice [5]. The importance of this protein for virulence both in macrophages and mice has been reported in *Francisella*, which further implies that its importance for pathogenesis is conserved in multiple *Francisella* species [14]. In our previous study, *B. abortus* Ohr showed potential as a potent immunogenic antigen as revealed by 2DE and western blot assays using *B. abortus*-infected bovine serum [12]. Consequently, we constructed pMAL-Ohr to further investigate the efficacy of rOhr in inducing an immune response against *Brucella* infection in a mouse model.

Innate immunity plays an important role during *B. abortus* infection, helping to decrease the initial number of bacteria and influencing the development of a protective adaptive immunity. Furthermore, the chronic nature of the disease caused by this pathogen requires an effective adaptive immune response, and although several components of the immune system contribute to protect against intracellular pathogens, it is well documented that the cell-mediated immune response plays a major role in the mediated immune protection against *Brucella* and other intracellular pathogens [8, 20]. Host immunity involves cell-mediated (Th1) and humoral (Th2) immunities; the former is characterized by the production of IgG2a antibodies, whereas the latter is characterized by the production of IgG1 antibodies [9, 16, 20]. Interestingly, significant increased anti-Ohr IgG1 and IgG2a titers were observed, which suggested the induction of both humoral and cell-mediated protective immune responses. However, IgG2a production was higher than that of IgG1, which indicated the induction of a predominantly Th1 response. Immunization with rOhr conferred protection against infection in BALB/c mice as confirmed by the significant reduction of the bacterial load in the spleen (1.29 protection unit). Because

the rOhr protein conferred significant protection against a virulent *B. abortus* infection in BALB/c mice specifically by a dominant Th1-cell immunity, this protein can be considered as a potential candidate vaccine for protection against brucellosis.

In the pathogenesis of brucellosis, cytokines play a critical role in the immune response, which includes mediating innate and adaptive immunity and directing the immune response among immune-associated cells [11]. The cytokine content in the serum reflects cytokine production and secretion from spleen cells [1]; hence, we analyzed the cytokine content in the serum of mice. In the present study, the levels of different cytokines were measured, and a significant increases in the cytokines TNF, IFN-γ, and IL-10 and the chemokine MCP-1 in the sera were observed, which suggests further protection against brucellosis predominantly via Th1 immunity. At 5 weeks after the first immunization (which consisted of two immunizations with the recombinant protein), a significant increase in TNF ($p < 0.01$) was observed, and this level continued to increase significantly 2 weeks after the third immunization. This cytokine is one of the key players in brucellosis and is believed to be an important mediator of acquired cell-mediated resistance, maximizing the phagocytic role of macrophages and mediating resistance to primary and secondary *B. abortus* infections [11, 22]. Additionally, mutations disturbing TNF-α generation in mice influenced clearance and favored *Brucella* replication [10]. Other cytokines, such as IFN-γ, IL-10, and MCP-1, were observed to increase significantly 2 weeks after the third immunization. IFN-γ is generally the major essential effector cytokine that activates the bactericidal function of macrophages and inhibits microbial replication [11, 20]. IL-10 is an immunoregulatory cytokine that acts on antigen-presenting cells such as macrophages to inhibit the development of the cell-mediated response in order to prevent further tissue damage [21]. Interestingly, the level of IL-10 in the present study was distinctly elevated (23.66-fold increase), which might support the presence of a humoral-mediated response. The chemokine MCP-1 has been implicated in human and mouse host immunity to *Brucella*.

In conclusion, our results indicate that immunogenic Ohr could be a potential protective antigen for the development of subunit vaccines against brucellosis, since it elicits both humoral and cellular immune responses and confers protection against virulent *B. abortus* challenge. Further studies will be focused on the development of efficient adjuvant or antigen delivery systems to further evaluate the protective immunity of rOhr alone or in combination with other antigens.

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