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Running title: Protection of 5 recombinant proteins against brucellosis

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Abstract

Chronic infection with intracellular *Brucella abortus* (*B. abortus*) in livestock remains to be a major problem worldwide. Thus, the search for an ideal vaccine is still ongoing. In this study, we evaluated the protection efficacy of a combination of *B. abortus* recombinant proteins; superoxide dismutase (rSodC), riboflavin synthase subunit beta (rRibH), nucleoside diphosphate kinase (rNdk), 50S ribosomal protein (rL7/L12) and malate dehydrogenase (rMDH) cloned and expressed into a pMal vector system and DH5α, respectively and further purified and applied intraperitoneally into BALB/c mice. After first immunization and two boosters, mice were infected intraperitoneally (IP) with 5×10^4 CFU of virulent *B. abortus* 544. Spleens were harvested and bacterial loads were evaluated at two-week post-infection. Results revealed that this combination showed significant reduction in bacterial colonization in the spleen with a log protection unit of 1.31 which is comparable to the average protection conferred by the widely used live attenuated vaccine RB51. Cytokine analysis exhibited enhancement of cell-mediated immune response as IFN-γ is significantly elevated while IL-10 which is considered beneficial to the pathogen's survival was reduced compared to control group. Furthermore, both titers of IgG1 and IgG2a were significantly elevated at three and four-week time points from first immunization. In summary, our in vivo data revealed that vaccination with a combination of five different proteins conferred a heightened host response to *Brucella* infection through cell-mediated immunity which is desirable in the control of intracellular pathogens. Thus, this combination might be considered for further improvement as potential candidate vaccine against *Brucella* infection.

Keywords

B. abortus, recombinant proteins, vaccination, cytokines, protection

Introduction

B. abortus is a Gram-negative facultative intracellular parasite that infects a wide range of domestic animals such as cattle, small ruminants and humans [1]. Brucellosis still remains to be an important zoonosis in developing countries and in particular causes about 500,000 cases worldwide [2, 3]. Among the species of *Brucella, B. abortus* which is responsible for brucellosis in cattle is considered to be a major pathogen that provokes clinical brucellosis in humans [4]. This zoonotic pathogen proliferates within professional phagocytes that often lead to chronic infection [5, 6]. Its ability to circumvent the bactericidal mechanisms of macrophages is key to its virulence [6, 7].

Recent advances in the development of brucellosis vaccine are still underway. The availability of current live attenuated vaccines for animals such as RB51 and *B. abortus* strain 19 pose risk in humans, thus handling and strict adherence to vaccination protocols are required [8, 9]. Another approach proven to be beneficial in the elimination of brucellosis in some states of America is identification of infected animals proceeded by culling and immunization of uninfected animals which may not be applicable in other countries due to cultural reasons [8, 9]. The search for the ideal vaccine will continue to be an imminent goal in the field of vaccine research for the eradication of brucellosis. More importantly, the absence of human vaccines raises the need to control brucellosis in reservoir animals which can be achieved by development of vaccines that offer efficacy and safety in both animals and humans [10, 11]. Many efforts have been raised in the development of vaccine strategies intended for intracellular pathogens which proved to be more difficult. One approach has been the use of recombinant vaccine of which success is highly dependent on the ability of individual purified antigens of specific pathogens to elicit appropriate cell-mediated immune responses to eradicate intracellular infections [12].

Preliminary evaluation in the selection of potential *Brucella* vaccine in livestock includes categories that manifest cytokine profiles reflective of Th1 dominance as previously reported. This includes predominance in the production of TNF and IFN-γ as crucial cytokines in the intracellular killing of *B. abortus*. Although selection of potential vaccines is based upon results obtained from the mouse model and mouse responses may not always be reflective of other animals, considering this index may still be considered helpful [13, 14].

In this study, we evaluated the protective efficacy of a combination of five selected *B. abortus* recombinant proteins that were previously reported to be individually immunogenic and able to induce protective immunity using various adjuvants in a murine model. We have previously characterized the immunogenicity of two of the components of this vaccine combination; recombinant protein Ndk (nucleoside diphosphate kinase) and rMDH (malate dehydrogenase) which were cloned into a pMal vector system [1, 15]. These proteins showed immunoreactivity with *B. abortus*-positive mouse sera while the protein MBP (Maltose-binding protein) encoded by the vector system is non-reactive with both of *B. abortus*-negative and positive mouse sera. Previously identified immunogenic recombinant proteins were cloned and added to this combination namely; recombinant RibH (riboflavin synthase subunit beta) which has been depicted to be highly immunogenic through a three-dimensional analysis revealing a pentameric structure that is specifically suggestive of immunogenicity [16], rSodC (Cu-Zn superoxide dismutase) previously evaluated as a peptide vaccine [17] and rL7/L12 (50S ribosomal protein) which displayed a T-helper 1 immune response upon evaluation with murine CD4+ T cells [18]. Although the protective efficacy of rMDH has not yet been proven in vivo, its high immunogenicity in *Brucella*-positive bovine and mouse sera at different stages of infection was considered for its inclusion in this combination vaccine [15].

Materials and Methods

Bacterial strains

Wild-type strains derived from *B. abortus* 544 (ATCC 23448), a smooth, virulent *B. abortus* biovar 1 strain was cultivated in either Brucella broth (Becton Dickinson, MD, USA) or on Brucella agar. Bacteria were cultured at 37˚C with vigorous shaking until they reached stationary phase. *Escherichia* (*E.*) *coli* DH5α was purchased from Invitrogen (USA) and used for creating plasmid constructs. *E. coli* DH5α culture was routinely grown at 37˚C in LB broth or agar supplemented with 100 µg/mL of ampicillin (Sigma, USA).

Recombinant expression and Protein purification

The sequences of the five *B. abortus* genes were amplified using PCR (Table1). Cloning and expression of the recombinant proteins were conducted according to a previous study into a pMAL vector system [1]. Recombinant proteins were expressed in *E. coli* DH5α, induced in LB broth supplemented with 100 µg/mL ampicillin, 0.2% glucose and varying IPTG concentrations of 0.1-0.5 mM at 37˚C for 4 h. *E. coli* cells were harvested at 5,000 x *g* for 10 min and resuspended in 25 mL column buffer (20 mM Tris HCl, 1 mM EDTA, 200 mM NaCl, 10% glycerol, pH 7.4). The lysates were subjected to a freeze-thaw cycle thrice and sonicated (Bandelin electronic, Germany) at 10,000 Hz on ice for 15 min. The supernatant was centrifuged at 5,000 x *g* for 20 min, filtered through 0.45 µm (Minisart, Sigma Aldrich, USA) and loaded into a maltose resin column (Bio-Rad Laboratories, USA). Finally, purified proteins were eluted with 10 mM maltose in column buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Induced cells were lysed and purified products were boiled at 100˚C for 5 min in 2x SDS buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8) subjected to SDS-PAGE [19, 20]. Following gel electrophoresis, separated proteins were subjected to western blot analysis. Proteins were transferred to Immobilon-P membranes (Milipore, USA) emerged in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3) for 30 min using ATTO Semi-dry transfer machine (WSE-7210, Japan). Membranes were blocked in 5% skim milk at room temperature (Difco, USA) for 30 min, washed with 0.05% Tween-20 (PBS-T) and incubated with *Brucella*-positive or *Brucella*-negative mouse sera (sera collected from mouse infected with virulent *B. abortus* (1:10000 dilution) at 4˚C, overnight. Following incubation with mouse sera, the membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:1000 dilution) (Sigma, USA) for 1 h at room temperature, washed, detected with ECL solution (Thermo Scientific, USA) and finally analyzed with a Molecular Imager ChemiDoc XRS+ system machine (Bio-Rad Laboratories).

Immunization and Bacterial challenge

The protective effect of a combination of recombinant proteins; SodC, RibH, Ndk, L7/L12 and MDH was evaluated. Twenty, ten-week-old female BALB/c mice (Japan SLC, Japan) were allocated to four groups. All groups of mice were intraperitoneally injected with 1:1 volume ratio of recombinant protein and incomplete Freund's adjuvant (IFA) (Sigma, USA) in a total volume of 200 µl. The groups of five mice each were immunized IP with PBS, maltose-binding protein (MBP) (100 µg), RB51 (1 x 10^6 CFU) or a combination of five *B. abortus* recombinant proteins (20 µg each for a total of 100 µg) on weeks 0, 2, and 3. Serum samples were collected via tail

vein on weeks 3 and 4 after the first immunization. A week after the last immunization, mice were IP challenged with approximately 5 x 10^4 CFU of *B. abortus* in 100 µl PBS.

Ethical approval

The method of handling and sacrifice conducted in this experiment was approved by the Animal Ethical Committee of Gyeongsang National University (Authorization Number GNU-170331-M0017).

Cytokine and Humoral immune responses

The levels of IL-12p70, IL-10, IFN-γ, TNF, MCP-1 and IL-6 in sera were determined by cytometric bead array (BD CBA Mouse Inflammation Kit, USA) and analyzed using a FACS Calibur flow cytometer (BD Biosciences, CA, USA). On the other hand, IgG1 and IgG2a isotypes were measured through indirect ELISA. Briefly, purified proteins were diluted in coating buffer (carbonate buffer; pH 9.6) comprising of 1.5 µg/ml of each protein, coated into 96-well plate (Maxibinding, SPL Life Sciences, Korea) in 100 µl per well followed by overnight incubation at 4˚C. The wells were washed with 0.5% PBS-T and blocked with 5% skim milk in PBS-T at room temperature for 2 h. The wells were then incubated with sera in blocking buffer at dilutions reaching cut off values. The wells were washed and incubated with HRP-conjugated IgG1 or IgG2a (Abcam, USA) for 1 h. Finally, the wells were washed and O-phenyldiamine (OPD) was added into the wells and the absorbance was read after 15 min at 450 nm through an ELISA reader (Biotek, Seoul Korea). Cut-off value was computed as the mean specific OD plus standard deviation for non-immunized mice diluted at 1:100 [1]. Titers were determined as the reciprocal value of the dilution that yields an absorbance higher than the cut off value.

Bacterial clearance efficiency

158 The protective effect of the combined vaccine was evaluated as previously reported [1]. Mice
159 were sacrificed via cervical dislocation at two-week post-infection. Spleens were collected,
160 weighed and homogenized in PBS. Homogenized spleens were serially diluted, plated on
161 Brucella agar and incubated at 37˚C for 3 days. Log protection was computed as the mean log_{10}
162 CFU of PBS group minus Log_{10} CFU of the vaccinated group. Finally, the log_{10} number of
163 CFUs/g was calculated.

164 **Statistical analysis**

165 The results for each of experiment are expressed as the mean ± SD. One-way ANOVA
166 program was utilized in all assays.

167 # Results

168 **Protein purification and immunoreactivity of recombinant proteins**

169 After induction and purification steps, purified proteins were analysed via SDS to check for
170 target molecular masses. The approximate molecular masses for recombinant proteins; SodC,
171 RibH, Ndk, L7/L12 and MDH were approximately 68.1, 67.3, 65.27, 62.5 and 73 kDa,
172 respectively (Fig. 1A). Immunoblot analysis showed immunoreactivity of rSodC, rRibH, rNdk,
173 rL7/L12 and rMDH with both *Brucella*-positive (Fig. 1B), or -negative mouse sera (Fig. 1C).
174 Consistent with previous data, MBP did not react with either *Brucella*-positive or -negative
175 mouse sera (Fig. 1B, Fig. 1C).

176 **Combined recombinant vaccine engendered significant protection against *Brucella* infection**

177 The rate of infection was assessed by measuring CFU in the spleen two-week post-infection.
178 The mean log_{10} CFU of the spleen of the combined protein-immunized group was significantly

179 reduced than with PBS (P<0.01) or MBP group (P<0.05) (Fig. 2A), demonstrating a log
180 protection unit of approximately 1.31 over PBS or MBP group. The CFU of MBP group was not
181 significantly different from the PBS group. Furthermore, the weight of the spleen was also
182 reduced compared with the PBS or MBP group (P<0.05) (Fig. 2B). Although higher log
183 protection units are established in previous studies, the log protection unit provided by the
184 attenuated live RB51 vaccine in the current study is consistent with other studies demonstrating a
185 1.4 log protection unit in BALB/c mice with *B. abortus* 544 infection [21] and 1.26 with *B.*
186 *abortus* 2308 infection [22].

187 **Combined antigens induce substantial Th1 immune response**

188 The production of (Th1) type cell-mediated immunity cytokines that is responsible for anti-
189 *Brucella* activities such as macrophage and lymphocyte activation for intracellular killing is an
190 established protective immunity mechanism in the murine model [23, 24]. In this study, cytokines
191 in the sera were measured and have shown that PBS and MBP group did not elicit production of
192 IFN-γ and exhibited increased levels of IL-10 while combined protein-immunized group
193 substantially produced 2.3-fold (P<0.05) and 10-fold (P<0.001) increase of IFN-γ over PBS and
194 MBP, respectively at three-week post-immunization (Fig. 3). Interestingly, vaccination with
195 RB51 resulted in an extreme elevation of 23.5-fold and 106-fold increase in IFN-γ compared with
196 PBS and MBP groups, respectively at three-week post-immunization and 24.8-fold and 76.5-fold
197 increase compared with PBS and MBP groups, respectively at four-week post-immunization (Fig.
198 3). On the other hand, a 1.6-fold and 1.7-fold decreased level of IL-10 over PBS and MBP,
199 respectively (Fig. 4) was observed at three-week post-immunization (P<0.01). At four-week
200 post-immunization, levels of IL-10 declined significantly compared with PBS group (P<0.0001).
201 IL-12p70, IL-6, MCP-1, and TNF were not significantly different across all groups.

Humoral immune response in combination protein immunized mice

The presence of rSodC, rRibH, rNdk, rL7/L12 and rMDH-specific antibodies in the sera of mice collected at three- and four-week post-immunization was analyzed through ELISA. As shown in Fig. 5A and B, combined antigens elicited significant levels of both IgG1 (P<0.05) and IgG2a (P<0.001) at three-week post-immunization. Similarly, at four-week post-immunization, both IgG1 (P<0.05) and IgG2a (P<0.01) were shown to be elevated compared to MBP group. The ratio of IgG2a/IgG1 at three-week and four-week post-immunization was approximately 1.0. The titers for the live vaccine strain RB51 was also found to induce a lower humoral immune response compared to the combined protein-immunized group. Consistent with previous studies, PBS group did not induce humoral immunity (data not shown).

Discussion

The search for the ideal vaccine will continue to be the primary goal in the effort of eradicating brucellosis as vaccination still remains to be the most efficient method in the control of this infectious agent. RB51, a widely used live attenuated vaccine offers major advantages such as non-interference in the diagnosis of brucellosis, however it also causes residual virulence in both animals and humans [25]. Several antigenic proteins such as periplasmic, cytoplasmic, inner and outer membranes have been previously identified according to previous studies.

It is our goal in this study to evaluate the potential of these single immunogenic recombinant proteins hypothesized to confer a superior protection to *Brucella* infection when given as a combination using a mouse model. Constituents of the vaccine combination have been selected based on the protective efficacy of individual proteins as previously reported. Thus, five recombinant proteins encoded by *B. abortus* SodC, ribH, ndk, L7/L12 and mdh genes were

selected and combined. Immunization with recombinant L7/L12 ribosomal protein had been characterized earliest to be an important immunogenic antigen that elicits primarily Th1 response specifically manifested with an elevated level of IFN-γ [18]. Substantially, immunization with rL7/L12 led to an average protective log reduction of 1.0 when compared to *B. abortus* S19 vaccinated group [18]. Another important feature of this protein is that it is capable of eliciting delayed-type hypersensitivity [26]. On the other hand, rSodC has been conclusively established to be responsible for bacterial survival against oxidative killing by catalyzing dismutation of superoxide as a by-product of the process of phagocytosis in host macrophages [27]. Given the function of this molecule, SodC is considered to be an important virulence determinant in *Brucella* infection [28]. This has also been found to be consistent with other bacteria including *Mycobacterium tuberculosis* [29] and *Salmonella enterocolitica* serovar Typhimurium [30]. RibH is a cytoplasmic protein that induces Th1-type responses leading to a notable protection upon *B. abortus* infection in mice when utilized as a DNA and recombinant vaccine [31, 16]. *B. abortus* rMDH and rNdk were determined as immunogenic antigens through a two-dimensional electrophoresis (2DE) assay against *B. abortus*-infected cattle sera and immunoblot assay against *B. abortus*-infected mouse sera. The latter was further investigated as recombinant vaccine in a mouse model revealing a significant protective efficacy [1]. In this study, all the recombinant *Brucella* proteins individually demonstrated immunoreactivity with *Brucella*-positive mouse sera. We have also shown that immunization with a combination of rSodc and rRibH revealed a marked protective immunity in *Brucella*-infected BALB/c mice (unpublished data). Obtaining an ideal vaccine for the control of brucellosis requires an immune response that is geared towards a cell-mediated (Th1) type immunity that involves CD8+ and CD4+ T lymphocytes, T-helper cells producing key cytokines IFN-γ, IL-12 and TNFα and activated macrophages [32, 33]. The current study exhibited substantial levels of 2.3-fold increase of IFN-γ in the combined protein-

immunized group which is considered principal for the activation of macrophages leading to an enhanced bactericidal killing [34, 35]. The anti-*Brucella* ability of IFN-γ-activated macrophages is mainly attributed to a magnified production of reactive oxygen intermediates in vitro [36]. Other than activation of macrophages, IFN-γ also activates cytotoxic CD8+ T-cells and stimulates activated plasma B cells to produce IgG2a isotypes [37, 38]. In reference to an established immune response to RB51 which is also consistent with our data showing an approximate 100-fold increase in IFN-γ, we can conclude that the significant elevation of IFN-γ mounted in the combined protein-immunized group at three-week post-immunization basically demonstrates a Th1 type response which is ideal and desirable in the control of facultative intracellular pathogens. Th1 immunity is also characterized by IgG2a production while Th2 immunity is distinguished by IgG1 and IgE responses [16, 39, 40]. Although humoral (Th2) immunity is of lesser importance in brucellosis control [32, 33], combined protein-immunized group elicited high levels of both IgG1 and IgG2a with IgG2a/IgG1 ratio of approximately 1.0 at three- and four-week post-immunization which is highly suggestive of Th1 immunity. A previous study had demonstrated a mixed Th1 and Th2 responses upon immunization with recombinant *Brucella* lumazine synthase which led to decreased bacterial susceptibility in BALB/c mice [16]. In this study, production of IL-10 is significantly attenuated in the combined protein-immunized group at three- and four-week post-immunization. IL-10 is a cytokine of the Th2 branch with a bivalent function. It regulates excessive proinflammatory responses that is characterized in Gram-negative infections but also inhibits responses of the CD8+ and Th1 immunity [41]. Neutralization of IL-10 reduced bacterial proliferation in the spleens 10-fold lower in BALB/c mice challenged with *B. abortus* strain 2308 and furthermore dampens the anti-*Brucella* functions of activated macrophages despite the abundance of IFN-γ [42]. In addition, it also diminishes the ability of spleen cells to produce IFN-γ upon stimulation of antigens in

Brucella [42] and consequently stimulates generation of IFN-γ, IL-17 as well as TNF-α in splenic cells [43]. The 1.6-1.7-fold inhibition in the levels of IL-10 in the combined protein-immunized group could have promoted induction of significant levels of IFN-γ that heightened its resistance against *Brucella* infection. The combination of *B. abortus* recombinant proteins: SodC, RibH, Ndk, L7/L12 and MDH clearly exhibited a cell-mediated immune response which is a primary category in the initial screening of candidate vaccines. Although higher log protection units of RB51 are established in previous studies, the log protection unit provided by the live attenuated RB51 vaccine in our current and previous data is within 1.07-1.27 which is consistent with other studies demonstrating 1.4-log protection unit in BALB/c mice with *B. abortus* 544 infection [24] and 1.26- log protection unit with *B. abortus* 2308 infection [32]. The protective efficacy of RB51 is very variable and could even reach up to 2.31-log reduction [18]. Thus, this protective immunity is equivalent when compared to the lower range but does not surpass the higher range of protection conferred by RB51. Further investigation is warranted in improving the protective efficacy of this combination such as the type of adjuvant to be used that can boost cell-mediated immunity, the dose of each *Brucella* antigen and the frequency of vaccination.

In summary, the combination of these five antigens predominantly boosted cellular immune responses and humoral immunity in a mouse model through upregulation of IFN-γ and inhibition of IL-10 production that possibly contributed to protective immunity against brucellosis. Thus, this vaccine combination might be considered as potential effective vaccine candidate for further development in the future.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Table

Table1. Primer sequences used for cloning *B. abortus* recombinant proteins

Gene	Forward primer	Reverse primer	Restriction enzyme (forward)	Restriction enzyme (reverse)
ribH	5' ACC GGA TCC ATG AAC CAA AGC TGT CCG AAC 3'	5' AGC CTG CAG CGG CTG CGC TCG CTC ACG ATC 3'	*Bam*/HI	*PstI*
SodC	5' ACC GGA TCC ATG AAG TCC TTA TTT ATT GCA 3'	5' AGC CTG CAG TTA TTC GAT CAC GCC GCA GGC 3'	*Bam*/HI	*PstI*
ndk	5′ CGC GGA TCC ATG GCA ATT GAA CGTACGC 3′	5′ GGC CTG CAG TCA GCC AAC GAT TTC GGT 3′	*Bam/HI*	PstI
L7/L12	5’ AGC TCT AGA ATG GCT GAT CTC GCA AAG ATC 3’	5’ ATC CTG CAG C TTA CTT GAG TTC AAC CTT GGC 3’	*XBaI*	*PstI*
mdh	5' ATT TC GGA TCC ATG GCA CGC AAC AAG ATT 3'	5' AGGC GTC GAC TTA TTT CAG CGA CGG ACG 3'	*Bam*/HI	*SalI*

Figure Legend

Fig. 1. Expression and immunoreactivity of purified *B. abortus* recombinant proteins. SDS-PAGE analysis of purified proteins stained with Coomasie Brilliant Blue (A) Immunogenicity of each

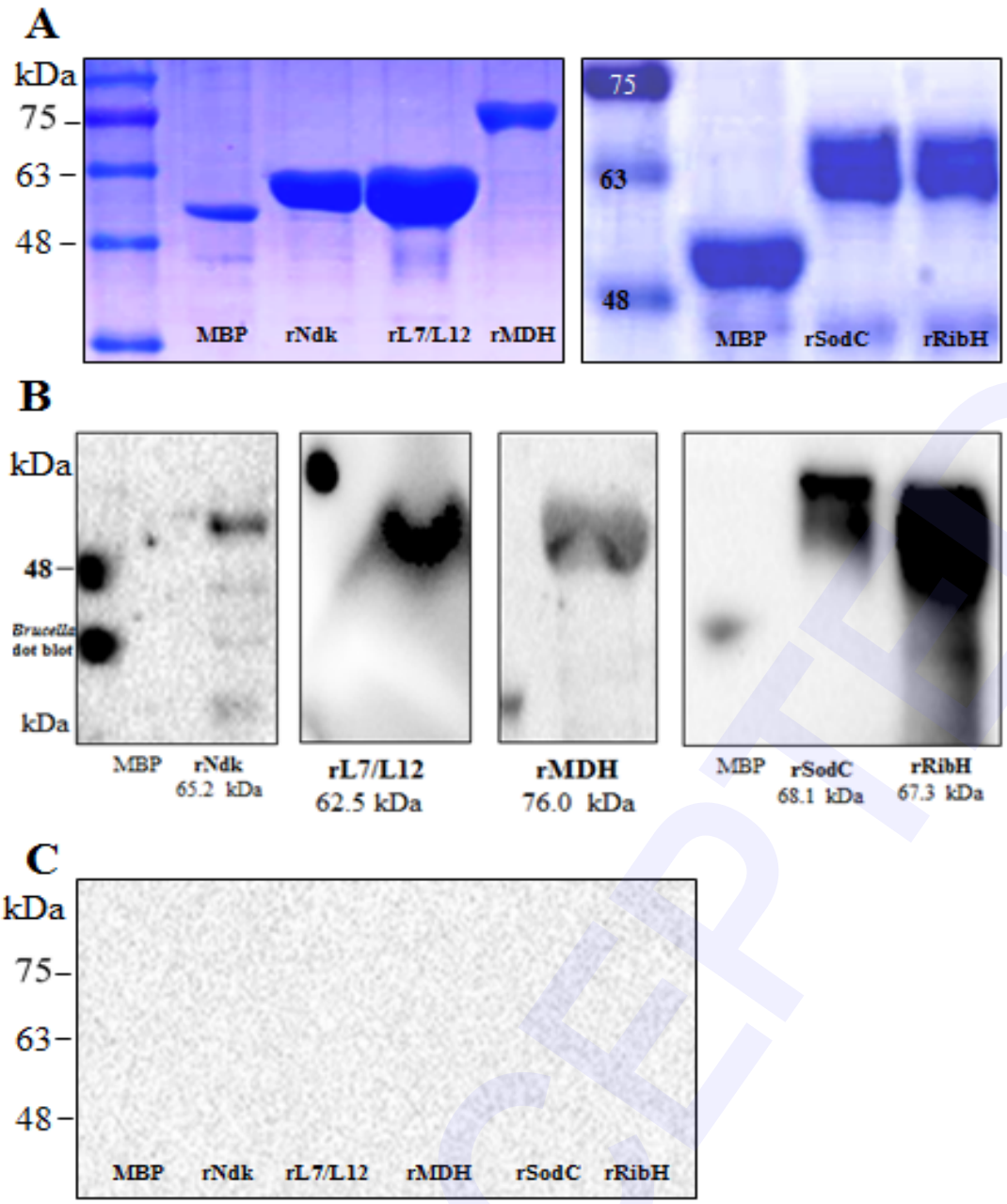
recombinant protein against *Brucella*-positive mouse sera (B) or -negative mouse sera (C). *B. abortus* dot blots are provided as reference for determining molecular weight.

Fig. 2. Degree of protection conferred by the administration of combined recombinant proteins against a virulent strain of *B. abortus*. Protection log unit in the combined protein-immunized group (A). Reduction in the weight of the spleen in the combined immunized group (B). Statistical differences are analyzed from the values of the PBS (non-immunized group). Asterisks indicate significant difference: **P*<0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Fig. 3. IFN-γ concentration (pg/ml) in the sera of mice challenged with *B. abortus* infection at 3- and 4-week time point after first immunization. Asterisks indicate significant difference:* *P*<0.05, ***P*< 0.01, ****P*< 0.001.

Fig. 4. IL-10 concentration (pg/ml) in the sera of mice challenged with *B. abortus* infection at 3- and 4-week time point after first immunization. Asterisks indicate significant difference:* *P*<0.05, ***P*< 0.01, ****P*< 0.001, *****P* < 0.0001.

Fig. 5. Humoral immune response in the combined protein-immunized group. The levels of IgG1 (A) and IgG2a (B) elicited by the combined protein-immunized group after 3- and 4-week time point after the first immunization were determined by ELISA. Mice infected with 1×10^6 RB51 were included as positive control. Data are represented as the mean ± SD (n=5 mice/group). Asterisk indicates significant difference: * *P*<0.05, ***P*< 0.01, ****P*< 0.001, *****P* < 0.0001.



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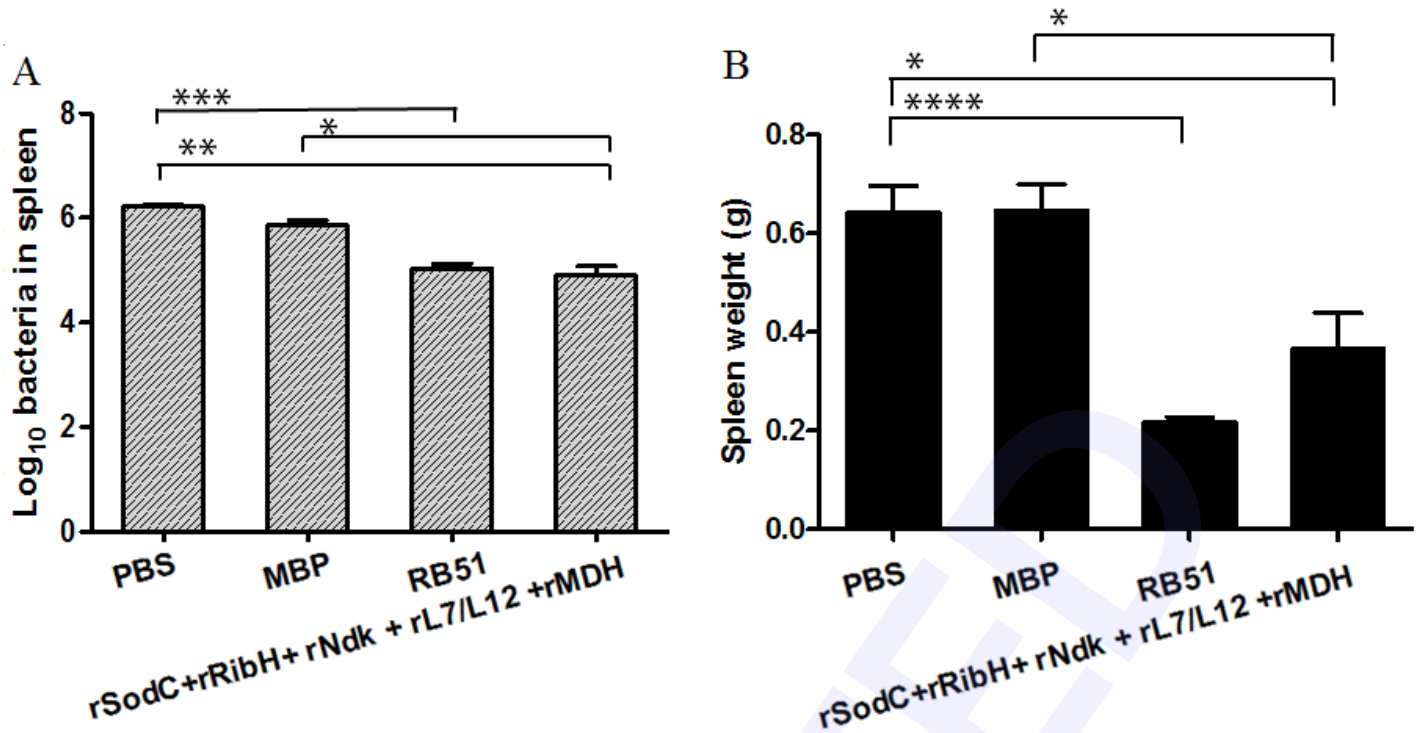

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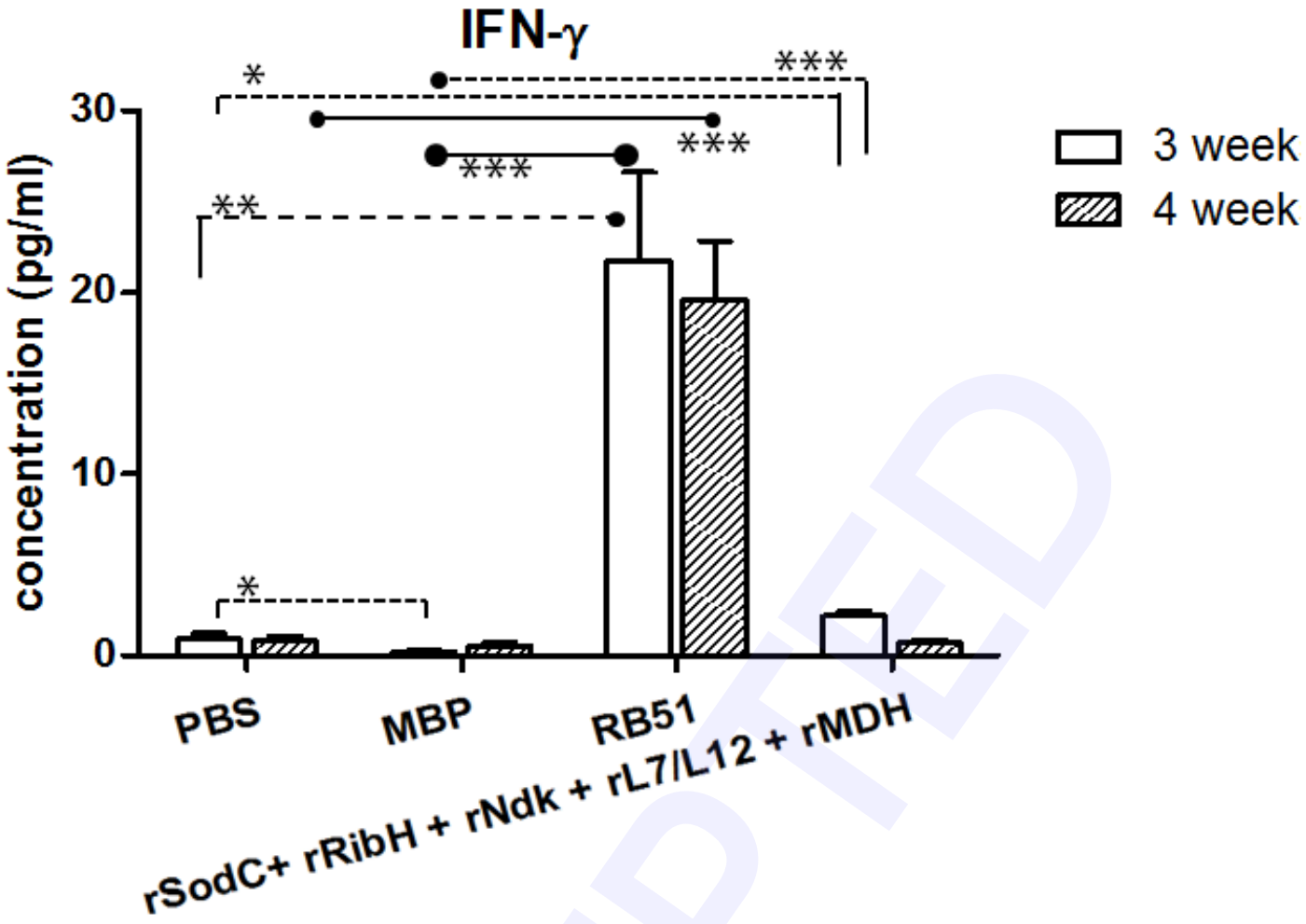

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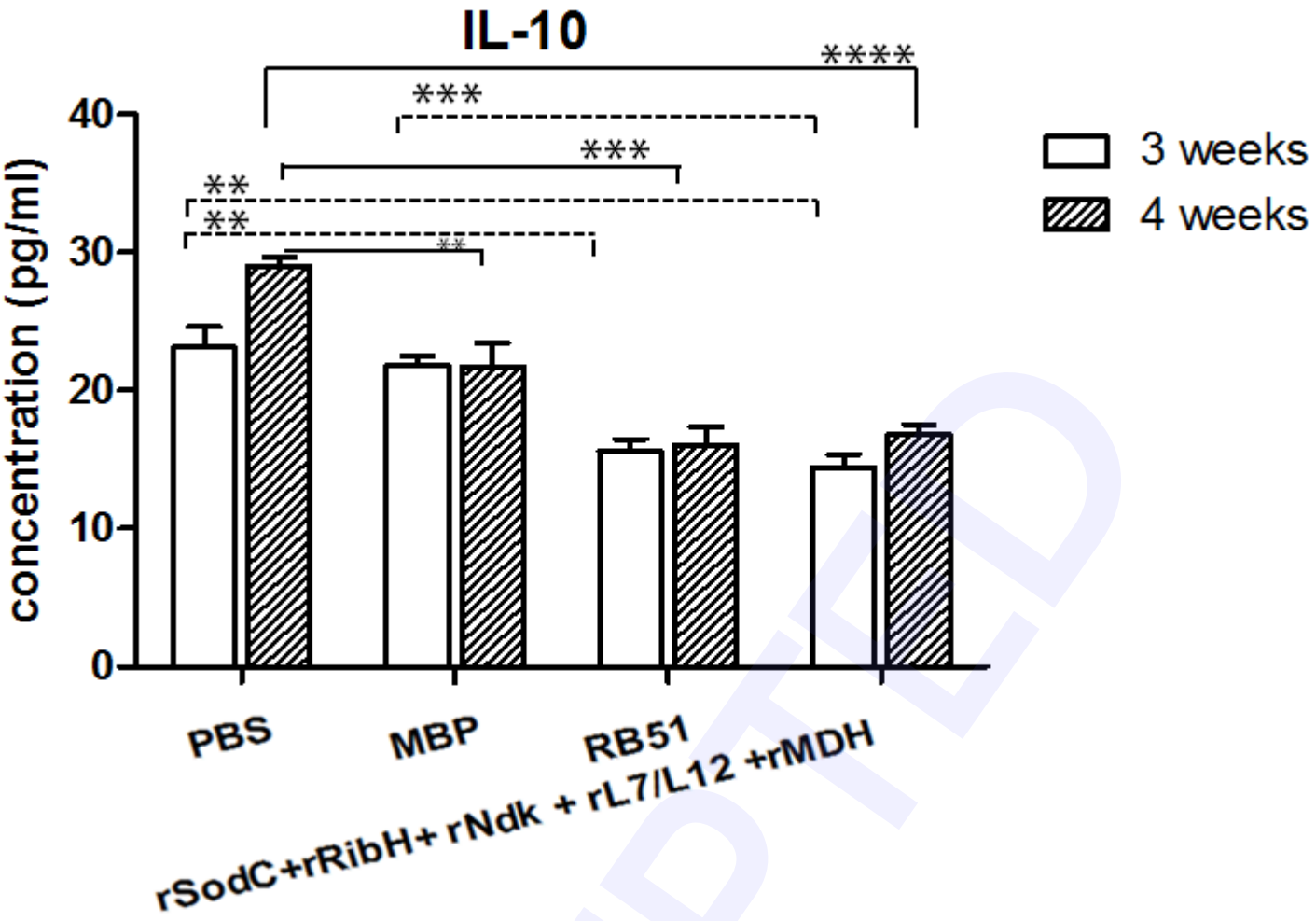
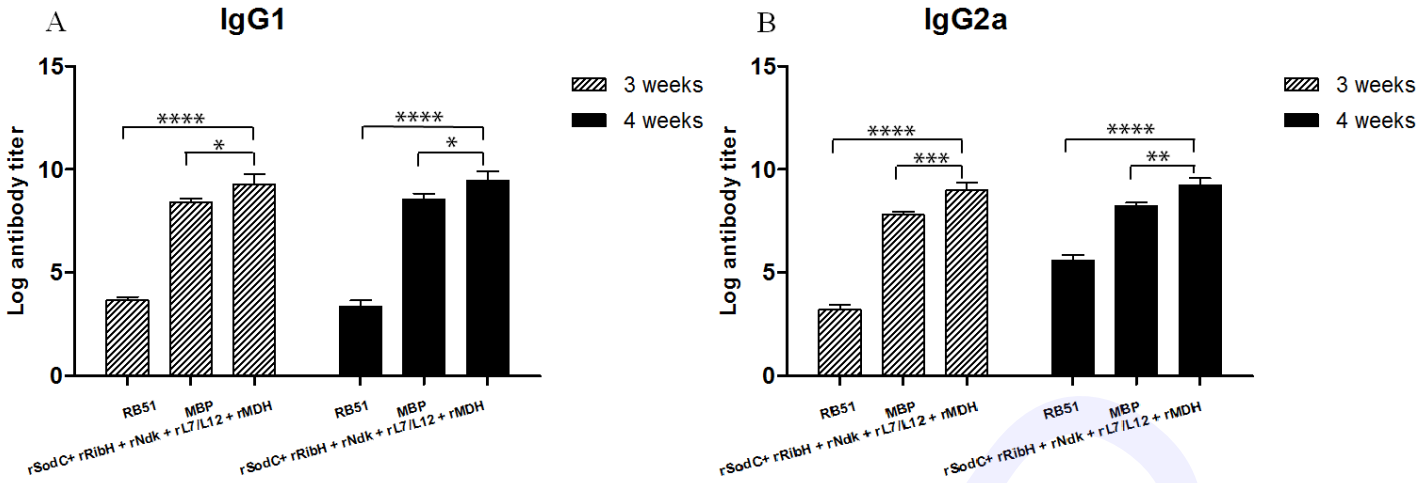


Fig. 4. Fig. 4. IL-10 concentration (pg/ml) in the sera of mice challenged with B. abortus infection at 3- and 4-week time point after first immunization. Asterisks indicate significant difference:* P<0.05, **P< 0.01, ***P< 0.001, ****P < 0.0001.



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