Substantial Protective Immunity Conferred by a Combination of \textit{Brucella abortus} Recombinant Proteins against \textit{Brucella abortus} 544 Infection in BALB/c Mice

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**Introduction**

\textit{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 an important zoonosis in developing countries and in particular, causes about 500,000 cases worldwide each year [2, 3]. Among the species of \textit{Brucella}, \textit{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 \textit{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 U.S. states 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 vaccines 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, 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 B. abortus-negative and -positive mouse sera. Previously identified immunogenic recombinant proteins were cloned and added to this combination, namely: recombinant RibH 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), previously evaluated as a peptide vaccine [17], and rL7/L12, 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, were cultivated in either Brucella broth (Becton Dickinson, 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 (Table 1). 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Restriction enzyme (forward)</th>
<th>Restriction enzyme (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ribH</td>
<td>5’ ACC GGA TCC ATG AAC CAA AGC TGT CCG AAC 3’</td>
<td>5’ AGC CTC CAG CGG CTC CAG CTC TCG CTC ACG ATC 3’</td>
<td>Bam/Hi</td>
<td>PstI</td>
</tr>
<tr>
<td>SodC</td>
<td>5’ ACC GGA TCC ATG AAG TCC CCA TTT ATT GCA 3’</td>
<td>5’ AGC CTC CAG TTA TTC GAT CAC GCC GCA GGC 3’</td>
<td>Bam/Hi</td>
<td>PstI</td>
</tr>
<tr>
<td>ndk</td>
<td>5’ CGC GGA TCC ATG GCA ATT GAA CGT ACGG 3’</td>
<td>5’ GCC CTC CAG TCA GCC AAC GAT TTC GGT 3’</td>
<td>Bam/Hi</td>
<td>PstI</td>
</tr>
<tr>
<td>L7/L12</td>
<td>5’ AGC TCT AGA ATG GCT GAT CTC GCA AAG ATC 3’</td>
<td>5’ ATC CTC CAG C TTA CTT GAG TTC AAC CTT GGC 3’</td>
<td>XbaI</td>
<td>PstI</td>
</tr>
<tr>
<td>mdh</td>
<td>5’ ATT TC CGA TCC ATG GCA CGG AAC AAG ATT 3’</td>
<td>5’ AGGC CTC CAC TTA TTT CAG CGA CGG ACG 3’</td>
<td>Bam/Hi</td>
<td>SalI</td>
</tr>
</tbody>
</table>

*Footnote-Enzyme recognition sequences are underlined.*
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 xg 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 xg 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) and incubated with 100 μg/ml of each protein, coated into a 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 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 (1:10000 dilution) at 4°C 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) in a total volume of 200 μl. The groups of five mice each were immunized IP with PBS, maltose-binding protein (MBP) (100 μg), RBSI (1 x 10^7 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 10^7 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-12p40, 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, 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 1.5 μg/ml of each protein, coated into a 96-well plate (Maxi-binding, 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, 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**

The protective effect of the combined vaccine was evaluated as previously reported [1]. Mice were sacrificed via cervical dislocation at two weeks post-infection. Spleens were collected, weighed and homogenized in PBS. Homogenized spleens were serially diluted, plated on Brucella agar and incubated at 37°C for 3 days. Log protection was computed as the mean log₁₀ CFU of PBS group minus log₁₀ CFU of the vaccinated group. Finally, the log₁₀ (number of CFUs/g) was calculated.

**Statistical Analysis**

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

**Results**

**Protein Purification and Immunoreactivity of Recombinant Proteins**

After induction and purification steps, purified proteins were analyzed via SDS to check for target molecular masses. The approximate molecular masses for recombinant proteins; SodC, RibH, Ndk, L7/L12 and MDH were analyzed via SDS to check for target molecular masses. The approximate molecular masses for recombinant proteins; SodC, RibH, Ndk, L7/L12 and MDH were approximately 68.1, 67.3, 65.27, 62.5, and 73 kDa, respectively (Fig. 1A). Immunoblot analysis showed immunoreactivity of rSodC, rRibH, rNdk, rL7/L12, and rMDH with both Brucella-positive (Fig. 1B), and -negative mouse sera (Fig. 1C).

Consistent with previous data, MBP did not react with either Brucella-positive or -negative mouse sera (Figs. 1B and 1C).

**Combined Recombinant Vaccine Engendered Significant Protection against Brucella Infection**

The rate of infection was assessed by measuring CFU in the spleen two weeks post-infection. The mean log₁₀ CFU of the spleen of the combined protein-immunized group was significantly reduced than with the PBS ($p < 0.01$) or MBP groups ($p < 0.05$) (Fig. 2A), demonstrating a log protection unit of approximately 1.31 over the PBS or MBP groups. The CFU of the MBP group was not significantly different from the PBS group. Furthermore, the weight of the spleen was also reduced compared with the PBS or MBP groups ($p < 0.05$) (Fig. 2B). Although higher log protection units are established in previous studies, the log protection unit provided by the attenuated live RB51 vaccine in the current study is consistent with other studies demonstrating a 1.4 log protection unit in BALB/c mice with *B. abortus* 544 infection [21] and 1.26 with *B. abortus* 2308 infection [22].

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**Fig. 1.** Expression and immunoreactivity of purified *B. abortus* recombinant proteins.
SDS-PAGE analysis of purified proteins stained with Coomassie 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). rSodC+rRibH+rNdk+rL7/L12+rMDH: Combination of *B. abortus* recombinant proteins (Cu-Zn superoxide dismutase, riboflavin synthase subunit beta, nucleoside diphosphate kinase, 50S ribosomal protein and malate dehydrogenase). Statistical differences are analyzed from the values of the PBS (non-immunized group). Asterisks indicate significant difference: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. 
Combined Antigens Induce Substantial Th1 Immune Response

The production of (Th1)-type, cell-mediated immunity cytokines that is responsible for anti-Brucella activities such as macrophage and lymphocyte activation for intracellular killing is an established protective immunity mechanism in the murine model [23, 24]. In this study, cytokines in the sera were measured and have shown that the PBS and MBP groups did not elicit production of IFN-γ and exhibited increased levels of IL-10 while the combined protein-immunized group substantially produced 2.3-fold ($p < 0.05$) and 10-fold ($p < 0.001$) increases of IFN-γ over PBS and MBP groups.

**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. *rSodC*rRibH+rNdk+rL7/L12+rMDH: Combination of B. abortus recombinant proteins (Cu-Zn superoxide dismutase, riboflavin synthase subunit beta, nucleoside diphosphate kinase, 50S ribosomal protein and malate dehydrogenase). 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. *rSodC*rRibH+rNdk+rL7/L12+rMDH: Combination of B. abortus recombinant proteins (Cu-Zn superoxide dismutase, riboflavin synthase subunit beta, nucleoside diphosphate kinase, 50S ribosomal protein and malate dehydrogenase). 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 points after the first immunization were determined by ELISA. Mice infected with $1 \times 10^6$ RB51 were included as positive control. *rSodC*rRibH+rNdk+rL7/L12+rMDH: Combination of B. abortus recombinant proteins (Cu-Zn superoxide dismutase, riboflavin synthase subunit beta, nucleoside diphosphate kinase, 50S ribosomal protein and malate dehydrogenase). Data are represented as the mean ± SD ($n = 5$ mice/group). Asterisks indicate significant difference: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.
MBP, respectively at three weeks post-immunization (Fig. 3). Interestingly, vaccination with RB51 resulted in an extreme elevation of 23.5-fold and 106-fold increases in IFN-γ compared with the PBS and MBP groups, respectively, at three weeks post-immunization and 24.8-fold and 76.5-fold increases compared with PBS and MBP groups, respectively, at four weeks post-immunization (Fig. 3). On the other hand, 1.6-fold and 1.7-fold decreased levels of IL-10 over PBS and MBP, respectively, (Fig. 4) were observed at three weeks post-immunization (p < 0.01). At four weeks post-immunization, levels of IL-10 declined significantly compared with the PBS group (p < 0.0001). 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 weeks post-immunization was analyzed through ELISA. As shown in Figs. 5A and 5B, combined antigens elicited significant levels of both IgG1 (p < 0.05) and IgG2a (p < 0.001) at three weeks post-immunization. Similarly, at four weeks post-immunization, both IgG1 (p < 0.05) and IgG2a (p < 0.01) were shown to be elevated compared to the MBP group. The ratio of IgG2a/IgG1 at three and four weeks post-immunization was approximately 1.0. The titer 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, the 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 to eradicate brucellosis as vaccination still remains 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 superior protection against 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 earlier 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 the 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 TNFa 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 weeks 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], the combined protein-immunized group elicited high levels of both IgG1 and IgG2a with IgG2a/IgG1 ratio of approximately 1.0 at three and four weeks post-immunization which is highly suggestive of Th1 immunity. A previous study had demonstrated 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 weeks post-immunization. IL-10 is a cytokine of the Th2 branch with a bivalent function. It regulates excessive proinflammatory responses that are 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 and 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 a potential effective vaccine candidate for further development in the future.

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

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