Original Article
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Vaccination of goats with a combination *Salmonella* vector expressing four *Brucella* antigens (BLS, PrpA, Omp19, and SOD) confers protection against *Brucella abortus* infection

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Salmonella is an intracellular pathogen with a cellular infection mechanism similar to that of *Brucella*, making it a suitable choice for use in an anti-*Brucella* immune boost system. This study explores the efficacy of a *Salmonella* Typhimurium delivery-based combination vaccine for four heterologous *Brucella* antigens (*Brucella* lumazine synthase, proline racemase subunit A, outer-membrane protein 19, and Cu/Zn superoxide dismutase) targeting brucellosis in goats. We inoculated the attenuated *Salmonella* delivery-based vaccine combination subcutaneously at two different inoculation levels; 5×10^9 colony-forming unit (CFU)/mL (Group B) and 5×10^{10} CFU/mL (Group C) and challenged the inoculations with virulent *Brucella abortus* at 6 weeks post-immunization. Serum immunoglobulin G titers against individual antigens in *Salmonella* immunized goats (Group C) were significantly higher than those of the non-immunized goats (Group A) at 3 and 6 weeks after vaccination. Upon antigenic stimulation, interferon-γ from peripheral blood mononuclear cells was significantly elevated in Groups B and C compared to that in Group A. The immunized goats had a significantly higher level of protection as demonstrated by the low bacterial loads in most tissues from the goats challenged with *B. abortus*. Relative real-time polymerase chain reaction results revealed that the expression of *Brucella* antigens was lower in spleen, kidney, and lung of immunized goats than of non-immunized animals. Also, treatment with our combination vaccine ameliorated histopathological lesions induced by the *Brucella* infection. Overall, the *Salmonella* Typhimurium delivery-based combination vaccine was effective in delivering immunogenic *Brucella* proteins, making it potentially useful in protecting livestock from brucellosis.

Keywords: *Brucella* vaccine, *Salmonella* vector, brucellosis, goats, protective efficacy

Introduction

Brucellosis is a severe and acute febrile disease caused by infection with a *Brucella* species, a gram-negative bacterium of the genus *Brucella* [5,21]. It remains one of the important worldwide zoonotic diseases [3]. In 1955, bovine brucellosis was reported among dairy cattle imported to the Republic of Korea [17,25]. Thereafter, a total of 85,521 *Brucella* reactor animals have been identified in 14,215 outbreaks between 2001 and 2011. The numbers of brucellosis cases at both the individual animal and farm levels increased after 2003, peaked in 2006, and slightly decreased thereafter because of implementation of a 'test-and-slaughter' policy [16,23].

It is important to control brucellosis in animal population because humans can be directly or indirectly infected by infected animals; thus immunization against *Brucella* in animals has a critical role in human health. *Brucella abortus* S19 and *Brucella melitensis* Rev. 1 vaccines have been widely used in developed countries. However, those vaccines have induced abortions in pregnant animals [18,22]. Moreover, they elicit anti-*Brucella* antibodies that interfere with serodiagnosis [13].

Antigen delivery systems become necessary when antigens are inefficiently transported to appropriate sites or presented to the immune system [20]. *Brucella* protective immunogens can be delivered to critical immunological sites by using a *Salmonella*-based vector. Therefore, an intracellular pathogen, such as an attenuated *Salmonella* strain, can be licensed as a

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vector to deliver *Brucella* antigens. As *Salmonella* can produce infection in a manner similar to that of *Brucella*, it is a pragmatic choice for an anti-*Brucella* vaccine-delivery platform. The attenuated *Salmonella* Typhimurium delivery-based *Brucella* vaccine used in this study was previously developed and has proven to be a suitably vectored *Brucella* vaccine when applied through different routes of immunization in mice [11]. That vaccine's protective efficiency was improved by combining four heterologous *Brucella* antigens: *Brucella* lumazine synthase (BLS), proline racemase subunit A (PrpA), outer-membrane protein 19 (Omp19), and Cu/Zn superoxide dismutase (SOD) proteins [9]. However, a livestock vaccine trial to show the actual response of the natural host to the *Brucella* vaccine delivered by an attenuated *Salmonella* vector has not been reported. Although large-animal experimental trials are uncommon because of limited resources, they are important for characterization of the equivalent immune responses in livestock hosts (natural host) in order to consider the commercial use of a developed vaccine. Therefore, this study reports on the use of a novel *Salmonella* system delivering four *Brucella* antigens (SOD, BLS, PrpA, and Omp19) to determine whether combinations of individual vaccines can efficiently regulate brucellosis in goats.

Materials and Methods

Ethics and biosafety statement

All animal experimental procedures were approved (CBNU 2016-98) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law (2007, Article 13: Experiments with animals). All goats used in the study were housed and maintained humanely. The biosafety level-3 organism *B. abortus* strain 544 was handled with the required safety precautions and under the supervision of the Ministry of Health & Welfare, South Korea.

Bacteria strains, plasmids, and construction of vaccine

B. abortus strain 544 (ATCC23448) was used as the virulent challenge strain [7,12]. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Construction and validation of *Salmonella* Typhimurium strains expressing *Brucella* immunogenic frames were undertaken as previously described [8,9,11]. The challenge strain (*B. abortus* strain 544) was prepared for the challenge experiment by brief culture in *Brucella* broth (Becton, Dickinson and Company, USA) at 37°C for 24 h and then resuspended to approximately 5×10^8 CFU/mL. Goats were conjunctively challenged with 100 μL of the challenge strain in saline.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Source
Strain		
Escherichia coli		
BL21(DE3)	F^-,*ompT*, *hsd*$S_B(r_B^-,m_B^-)$,*dcm*, *gal*, λ(DE3)	Lab stock
JOL1922	BL21 with pET28a-Omp19	[9]
JOL1923	BL21 with pET28a-BLS	[9]
JOL1924	BL21 with pET28a-PrpA	[9]
HJL208	BL21 with pET28a-SOD	[7]
Salmonella Typhimurium		
JOL1800	JOL912 *Δffal*; O-antigen deficient rough strain, bacteria delivery strain	[9]
JOL1818	JOL1800 with pJHL65	[9]
JOL1878	JOL1800 with pJHL65-SOD	[9]
JOL1879	JOL1800 with pJHL65-Omp19	[9]
JOL1880	JOL1800 with pJHL65-BLS	[9]
JOL1881	JOL1800 with pJHL65-PrpA	[9]
Brucella abortus		
HJL254	*Brucella abortus* strain 544 (ATCC23448)	[7]
Plasmid		
pET28a	IPTG-inducible expression vector; Km^r	Novagen
pJHL65	Asd^+, pBR *ori*, β-lactamase signal sequence-based periplasmic secretion plasmid, 6× His tag	[8]

Omp19, outer-membrane protein 19; BLS, *Brucella* lumazine synthase; PrpA, proline racemase subunit A; SOD, superoxide dismutase.

Immunization and challenge of *B. abortus* biotype 1

A total of 10-month-old goats (n = 18) that were seronegative for brucellosis based on a Rose-Bengal plate agglutination test were used in the study. The animals were divided into 4 groups, fasted for 24 h, and immunized according to the scheme in Table 2. Upon vaccination, animals were monitored for immunization-induced morbidity or mortality and underwent immunological profiling. The negative control (NC) group (n = 3) was subcutaneously (SC) inoculated with 0.1 mL of saline. Group A (n = 5), as the vector control group, was inoculated SC with approximately 5×10^9 colony-forming units (CFU/mL) of *Salmonella* Typhimurium delivery strain containing pJHL65 only in 1 mL. Group B (n = 5) and Group C (n = 5) were immunized SC using the *Salmonella* Typhimurium system with inoculations of approximately 5×10^9 CFU/mL and 5×10^{10} CFU/mL, respectively, of mixtures containing four different delivery strains in 1 mL. At 6 weeks post-immunization, Groups A, B, and C were conjunctively challenged with goat-passaged virulent *B. abortus* strain 544 at a dosage of 5×10^8 CFU/mL in 100 μL (50 μL of inoculum per eye). Animals were kept in a restricted large-animal isolation facility with no contact between groups and were observed daily.

Humoral immune responses of the vaccinated goats

To determine the level of anti-*Brucella* antibodies generated, the humoral response of the control and immunized goats were investigated by performing goat immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA). Serum samples were collected at 3-week intervals after immunization. A standard ELISA was carried out in serum to evaluate the immune response against the BLS, PrpA, Omp19, and SOD antigens in goats according to the modified method previously reported [6]. Briefly, 96-well microtiter plates (Nunc, Denmark) were coated overnight with pre-titrated recombinant BLS (5 μg/mL), Omp19 (5 μg/mL), PrpA (5 μg/mL), or SOD (5 μg/mL) proteins in phosphate-buffered saline (PBS), blocked for 30 min using PBS containing 1% bovine serum albumin (diluent; 200 μL/well), and washed with PBS containing 0.05% Tween-20. Serum samples were diluted at 1:100 in diluent. The plate was treated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG-Fc detection antibody (1:150,000; Bethyl Lab, USA). For IgG samples, colorimetric changes resulting from the action of HRP on o-phenylenediamine (Sigma-Aldrich, USA) were measured through an automated ELISA spectrophotometer (Multiskan GO; Thermo Fisher Scientific, Finland) at 492 nm, 10 min after development. The values for binding of IgG to the respective antigens in each group were expressed as the mean optical density (OD) ± SE.

Interferon (IFN)-γ quantification by goat IFN-γ ELISA kit

Measurement of IFN-γ from peripheral blood mononuclear cells (PBMCs) was done at 6 weeks post-immunization. PBMCs were prepared by modifying a previously described method [4]. PBMCs were prepared using Ficoll sodium diatrizoate gradient (Sigma-Aldrich), washed twice with PBS and resuspended in RPMI 1640 medium to a level of 2.5×10^6 viable cells per milliliter as determined by trypan blue dye exclusion. The PBMCs (5×10^5 cells/well) were stimulated *in vitro* with BLS, PrpA, SOD, or Omp19 antigen (4 μg/well) for 24 h. The supernatants were collected and used for cytokine measurement as previously described [1,9]. Antigen-specific induction of IFN-γ was measured by using an IFN-γ ELISA kit with biotin-conjugated anti-IFN-γ primary antibody. HRP-avidin (1:1,000; Cusabio Biotech, China) was used to bind the primary antibody. The OD of each well was measured by using a microplate reader set to 450 nm.

Bacterial culture

At 8 weeks post-infection, the experimental animals were humanely sacrificed, and tissues from each animal were freshly collected. Sampled tissues included spleen, liver, lung, kidney, heart, testis, epididymis, mandibular lymph node (LN), parotid LN, retropharyngeal LN, superficial cervical LN, bronchial LN, portal LN, and mesenteric LN. Each sample (5 g) was collected aseptically in 5 mL of PBS and immediately homogenized with a sterile wooden applicator. Inoculum (200

Table 2. Immunization and challenge scheme

Group	Strain and immunogen	Formulation and dose	Goat challenge (conjunctival inoculation)
NC	Non-immunized negative control	Phosphate-buffered saline	–
A	Vector control group	5×10^9 each (pJHL65)	6 weeks post-immunization (5×10^8 CFU/mL in 100 μL)
B	RSrBL*	5×10^9 each (4 strains)	
C	RSrBL*	5×10^{10} each (4 strains)	

*RSrBL formulation, four rough strains JOL1800 *Salmonella* live vectors—JOL1878, JOL1879, JOL1880, JOL1881 each constitutively expressing *Brucella* immunogenic proteins, *i.e.*, superoxide dismutase (SOD), outer-membrane protein 19 (Omp19), *Brucella* lumazine synthase (BLS), and proline racemase subunit A (PrpA).

μL) was seeded and spread on *Brucella* agar media containing an antibiotic supplement and incubated. Cultures were examined daily for presence of colonies and counted after incubation for 7 days at 37°C in a 5% CO_2 atmosphere.

Table 3. Oligonucleotide primers used for real-time polymerase chain reaction assay of *Brucella abortus* with corresponding length, temperature (Tm), and guanine cytosine (GC) content

Genome	Primer sequence	Length (bp)	Tm (°C)	GC (%)
IS711 genetic element	Forward: 5′-CATGCGCTATGTCTGGTTAC-3′	20	55.8	50.0
	Reverse: 5′-GGCTTTTCTATCACGGTATTC-3′	21	53.5	42.9
Goat GAPDH	Forward: 5′-ATCTCGCTCCTGGAAGATG-3′	19	55.3	52.6
	Reverse: 5′-TCGGAGTGAACGGATTCG-3′	18	55.9	55.6

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

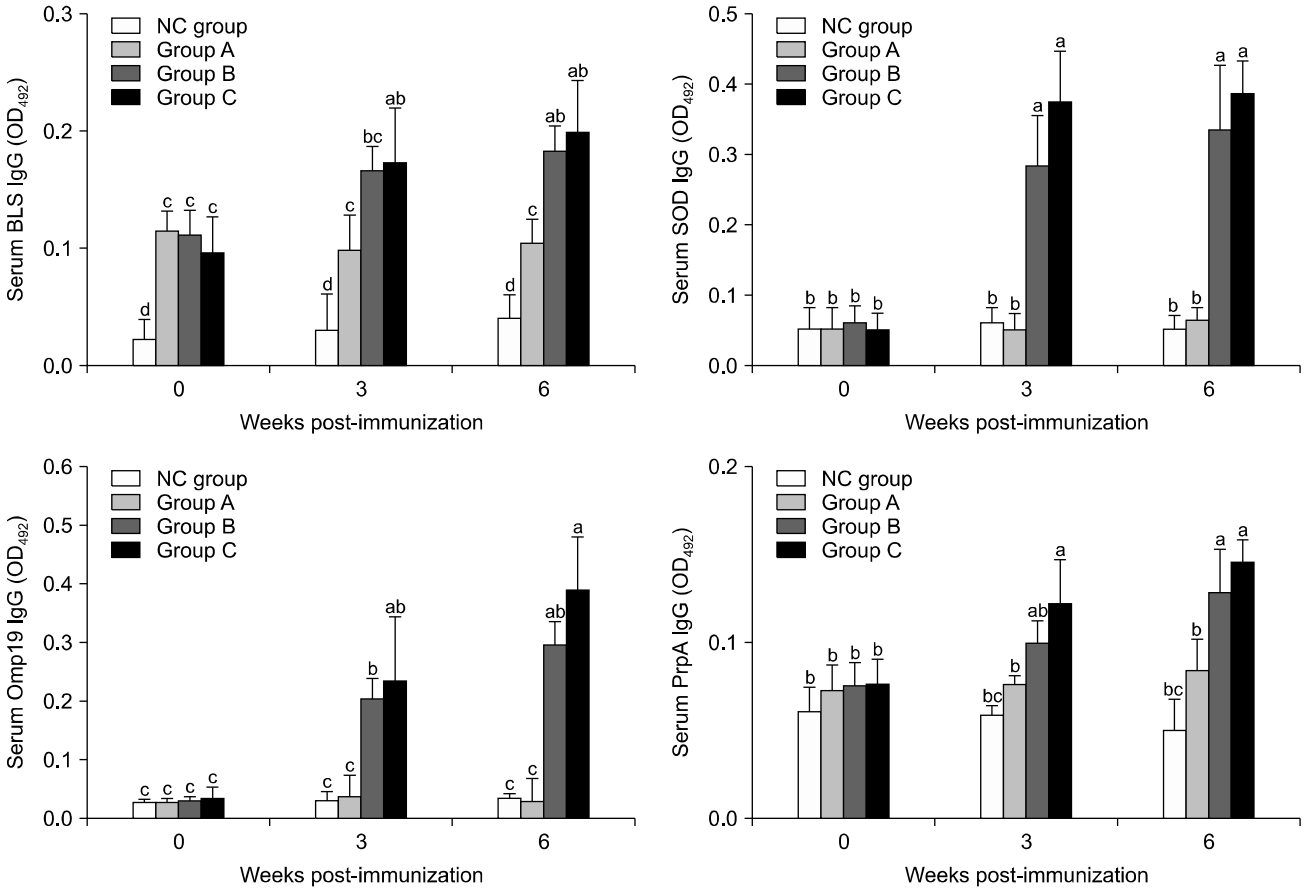

Fig. 1. Humoral immune responses. The systemic humoral responses elicited by the four vaccine strains were investigated by measuring the relative levels of plasma immunoglobulin G (IgG) from samples obtained from non-immunized negative control (NC) group, Group A (vector control containing pJHL65), Group B (*Salmonella* delivery vaccine inoculated at 5×10^9 CFU/mL; CFU, colony-forming unit), and Group C (*Salmonella* delivery vaccine inoculated at 5×10^{10} CFU/mL). Serum IgG titers against *Brucella* lumazine synthase (BLS), *Brucella* superoxide dismutase (SOD), *Brucella* outer-membrane protein 19 (Omp19), and *Brucella* proline racemase subunit A (PrpA) in Group C goats were significantly increased compared to those of the non-immunized NC and vector control groups at 3 and 6 weeks post-immunization. Antibody levels are expressed as mean optical density at 492 nm (OD_{492}) ± SE values. Bars labeled with a different letter on the graph are significantly different from each other ($p < 0.05$) based on results of one-way ANOVA with Duncan's multiple range test.

DNA extraction and relative real-time polymerase chain reaction (PCR) measurement of *B. abortus* antigens in tissue samples

DNA was extracted from tissue by using the GeneAll genomic DNA (gDNA) extraction kit (Seoul, Korea) according to the manufacturer's recommendation. The total gDNA concentration and purity (A_{260}/A_{280}) were measured by using an *e*-spect Malcom spectrophotometer (ES-2 model; Malcom, Japan) at a 250 nm wavelength and using the Nanodrop method. An A_{260}/A_{280} absorbance ratio of 1.8 to 2.0 was regarded as indicating pure gDNA. A 20 μL reaction mixture containing 2 μL gDNA was used as a template in the assay, which used 500 nmol *B. abortus*-specific primers. Real-time PCR conditions were set as follows: denaturation and polymerase activation step at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, concluding with derivation of dissociation curves on a CFX96 real-time PCR detection system (Bio-Rad Laboratories, USA) using SYBR green I as the double-strand DNA-specific binding dye. After reaction completion, specificity was verified by performing melting curve analysis. Quantification was performed by comparing cycle threshold (Ct) values of each sample with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the real-time PCR primers are summarized in Table 3. The oligonucleotide primers used in the study were purchased from Bioneer (Korea).

Microscopic observation of tissue sections

Tissues in 10% neutral buffered formalin were routinely processed by using a Shandon Citadel 1000 tissue processor and were embedded in paraffin. Tissue sections (5 μm) were obtained by using a microtome (HM-340E; Thermo Fisher Scientific, USA) and placed on glass slides. Hematoxylin and eosin staining was performed according to standard techniques [2]. A microphotograph was taken by using an Olympus microscope (BX53F; Olympus, Japan) and digital imaging software (Olympus). Goat liver sections were examined for hepatic microgranulomas by counting the number of foci in 10 microscopic fields in each tissue sample at 100× magnification.

Statistical analysis

All data are expressed as mean ± SE values. Differences between multiple groups were compared by using one-way analysis of variance (ANOVA) by using SAS (ver. 9.1; SAS Institute, USA). Individual comparisons were made by applying Duncan's multiple range test.

Results

Brucella antigen-specific humoral immune responses increased in vaccinated goats

The systemic humoral immune responses elicited by the combined vaccine strains were investigated. As shown in Fig. 1, all immunized goats (Groups B and C) developed serum IgG antibodies production against the individual antigens, including *Brucella* BLS, *Brucella* SOD, *Brucella* Omp19, and *Brucella* PrpA. Serum IgG titers against the individual antigens in Group C were significantly higher than those in the non-immunized NC group and vector control Group A at 3 and 6 weeks after the vaccine injection. Individual antigen-specific serum IgG titers in Group B were also significantly higher than those of the non-immunized NC group and vector control Group A at 6 weeks after vaccine injection, but only the SOD and Omp19 antigen-specific IgG levels were significantly increased at 3 weeks after vaccine injection. However, relative serum IgG titers against all antigens in Group C were not statistically different from those of Group B at 3 and 6 weeks after the vaccine injection. There were no statistical differences between the non-immunized NC group and the vector control Group A. These results indicate that treatment by our combination vaccine at each of the two dosages tested can fully induce humoral immune responses targeting individual *Brucella*

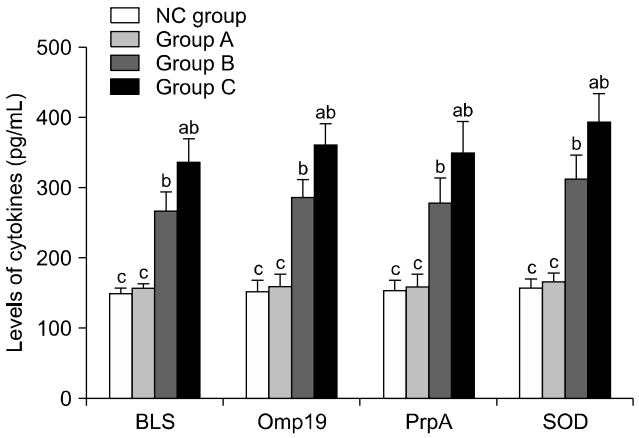

Fig. 2. Bar graphs of the enzyme-linked immunosorbent assay analysis results for the levels of goat interferon (IFN)-γ in peripheral blood mononuclear cells re-stimulated with each antigen at 6 weeks after immunization. The levels of IFN-γ (pg/mL) from the non-immunized negative control (NC) group, Group A (vector control containing pJHL65), Group B (*Salmonella* delivery vaccine inoculated at 5×10^9 CFU/mL; CFU, colony-forming unit), and Group C (*Salmonella* delivery vaccine inoculated at 5×10^{10} CFU/mL) were significantly elevated in Group C, and Group B levels were higher than those of Group A and the NC group. Optical density of each well was measured by using a microplate reader set to 450 nm. Values are presented as mean ± SE. Bars labeled by different letters on the graph are significantly different from each other ($p < 0.05$) based on one-way ANOVA and Duncan's multiple range test. BLS, *Brucella* lumazine synthase; Omp19, outer-membrane protein 19; PrpA, proline racemase subunit A; SOD, superoxide dismutase.

antigens at 6 weeks post-vaccination.

Treatment with the combination vaccine increased the secretion of IFN-γ from PBMCs

Antigen-specific induction of IFN-γ production from PBMCs stimulated with individual antigens (BLS, Omp19, PrpA, or SOD) was measured by using an ELISA at 6 weeks post-vaccination. As shown in Fig. 2, significantly higher IFN-γ production occurred in PBMCs of goats in Groups B and C than in the non-immunized NC group and vector control Group A ($p < 0.05$). Although levels of IFN-γ were higher in Group C than in Group B after stimulation with the individual antigens, no significant differences were noted between those two groups. These results indicate that treatment with our combination vaccine at two different dosages can fully induce cell-mediated immune responses against individual *Brucella* antigens at 6 weeks after immunization.

Salmonella delivery vaccine immunization protected against the challenge of *B. abortus*

The protective efficacy of immunization with the *Salmonella* Typhimurium-based *Brucella* antigen vaccine was investigated in a goat model. At 8 weeks after infection, the magnitude of the challenge bacteria load in the spleen, heart, kidney, liver, lung, testis, epididymis, parotid LN, portal LN, retropharyngeal LN, superficial cervical LN, mandibular LN, mesenteric LN, and bronchial LN reflected the efficacy of the immunization. Compared to the Group A vector control goats, the immunized goats showed significantly higher protection and a lower bacterial load in most of the above-mentioned tissues of goats challenged with *B. abortus* virulent strain 544 (Fig. 3). Generally, the degree of protection in the Group C goats was higher than that in the Group B goats, but the bacteria load in Group B was still lower in most of the collected tissue types than that of Group A. As expected the non-infected NC group showed no bacterial loads.

Relative real-time PCR technique was used to compare the presence of *B. abortus* antigens in all collected samples. As shown in Fig. 4, expression of *B. abortus* antigens was lower in the spleen, liver, kidney, and lung of Groups B and C than those of Group A, although no significant difference was noted between Groups B, C, and the NC group. Such a pattern of decreased antigen expression in the vaccine-treated groups was not observed in the other collected tissues.

Treatment with combination vaccine ameliorated histopathological lesions induced by *B. abortus* strain 544 infection

At 8 weeks after infection with *B. abortus* strain 544, aggregations of inflammatory cells that consisted predominantly of lymphocytes (microgranulomas) in the liver and distinctly visible trabeculae in the spleen were microscopically observed in the examined goats (panel A in Fig. 5). As shown in panel B in Fig. 5, the incidence of microgranuloma foci lesions was

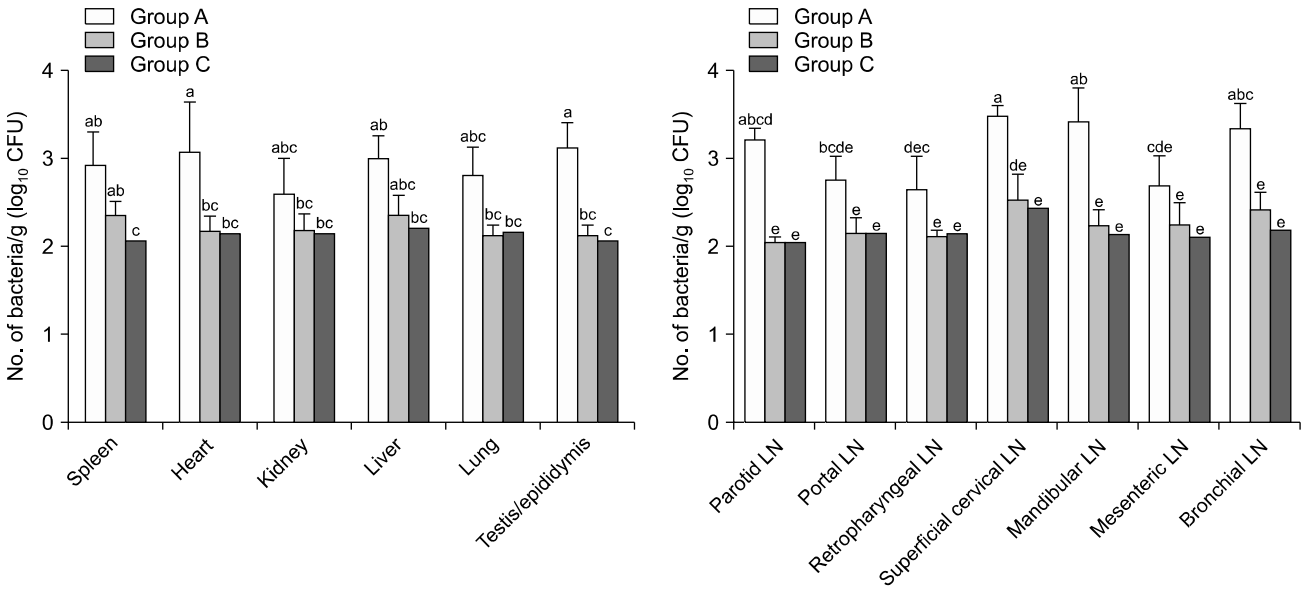

Fig. 3. Protective efficacy of the *Salmonella* delivery vaccine. Group A (vector control containing pJHL65), Group B (*Salmonella* delivery vaccine inoculated at 5×10^9 CFU/mL; CFU, colony-forming unit), and Group C (*Salmonella* delivery vaccine inoculated at 5×10^{10} CFU/mL) goats were challenged with virulent *Brucella abortus* strain 544 at 6 weeks after immunization and were euthanized at 8 weeks post-challenge. The challenge-bacteria load recovered from various organs and lymph nodes (LN) reflects the potency of immunization. The number of CFUs was counted to assess bacterial proliferation in tissues. Bars labeled by different letters on the graph are significantly different from each other ($p < 0.05$) based on one-way ANOVA and Duncan's multiple range test.

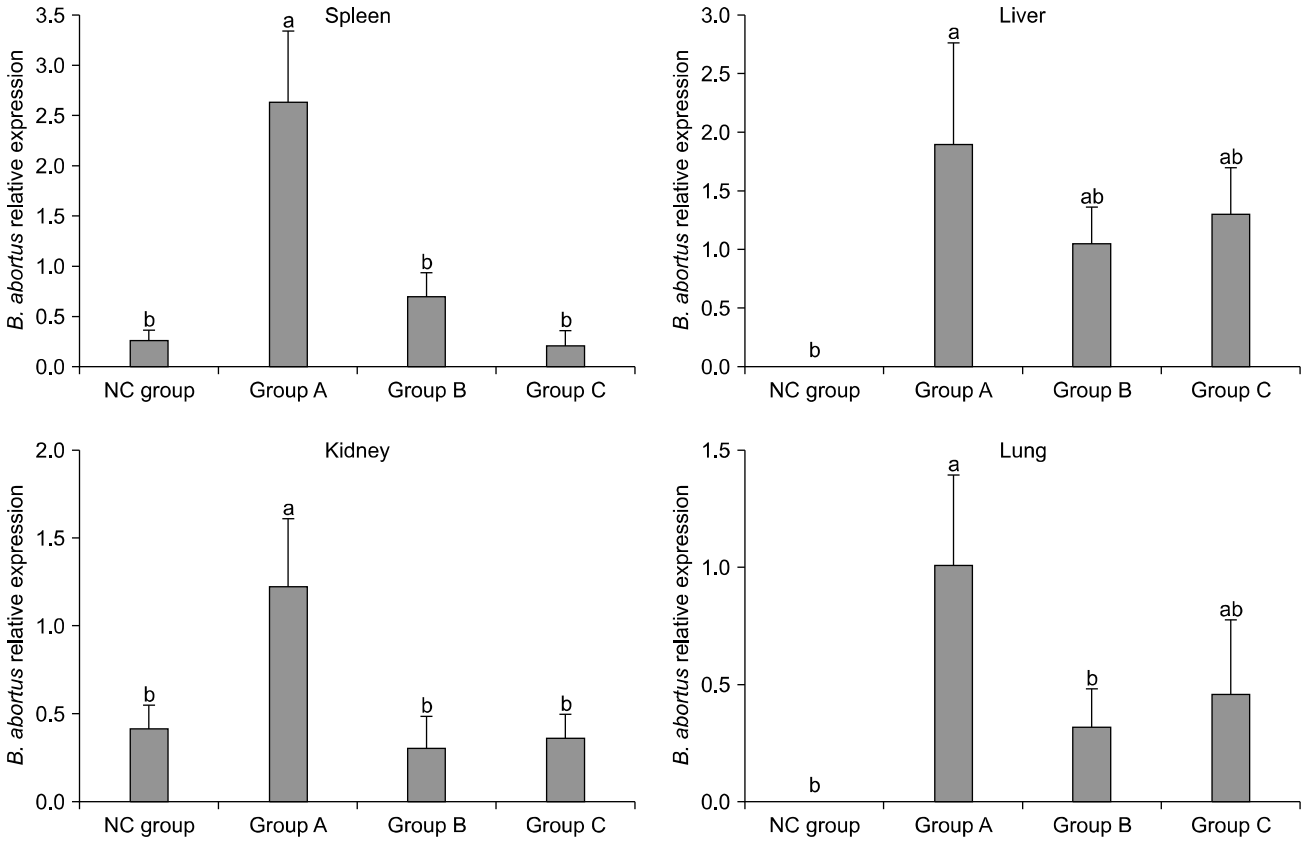

Fig. 4. Relative expression of *Brucella abortus* antigens in various tissues of goats. The expression levels of *Brucella* antigens in the non-immunized negative control (NC) group, Group B (*Salmonella* delivery vaccine inoculated at 5×10^9 CFU/mL; CFU, colony-forming unit), and Group C (*Salmonella* delivery vaccine inoculated at 5×10^{10} CFU/mL) were lower than that in Group A (vector control containing pJHL65) in the spleen, liver, kidney, and lung. No significant differences were noted between Groups B, C, and NC. Values are presented as mean ± SE. Bars labeled by different letters on the graph are significantly different from each other ($p < 0.05$) based on one-way ANOVA and Duncan's multiple range test.

significantly low in the livers of goats in Groups B and C. Levels of distinct visibility of trabeculae in the spleen were lower in Groups B and C than that in Group A, indicating lower septicemia associated with the vaccination. These results suggest that the combination vaccine treatment ameliorated *B. abortus* infection-related lesions.

Discussion

Immunization of animals and humans has been successfully practiced as a means to control infectious diseases for centuries [7]. In order to avoid the residual virulence of *Brucella* live vaccines, an alternative approach of delivering *Brucella* subunit components via live vaccine vectors has been used. Safety is the major concern when using attenuated live vaccine vectors delivering *Brucella* antigens compared to the use of live attenuated *Brucella* vaccine. Attenuated *Salmonella* Typhimurium live vector strains delivering various heterogeneous antigens to the immune system can be used as a combination vaccine vehicle against a variety of targeted pathogens at a relatively low cost [10].

The success of immunization with a mixture of antigens using attenuated *Salmonella* Typhimurium live vaccine was anticipated based on the results of previous studies. However, the immune response in livestock was based much on the precept that live attenuated *Salmonella* vaccine expressing recombinant rBL protein can induce significant protective effects in a mouse model [27]. In this study, the attenuated *Salmonella* Typhimurium Δlon, ΔcpxR, and Δasd bacterial delivery vector was used with the pBP65 plasmid encoding the *asd* gene to deliver the *Brucella* antigens at two different dosages. The goats inoculated with 5×10^{10} CFU/mL (Group C) showed significantly increased serum IgG titers against all antigens when compared to the vector control group. However, the lower inoculant concentration (5×10^9 CFU/mL) should not be overlooked, as it also produced higher serum IgG titers than that in Group A. Humoral response results of the two inoculation dosages showed the effectiveness of delivering

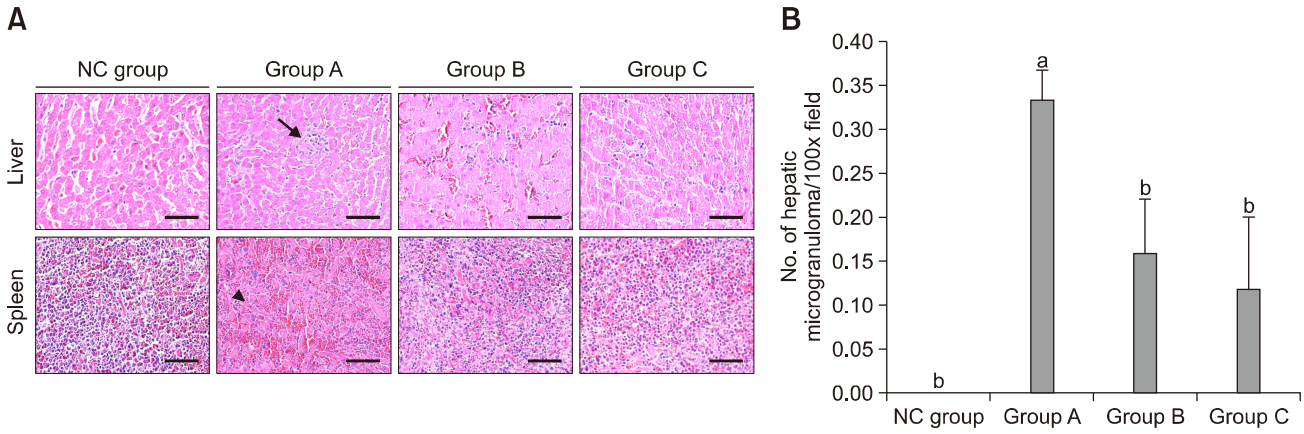

Fig. 5. Microphotograph of liver and spleen sections of from the non-immunized negative control (NC) group, Group A (vector control group), Group B (*Salmonella* delivery vaccine inoculated at 5×10^9 CFU/mL; CFU, colony-forming unit), and Group C (*Salmonella* delivery vaccine inoculated at 5×10^{10} CFU/mL) at 8 weeks post-challenge. (A) The liver sections revealed aggregation of inflammatory cells (arrow) that consisted predominantly of lymphocytes (microgranulomas). Spleen sections revealed distinctly visible trabeculae (arrowhead), suggesting a response to septicemia in the vector control group (Group A). H&E stain. Scale bars = 50 μm. (B) Comparison of microgranulomas based on counting the number of the foci in 10 microscopic fields per tissue sample showed that the microgranulomas per 100× fields in the vector control group was significantly higher than that in the immunized groups. Bars labeled by a different letter on the graph are significantly different from each other ($p < 0.05$) based on one-way ANOVA and Duncan's multiple range test.

antigens by the attenuated *Salmonella* Typhimurium vaccine. Furthermore, IFN-γ concentration was evaluated in supernatants of PBMCs following re-stimulation with heat-inactivated *B. abortus* antigens of goats immunized with vaccine candidates. The levels of IFN-γ were significantly elevated in both Group C (5×10^{10} CFU/mL) and Group B (5×10^9 CFU/mL) over that in Group A. The results suggest that a Th1-type immune response is strongly induced by both inoculation dosages. It seems logical to favor the higher inoculation for sufficient protection, but the lower inoculation dose also produces a significant immune response. In addition, economics must be considered because the lower dosage (5×10^9 CFU/mL) may offer a cost-effective alternative for the production of effective vaccines against brucellosis.

To examine cross-reactivity, *Brucella* spp. have been identified by performing amplification of a specific region of its genome, as has been done for other closely related bacteria [14,19]. The IS711 region of the *Brucella* genome has been used to identify *B. abortus*, *B. melitensis*, and *Brucella suis* biovar 1. Similar primers and probes exploiting the IS711 repetitive element have been used for real-time detection in some studies [14,15]. This study explored using real-time PCR and SYBR green I, a double-stranded DNA intercalating dye [14]. Real-time PCR was used as a confirmatory diagnostic alternative to problematic culturing of *Brucella* spp. The high detection sensitivity of real-time PCR can be explained by the fact that it detects DNA from bacteria that are damaged or nonviable and which therefore cannot be isolated by applying conventional culture techniques. In addition, relative real-time PCR can indicate which tissues have higher/lower levels of bacterial expression. Our results showed that the relative expression of *B. abortus* antigens was more decreased in spleen, kidney, and lung of immunized goats than in the non-immunized group. However, in contrast to the bacteria load recovery results, such a decrease in antigen expression in the vaccine groups was not observed in other collected tissues, especially not in the various LNs that were examined. Based on these results, we speculate that many nonviable *Brucella* in the LNs of immunized goats were measured by real-time PCR, since macrophages phagocytose antigens and then migrate to draining LNs. Therefore, we believe that the challenge strain-tissue recovery model is a gold standard for evaluating the efficacy of our combination vaccine, and that results obtained by using real-time PCR should be used for reference purposes only.

In this study, the experimental goats did not show any gross lesions, including splenomegaly, when necropsy was performed, thus indicating that severe brucellosis did not develop as a result of the experimental infection. Even though few gross lesions were observed, distinctly visible trabeculae was microscopically evident in the spleen of non-immunized goats after *Brucella* infection. This suggests that the spleen can respond to septicemia through active hyperemia [26], but the hyperactive spleen may not always be enlarged [8]. In addition, the experimentally infected goats revealed aggregations of inflammatory cells (microgranulomas), and fewer neutrophils

were observed in the liver. The recruitment of lymphocytes in the liver was evident based on the formation of microgranulomas, supporting the observations in a previous study that showed an inflammatory response in the liver of mice [24]. These two microscopic changes after *Brucella* infection were decreased in our vaccine-treated groups, indicating that such histopathological parameters can be used for evaluating the severity of brucellosis and the efficacy of a vaccine in goat.

In conclusion, we have shown that SC vaccination with an attenuated *Salmonella* Typhimurium live vector strain delivering a mixture of four *Brucella* immunogens (BLS, PrpA, Omp19, and SOD) can induce a protective immune response against infection by *B. abortus*. It was apparent that vaccination at the higher dose (Group C) was more efficacious than that at the lower dose (Group B); regardless, vaccination at the lower dose also had a significant protective effect. It is likely that a *Salmonella*-based delivery system could be adapted to develop multivalent recombinant *Salmonella* vaccines against brucellosis. Further work is needed to compare the efficacy of our combination vaccine with that of vaccines already in use, such as RB51.

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Conflict of Interest

The authors declare no conflicts of interest.

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