Protective efficacy of attenuated Salmonella Typhimurium strain expressing BLS, Omp19, PrpA, or SOD of Brucella abortus in goats

Mwense Leya 1,†, Won Kyong Kim 1,†, Enkhsaikhan Ochirkhuuyag 1,†, Eun-Chae Yu 2, Young-Jee Kim 2, Yoonhwan Yeo 2, Myeon-Sik Yang 1,†, Sang-Seop Han 2, John Hwa Lee 2,†, Dongseob Tark 2, Jin Hur 1,†, Bumseok Kim 1,†,*

1 College of Veterinary Medicine, Jeonbuk National University, Iksan 54596, Korea
2 Korea Zoonosis Research institute, Jeonbuk National University, Iksan 54531, Korea

ABSTRACT

Background: Attenuated Salmonella strain can be used as a vector to transport immunogens to the host antigen-binding sites.

Objectives: The study aimed to determine the protective efficacy of attenuated Salmonella strain expressing highly conserved Brucella immunogens in goats.

Methods: Goats were vaccinated with Salmonella vector expressing individually lipoprotein outer-membrane protein 19 (Omp19), Brucella lumazine synthase (BLS), proline racemase subunit A (PrpA), Cu/Zn superoxide dismutase (SOD) at 5 × 10^9 CFU/mL and challenge of all groups was done at 6 weeks after vaccination.

Results: Among these vaccines inoculated at 5 × 10^9 CFU/mL in 1 mL, Omp19 or SOD showed significantly higher serum immunoglobulin G titers at (2, 4, and 6) weeks post-vaccination, compared to the vector control. Interferon-γ production in response to individual antigens was significantly higher in SOD, Omp19, PrpA, and BLS individual groups, compared to that in the vector control (all \( p < 0.05 \)). Brucella colonization rate at 8 weeks post-challenge showed that most vaccine-treated groups exhibited significantly increased protection by demonstrating reduced numbers of Brucella in tissues collected from vaccinated groups. Real-time polymerase chain reaction revealed that Brucella antigen expression levels were reduced in the spleen, kidney, and parotid lymph node of vaccinated goats, compared to the non-vaccinated goats. Besides, treatment with vaccine expressing individual antigens ameliorated brucellosis-related histopathological lesions.

Conclusions: These results delineated that BLS, Omp19, PrpA, and SOD proteins achieved a definite level of protection, indicating that Salmonella Typhimurium successfully delivered Brucella antigens, and that individual vaccines could differentially elicit an antigen-specific immune response.

Keywords: Brucellosis; vaccine; Salmonella; goat
Individual Brucella antigens delivered by Salmonella in goats

INTRODUCTION

Brucellosis is a (zoonotic) disease that can transmit to humans, usually following exposure to infected animal fluids, or their products, such as unpasteurized milk [1-4]. Brucellosis is closely linked to low productivity in animals, resulting in economic loss [5]. The lowered reproduction in affected animals is related to the poorly understood mechanism of bacterial placentation localization, trophoblast tropism, and subsequent abortion, making it of the utmost importance [1].

Infectious diseases are mostly counteracted by vaccination, which is the most efficient and economic method [6]. A live attenuated Salmonella strain can be used as a vector transport system to convey Brucella immunogens to receptor binding sites [7,8]. Recently, Salmonella Typhimurium transport-based Brucella vaccine has been developed by mixing four heterologous antigens of Brucella, including proline racemase subunit A (PrpA), Cu/Zn superoxide dismutase (SOD), Brucella lumazine synthase (BLS), lipoprotein outer-membrane protein 19 (Omp19) proteins, and proline racemase subunit A (PrpA). Salmonella Typhimurium has been demonstrated by evidence to be a suitable vectored anti-Brucella immune amplifying system through multiple routes of immunization [9,10]. Subunit vaccine against Brucella, such as recombinant subunit vaccine that can achieve desirable protection against any species of Brucella in natural host, needs to be developed. To search for novel vaccines, we have focused on four heterologous antigens (BLS, Omp19, PrpA, and SOD), and used them individually to ascertain the protective efficacy of each antigen in a goat model.

A few critical steps are involved in the infection caused by Brucella species. First, it invades host epithelial cells to allow infection through mucosal surfaces [11,12]. Resistance to intracellular bacterial pathogens mostly relies on T-cell immune response [13] that involves activation of the antibacterial machinery of antigen-displaying cells (i.e., macrophages and dendritic cells), leading to augmentation of antigen-specific antibody and CD4+ and CD8+ T-cells response [14,15]. BLS, Omp19, PrpA, and SOD are efficient at inducing T-helper type 1 (Th1) cytokines. Adequate Th1 immune response, such as IFN-γ production, has a potent activity for Brucellosis clearance [16]. Among the identified Brucella antigens, BLS has been reported to elicit potent antigen-specific T-cell response that is important for antibody-mediated and cytotoxic immune defense [17]. PrpA is associated with persistent Brucella infections by motivating B-cell stimulation [18]. Therefore, immunization with PrpA would not only have immunomodulatory effects, but also reduce the chronicity of Brucella infection. Omp19 induces a Th-1 type response that confers protection against Brucella infection. It can also induce the maturation of murine dendritic cells in vivo [19]. Omps are proteins involved in the regulation of the delivery of bactericidal agents, as well as nutrients, among many other diverse functions [20]. Finally, SOD induces diverse immune responses by partitioning harmful superoxide (O2-) radicle into ordinary oxygen (O2) and hydrogen peroxide (H2O2), as a consequence of the phagocytosis of macrophages [21,22].

We evaluated the level of protection conferred by four Salmonella strains expressing highly conserved Brucella abortus (rB) antigen of BLS, Omp19, PrpA, or SOD as anti-Brucella vaccine candidate using goat model. Vaccines based on recombinant BLS, Omp19, PrpA, and SOD could individually elicit cellular immune response, and provide protection to host against Brucella infection. Such hypothesis was tested in the present work.
MATERIALS AND METHODS

Ethics guidelines
Consent for all experimental procedures was obtained from the Jeonbuk National University Animal Ethics Committee, supported by the Korean Council on Animal Care and Korean Animal Protection Law (Approval Number: CBNU2016-98), and all experimental procedures used on goats were conducted safely and humanely under their approved protocols. This study was performed under barrier conditions in a BL-3 laboratory during the period 2016–2017.

Vaccine system construction strains and plasmids and growth conditions
The challenge experiment employed the use of a highly virulent Brucella strain 544-HJL254 at a re-suspension of approximately $5 \times 10^8$ colony forming units (CFU)/mL [23]. A rough Salmonella Typhimurium subspecies appointed JOL1800 was electrophoresed with pET28A and pHJL65 plasmids as vectors overexpressing the target gene, as depicted in Table 1. Salmonella strains were grown, constructed, and validated for Salmonella Typhimurium strains expressing individual Brucella antigens-BLS, Omp19, PrpA, or SOD and delivery of plasmid pHJL-65, as described previously [9,24].

Study design, experimental animals, immunization, and challenge with Brucella abortus
Female goats with an average weight of (25–30) kg at 10 months of age that were seronegative of brucellosis were obtained from ImproAH farm in Gyeongsan-si, South Korea. Animals were randomly assigned to an experimental group by equally dividing them into 6 groups (n = 5 goats in each group), and inoculated as stated in Table 2. Upon inoculation, all goats were closely observed for vaccine-related safety assessment, such as immunization-induced morbidity, or mortality. Negative control group (NC) (n = 5 goats) were vaccinated with sterile phosphate buffered saline (PBS) subcutaneously (0.1 mL) as a non-vaccinated control. Female goats in groups B, C, D, and E were vaccinated with Salmonella vector expressing individually BLS, Omp19, PrpA, and SOD, respectively, at $5 \times 10^9$ CFU/mL in 1 mL. Challenge of all groups was done at 6 weeks after vaccination with Brucella strain- HJL254 ($5 \times 10^8$ CFU/mL) in 100 μL.

Table 1. The following bacterial strains and plasmid were employed in this study

| Strain/plasmid       | Description                                                                 | Source   |
|----------------------|-----------------------------------------------------------------------------|----------|
| **Escherichia coli** |                                                                             |          |
| BL21(DE3)            | $F^\prime$, ompT, hsdS$(r^\prime_m^\prime_w^\prime)$, 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                                                       | [10]     |
| **Salmonella**       |                                                                             |          |
| Typhimurium          |                                                                             |          |
| JOL1800              | JOL912 Δrfal; O-antigen deficient rough strain, bacteria delivery vector    | [9]      |
| JOL1818              | JOL1800 with pHJL65 plasmid; Salmonella vector control                      | [9]      |
| JOL1878              | JOL1800 with pHJL65-SOD                                                    | [9]      |
| JOL1879              | JOL1800 with pHJL65-Omp19                                                  | [9]      |
| JOL1880              | JOL1800 with pHJL65-BLS                                                    | [9]      |
| JOL1881              | JOL1800 with pHJL65-PrpA                                                   | [9]      |
| **Brucella abortus** |                                                                             |          |
| HJL254               | *Brucella abortus* strain 544 (ATCC23448)                                   | [10]     |
| **Plasmids**         |                                                                             |          |
| pET28a               | IPTG-inducible expression vector; Km'                                       | Novagen  |
| pHJL65               | Asd', pBR ori, β-lactamase signal sequence-based periplasmic secretion plasmid, 6 x His tag | [24]     |
conjunctively (50 μL of inocula/eye), respectively. All goats underwent daily observation to make sure no goat abstained from taking feed or showed any post-challenge effects, and were also given ample bedding (amount of bedding not estimated).

**Vaccine safety**

Evaluation of vaccine safety in goats was done by clinical observation of each goat’s performance during 8 weeks post-vaccination. At 8 weeks post-immunization, goats were aseptically euthanized, and various samples, including liver, spleen, uterus, lung, heart, kidney, mandibular lymph node (LN), superficial cervical LN, parotid LN, retropharyngeal LN, bronchial LN, portal LN, and mesenteric LN, were collected for histopathology examination and isolation of *Salmonella* delivery strains. Samples were homogenized, and plated onto Brilliant Green Agar (Becton, Dickinson and Company, USA). Polymerase chain reaction (PCR) was done by using OMPC- and TYPH-specific primer set to confirm isolation of *Salmonella* delivery strains.

**Antigen-specific IgG measurement**

To investigate the levels of anti-*Brucella* humoral response, goat immunoglobulin-G (IgG) enzyme linked immunosorbent assay (ELISA) quantitation set was employed. Serum samples were collected at (0, 1, 2, 4, and 6) weeks post-immunization. A standard ELISA was carried out to evaluate immune response against BLS, Omp19, PrpA, and SOD antigens in serum samples of goats compared to the vector control group, according to a modified method described in a previous study [8]. Microtiter plates (96-well; Nunc, Denmark) were incubated overnight with 5 μg/mL pre-titrated recombinant BLS, Omp19, PrpA, or SOD proteins each in PBS, blocked for 30 min using PBS containing 1% bovine serum albumin (diluent; 200 μL/well), and washed with 0.05% Tween-20. Thereafter, serum samples were diluted as 1:100 in diluent. Secondary horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG–Fc detection antibody was used at 1:150,000 (Bethyl Lab Inc., USA). Results shown are expressed as OD ± standard deviation upon colorimetric changes resulting from the action of HRP on o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, USA) at 10 min post-development for IgG samples.

**Interferon (IFN)-γ cytokine concentration measurement from peripheral blood mononuclear cells (PBMCs) of goats**

At 6 weeks post-vaccination, cytokine concentration measurement of PBMCs was investigated following the procedures previously described, using goat IFN-γ ELISA Kit [9,10]. Determination of the optical density of each microplate was set at 450 nm.

### Table 2. Vaccination and challenge strategy

| Group (n = 5) Strains & immunogen | Formulation and dose | Caprine challenge |
|-----------------------------------|----------------------|-------------------|
| NC Non-immunized negative control | Phosphate-buffered saline | - |
| A Vector group | 5 × 10⁷ CFU/mL each (pJHL-65) | 6 weeks post-immunization |
| B RSrBL* | 5 × 10⁷ CFU/mL each of BLS | (5 × 10⁸ CFU/mL in 100 μL conjunctival inoculation) |
| C RSrBL* | 5 × 10⁷ CFU/mL each of Omp19 | |
| D RSrBL* | 5 × 10⁷ CFU/mL each of PrpA | |
| E RSrBL* | 5 × 10⁷ CFU/mL each of SOD | |

Four rough strains JOL1800 *Salmonella* live vectors- JOL1878, JOL1879, JOL1880, and JOL1881, each form conveying *Brucella* immunogenic protein (RSrBL* formulation,) i.e., SOD, Omp19, BLS, Brucella lumazine synthase; PrpA, proline racemase subunit A.
Anaesthesia and euthanasia protocol
At 9 am 8 weeks post-challenge after 24 h of fasting, goats were euthanized in a painless and stress-free manner. Anaesthesia was first induced with Rompun Injection Xylazine (Bayer AG, Bayer Korea Ltd., Korea) intramuscularly (IM) (23.3 mg/kg), and goats were later euthanized by the use of Succinylcholine (Komipharm, Succipharm, Korea) injected via IM route (100 mg/kg).

Challenge experiment
All animal groups at 8 weeks post-challenge were euthanized, and liver, spleen, uterus, lung, heart, kidney, mandibular LN, superficial cervical LN, parotid LN, retropharyngeal LN, bronchial LN, portal LN, and mesenteric LN were freshly collected. Tissue samples for colony count and examination were weighed (5 g), and processed for immediate homogenization and inoculation. The 200 μL inocula seeded on Brucella agar media were observed, and colonies counted daily for 7 days.

Brucella antigen expression using real-time PCR
Firstly, tissue sample gDNA was extracted using GeneAll gDNA kit, following the manufacturer’s recommendation (Bioneer Corporation, Korea). Pure gDNA showed A260/A280 nm absorbance ratio of (1.8–2.0). The relative expression of Brucella was determined by Real-time PCR using Brucella-specific primers, each purchased from BIONEER, Korea, which Table 3 summarizes.

Histological analysis
Five μm thick tissue specimens were placed in 10% formalin, and prepared for eventual placement onto glass slides. Standard procedure was followed for Hematoxylin and eosin staining, and Microphotography was captured by Olympus microscope (BX53F model, Japan), and digital imaging software (analysis TS, Olympus Corp., Japan). Observed foci of hepatic microgranulomas were then counted in 10 microscopic fields/tissue samples (100× magnification).

Interpretation of data
Histograms were generated by Graph Pad Prism 5, and data expressed as mean ± SD. Data were analyzed by one-way analysis of variance using Duncan’s Multiple Range Test (DMRT) with means bearing different superscript letters differing significantly at \( p < 0.05 \). For multiple comparisons between two groups, Student’s \( t \)-test was used, and a \( p \) value of \( p < 0.05 \) was considered significant.

RESULTS

Vaccine safety
Goats vaccinated using Salmonella-based Brucella abortus vaccine strains administered subcutaneously showed no negative clinical sign (behaviour, appetite, body temperature-retained within normal range 38.5°C–40°C, etc.). Tissue organs collected showed no pathological changes in the vaccinated goats, and absence of Salmonella delivery strains in the isolates (data not shown).

Table 3. Primer sequence of real-time polymerase chain reaction

| Genome       | Primer sequence                                      | Length (bp) | Temp (°C) | GC (%) |
|--------------|------------------------------------------------------|-------------|-----------|--------|
| IS711 genetic element | F: 5’-CATGCGCTATGTCTGGTTAC-3’ | 20          | 55.8      | 50.0   |
|              | R: 5’-GGCTTTTCTATACGGGATTC-3’                     | 21          | 53.5      | 42.9   |
| Goat GAPDH   | F: 5’- ATCTCGCTCTGGAAGAGTG-3’                      | 19          | 55.3      | 52.6   |
|              | R: 5’- TCGGAGTGACCGTGAC-3’                         | 18          | 55.9      | 55.6   |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Omp 19 and SOD immunization induces specific humoral immune responses against Brucella abortus infected goats

Serum IgG titers based on ELISA were investigated at 2 weeks post-immunization, and Fig. 1 shows the data. Among the four individual antigens vaccinated at $5 \times 10^8$ CFU/mL in 1 mL, Omp19 (group C) and SOD (group E) revealed significantly ($p < 0.05$) higher titers at (2, 4, and 6) weeks post-vaccination, compared to vector control group A. Increasing trend of IgG production was only noticed against Omp19 antigen, while anti-SOD antibodies maintained approximately the same level at (2, 4, and 6) weeks post-vaccination. Weaker anti-PrpA and anti-BLS antibody induction were observed, compared to other antigen counterparts. They showed no significant differences compared to the vector control group.

Vaccination targeting individual antigens induces the secretion of IFN-γ

After re-stimulation with individual antigens at 4 weeks post-immunization, protein-specific IFN-γ concentrations in PBMCs in response to BLS (group B), Omp19 (group C), PrpA (group D), and SOD (group E) vaccines were compared to vector control (group A) (Fig. 2). IFN-γ production was only noticed against Omp19 antigen, while anti-SOD antibodies maintained approximately the same level at (2, 4, and 6) weeks post-vaccination. Weaker anti-PrpA and anti-BLS antibody induction were observed, compared to other antigen counterparts. They showed no significant differences compared to the vector control group.

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**Fig. 1.** Antibody responses against BLS, Omp19, PrpA, and SOD antigens. BLS, Omp19, PrpA, and SOD were inoculated with $5 \times 10^8$ CFU/mL of each of the four Brucella antigens in 1 mL, respectively. Omp19 and SOD showed significantly higher serum IgG titers at (2, 4, and 6) weeks post-vaccination, compared to vector control group A. The absorbance level of serum IgG was expressed as (OD492) ± SD of the mean, and dissimilar letters stipulate significant differences, as stated by Duncan’s multiple range test. A $p$ value of $p < 0.05$ was considered statistically significant.

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

**Fig. 2.** Vaccination targeting individual antigens induces secretion of goat IFN-γ in peripheral blood mononuclear cells at 4 weeks post-vaccination. SOD, Omp19, PrpA, and BLS individual groups in response to individual antigen showed significantly higher IFN-γ (pg/mL) production, compared to that in the vector control. Results represent all goat means in each group, and the error bars denote standard deviation.

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

*Significant value differences obtained for individual groups ($p < 0.01$).
production in response to individual antigens was the highest in SOD-stimulated goats. It was significantly higher in SOD, Omp19, PrpA, and BLS individual groups, compared to that in the vector group A (all \( p < 0.05 \)). Vaccinated groups showed no major statistical differences.

**Vaccination targeting individual antigens differentially confers protective efficacy in the goat model**

*Brucella* colonization rate to determine the effectiveness of vaccination was analyzed in spleen, lung, heart, kidney, liver, uterus, parotid LN, mandibular LN, retropharyngeal LN, superficial cervical LN, bronchial LN, portal LN, and mesenteric LN at 8 weeks post-challenge with *Brucella abortus* 544. Fig. 3 shows that most vaccine-treated groups (groups B, C, D, and E) exhibited significantly increased protection compared with vector group A, showing reduced numbers of *Brucella* in tissues collected from vaccinated groups, compared to those in control goats. Generally, no significant difference in bacterial burden was found in most tissues of individual antigen targeted vaccinated groups. These results indicate that all individual strains could confer a significant level of protection (\( p < 0.05 \)) by reducing bacterial tissue count correlated with vaccine protection efficacy.

![Graph showing bacterial proliferation in various organs and lymph nodes of goats challenged with 5 × 10⁸ CFU/mL of the highly infective *Brucella* 544-HU1254. *Brucella* colonization rates to determine the effectiveness of immunization were determined by colony-forming unit (log₁₀ CPU/g of tissue) at 8 weeks post-challenge. Results revealed that most of the vaccine-treated groups (groups B, C, D, and E) exhibited significantly greater protection than in the vector control, by showing reduced numbers of viable *Brucella* recovered from each tissue of vaccinated groups, compared to those in control goats. Dissimilar letters stipulate significant differences, as stated by Duncan’s multiple range test. A \( p \) value of \( p < 0.05 \) was considered statistically significant. BLS, *Brucella* lumazine synthase; Omp19, outer-membrane protein 19; PrpA, proline racemase subunit A; and SOD, superoxide dismutase; LN, lymph node.]
To determine the amounts of *Brucella* antigens expressed in the tissue samples, Real-time PCR was used (Fig. 4). *Brucella* antigens revealed a general decrease in the spleen, kidney, parotid LN, portal LN, superficial cervical LN, and mesenteric LN of goats immunized with individual antigen, than in vector group A. However, unlike bacterial load results, a decreased pattern of antigen relative expression was not shown in vaccine-treated groups of other organs.

**Vaccination with individual antigens vaccine ameliorates histopathological lesions as a result of infection**

The main pathological changes were only observable in the liver and spleen. Liver parenchyma showed infiltration of mononuclear cells, while the spleen showed an expansion of trabeculae (Fig. 5A). Fig. 5B shows that the liver revealed markedly decreased number of microgranuloma foci in all vaccine-treated groups (groups B, C, D, and E). Furthermore, expansion levels of splenic trabeculae were also decreased in groups B, C, D, and E, compared to goats in vector group A, suggesting reduction by immunization. Vaccination with individual antigen vaccine reduced *Brucella abortus* infection-related lesions.

**DISCUSSION**

*Brucella* infections in either human or animal depict a public health threat of global significance, with a consequence of severe economic losses [25]. Consistent with the search for a better vaccine, we explored the attenuated *Salmonella* Typhimurium delivery system already developed that revealed strong humoral and cytotoxic immune responses in mice [10]. This protection against *Brucella abortus* infection led to a vaccine trial in a natural host.
The main targets of immunization against Brucella abortus are usually cattle within control and eradication strategies. However, goat, bison, feral swine, elk, and other hosts can also harbour the infection. Other animals are even capable of sustaining the disease. This can be a major source of the re-emergence of bovine brucellosis.

Brucella vaccine candidates individually delivering BLS, Omp19, PrpA, and SOD antigens using attenuated Salmonella Typhimurium were used to investigate the protective effects. To come up with a robust protective immune response that can be close to the long-lived natural infection, the ideal mixture of a few proteins of the pathogen is a difficult and complex undertaking. In our recent study, we reported that immunization of goat with a combination Salmonella vector strain delivering a concoction of BLS, PrpA, Omp19, and SOD can induce an immune response against Brucellosis at two inoculations, a higher and a lower dose of (5 × 10^{10} and 5 × 10^{9}) CFU/mL, respectively. Therefore, the exploration of which conserved protein can effectively protect against Brucella abortus infection in a natural host is essential. Such results can be valuably used for economic viability and dose optimization.

In this study, in order to confirm the successful delivery of each antigen by the delivery vector, antigen specific antibodies were evaluated. Groups of vaccine delivering Omp19 showed the maximum level of IgG production in an increasing fashion. Furthermore, anti-SOD antibodies were also increased, starting at 2 weeks post-immunization. However, levels of anti-PrpA and BLS IgG were much weaker, even after subsequent weeks following immunization. The results were consistent with a similar study done to evaluate the...
antibody response and effectiveness of Brucella vaccine candidates, individually, or in a concoction, using mice model. In the same study, Salmonella Typhimurium-Omp candidate strain produced the highest level of IgG antibodies in serum samples; whereas, much lower levels of anti-PrpA antibodies was reported [29], as in our study. The role of T-cell response in brucellosis is very much more accepted among researchers than the role of antibody responses in the protection against brucellosis [30-32]. In the present study, the cytotoxic immune response generated by vaccination with Salmonella vector expressing Brucella antigens BLS, Omp19, PrpA, and SOD were investigated by evaluating IFN-γ productivity from antigen re-stimulated PBMCs. Our results demonstrated that vaccination targeting individual antigens showed a strong cell-mediated immune response. Brucella species are facultative intracellular pathogens that reside in macrophages [33]. Therefore, IFN-γ becomes the cytokine most essential effector for activating macrophages for more systematic killing and suppression of the replication of intracellular microbial pathogens [14]. In our study, although levels of anti-PrpA and anti-BLS IgG were weak to elicit humoral immunity in vaccine group delivering PrpA and BLS, they were able to stimulate Th1 product IFN-γ and bacterial loads similar to Omp19 and SOD groups. This indicates that cell-mediated immune response by our vaccine system has a more important role in regulating brucellosis, compared to humoral immune response.

Furthermore, we observed a significant level of protection when Brucella abortus individual antigens (BLS Omp19, PrpA, and SOD) were used in goats. Our results demonstrated that, in most tissues of immunized goats, significantly better protection was conferred than that in the vector control group. However, this was not at perfect consistency, because some LNs, including superficial cervical LNs, of immunized goats showed similar bacterial loads to those of control animals. Data collected from Real-time PCR revealed that Brucella abortus antigen levels were generally decreased in the spleen, kidney, portal LN, and parotid LN of goats immunized with individual antigens, compared to those in the vector control. However, unlike bacterial load results, such decreased pattern of antigen relative expression in other tissues was not shown in vaccine-treated groups. No statistical difference was noted between the immunized groups. Therefore, with the current experimental settings, the antigen among Salmonella-delivered BLS, Omp19, PrpA, and SOD that had the maximum protection was not determined.

Based on the above-mentioned results, regardless of having evidence suggesting significant protection and effectiveness achieved by the subunit vaccines, there are many limitations to the study. Firstly, there is the limited scope of efficacy of this vaccine of generally $< 2 \log_{10}$ units, and several boosts would be required to achieve long-lived immunity. So we could speculate that combining these antigens instead would induce slightly greater protection (favorable results). However to be efficacious, vector systems expressing Brucella immunogens still need perfect combination expressed in a high amount [27]; and attempting to add an adjuvant with vaccination using a live salmonella vector at this point, without prior demonstration of its preclinical benefit in immunogenic studies, would not be practical, as it might hamper the viability of the bacteria, and consequently affect the results. Second, information regarding the challenge in control animals, compared to animals inoculated with the vector, was not included in the present experimental design. If we have an additional group with no vector upon Brucella infection, we can infer any effect of the sole vector upon Brucella infection. However, such additional group (no vector upon Brucella infection) cannot be used directly with statistics for comparing our developed vaccine groups, because our vaccine-treated groups should be compared with vector-treated Brucella infection group, to demonstrate vaccine efficacy. Although in the current study, we tested the safety of the
vaccine in the uninfected goat, it is worthwhile in future study to determine any effect of the vector upon Brucella infection. Third, another limitation was the limited sample size used, due to facility (BL-3) issues. Study with 30 goats composing six groups should be performed at the same time in ABL-3 facility, since the virulent challenge strain 544 infection study should be performed in our ABL-3 facility with limited space. Furthermore, the main concern before the study was whether successful delivery of each of the individual Brucella antigens to the host would be attained, and so the sample size was not of major consideration. This study was the first attempt using selected individual vectored antigens (BLS, Omp19, PrpA, and SOD) in goat model; even though success (efficacy) was preconceived (following the study in mice model), similar results could not be assured in a goat model. Therefore, more additional studies are necessary to assess the immunodynamics of this vaccine in the natural host using the Salmonella delivery system at different doses and/or time-points of immunization, using a large sample size.

This study delineated that to some extent, a level of protection was reached by selected antigens (BLS, Omp19, PrpA, and SOD) in a goat model. This indicates that Salmonella Typhimurium delivered Brucella immunogens successfully, inducing an antigen-specific immune response. The efficacy of the vaccine candidate in the natural host (caprine) was also demonstrated. Future studies of different injection routes, dosages, and times of vaccine administration in the goat model should be considered in a larger sample size.

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