J Vet Sci. 2021 Mar;22(2):e15
https://doi.org/10.4142/jvs.2021.22.e15
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Original Article
Zoonotic Disease

Check for updates

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 Ochirkhuyag** [1], **Eun-Chae Yu** [2], **Young-Jee Kim** [2], **Yoonhwan Yeo** [2], **Myeon-Sik Yang** [1], **Sang-Seop Han** [2], **John Hwa Lee** [1], **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

OPEN ACCESS

Received: Sep 18, 2020
Revised: Nov 28, 2020
Accepted: Jan 4, 2021

***Corresponding author:**
Bumseok Kim
College of Veterinary Medicine, Jeonbuk National University, 79 Gobong-ro, Iksan 54596, Korea.
E-mail: bskims@jbnu.ac.kr

[†]Mwense Leya and Won Kyong Kim contributed equally to this work.

ORCID iDs
Mwense Leya
https://orcid.org/0000-0003-2304-4551
Won Kyong Kim
https://orcid.org/0000-0003-3015-0416
Enkhsaikhan Ochirkhuyag
https://orcid.org/0000-0001-5176-0868
Eun-Chae Yu
https://orcid.org/0000-0002-2237-0139
Young-Jee Kim
https://orcid.org/0000-0003-0836-6608

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

Yoonhwan Yeo
https://orcid.org/0000-0002-2018-6063
Myeon-Sik Yang
https://orcid.org/0000-0003-1858-3729
Sang-Seop Han
https://orcid.org/0000-0002-0809-6540
John Hwa Lee
https://orcid.org/0000-0002-2028-2207
Dongseob Tark
https://orcid.org/0000-0001-7499-4253
Jin Hur
https://orcid.org/0000-0003-2658-0747
Bumseok Kim
https://orcid.org/0000-0003-0392-2513

Funding

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant No.: HI16C2130) and supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2019R1A6A1A03033084).

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Hur J, Lee JH, Kim WK; Data curation: Kim WK, Mwense L; Formal analysis: Kim WK, Mwense L, Kim BS; Funding acquisition: Hur J, Lee JH, Kim BS; Investigation: Kim WK, Mwense L, Ochirkhuyag E, Yu EC, Kim YJ, Yeo Y, Yang MS, Tark D, Kim BS; Methodology: Kim WK, Mwense L; Project administration: Hur J, Lee JH, Kim BS, Tark D, Han SS; Resources: Hur J, Lee JH, Kim BS, Tark D; Software: Kim WK, Mwense L, Hur J; Supervision: Kim BS, Tark D, Hur J, Lee JH; Validation: Kim BS, Hur J; Writing - original draft: Mwense L; Writing - review & editing: Mwense L, Kim BS.

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 placenta localization, trophoblast tropism, and subsequent abortion, making it of the uttermost 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 (O_2^-) radicle into ordinary oxygen (O_2) and hydrogen peroxide (H_2O_2), as a consequence of the phagocytosis of macrophages [21,22].

We evaluated the level of protection conferred by four *Salmonell*a 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 5×10^8 colony forming units (CFU)/mL [23]. A rough *Salmonella* Typhimurium subspecies appointed JOL1800 was electrophoresed with pET28A and pJHL65 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 pJHL-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 5×10^9 CFU/mL in 1 mL. Challenge of all groups was done at 6 weeks after vaccination with *Brucella* strain- HJL254 (5×10^8 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^-, *ompT*, *hsdS*$_B$(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	[10]
Salmonella Typhimurium		
JOL1800	JOL912 *Δrfal;* O-antigen deficient rough strain, bacteria delivery vector	[9]
JOL1818	JOL1800 with pJHL65 plasmid; *Salmonella* vector control	[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)	[10]
Plasmids		
pET28a	IPTG-inducible expression vector; Km^r	Novagen
pJHL65	Asd^+, pBR *ori*, β-lactamase signal sequence-based periplasmic secretion plasmid, 6 x His tag	[24]

https://doi.org/10.4142/jvs.2021.22.e15

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	5×10^9 CFU/mL each (pJHL-65)	6 weeks post-immunization (5×10^8 CFU/mL in 100 μL conjunctival inoculation)
B	RSrBL*	5×10^9 CFU/mL each of BLS	
C	RSrBL*	5×10^9 CFU/mL each of Omp19	
D	RSrBL*	5×10^9 CFU/mL each of PrpA	
E	RSrBL*	5×10^9 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, and PrpA.
SOD, superoxide dismutase; Omp19, outer-membrane protein 19; BLS, *Brucella* lumazine synthase; PrpA, proline racemase subunit A.

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 3. Primer sequence of real-time polymerase chain reaction

Genome	Primer	Length (bp)	Temp (°C)	GC (%)
IS711genetic element	F; 5′-CATGCGCTATGTCTGGTTAC-3′	20	55.8	50.0
	R; 5′-GGCTTTTCTATCACGGTATTC-3′	21	53.5	42.9
Goat GAPDH	F; 5′- ATCTCGCTCCTGGAAGATG-3′	19	55.3	52.6
	R; 5′-TCGGAGTGAACGGATTCG-3′	18	55.9	55.6

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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 $A_{260}/A_{280\,nm}$ 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).

https://doi.org/10.4142/jvs.2021.22.e15

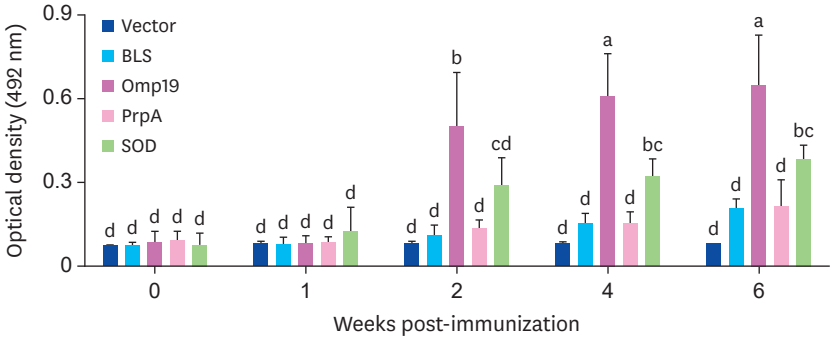

Fig. 1. Antibody responses against BLS, Omp19, PrpA, and SOD antigens. BLS, Omp19, PrpA, and SOD were inoculated with 5×10^9 CFU/mL of each of the four *Brucella* antigens in 1 mL, respectively. Omp19 and SOD showed significantly higher serum IgG titers at (2, 4, and 6) weeks post-vaccination, compared to vector control group A. The absorbance level of serum IgG was expressed as (OD_{492}) ± 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.

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 5×10^9 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 the vector 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

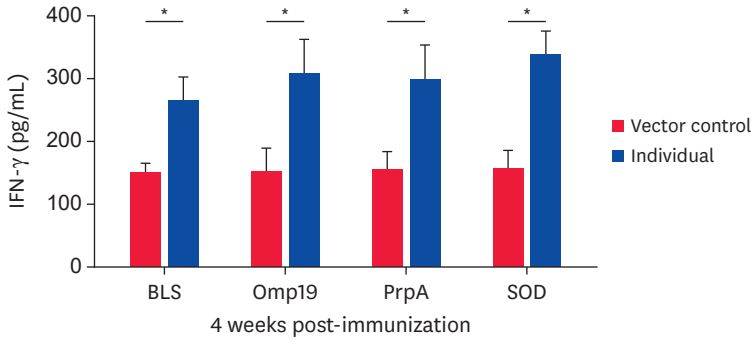

Fig. 2. Vaccination targeting individual antigens induces secretion of goat IFN-γ in peripheral blood mononuclear cells at 4 weeks post-vaccination. SOD, Omp19, PrpA, and BLS individual groups in response to individual antigen showed significantly higher IFN-γ (pg/mL) production, compared to that in 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 ($p < 0.01$).

https://doi.org/10.4142/jvs.2021.22.e15

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.

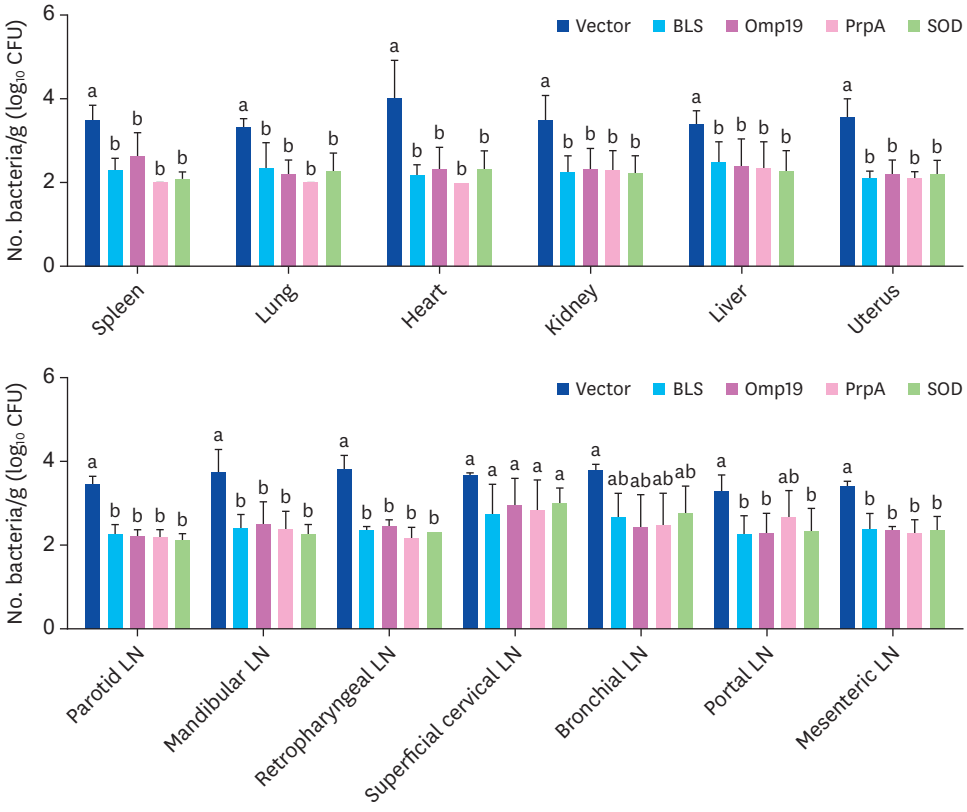

Fig. 3. Bacterial proliferation in various organs and lymph nodes of goats challenged with 5×10^8 CFU/mL of the highly infective *Brucella* 544-HJL254. *Brucella* colonization rates to determine the effectiveness of immunization were determined by colony-forming unit ($\log_{10}$ CPU/g of tissue) at 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.

https://doi.org/10.4142/jvs.2021.22.e15

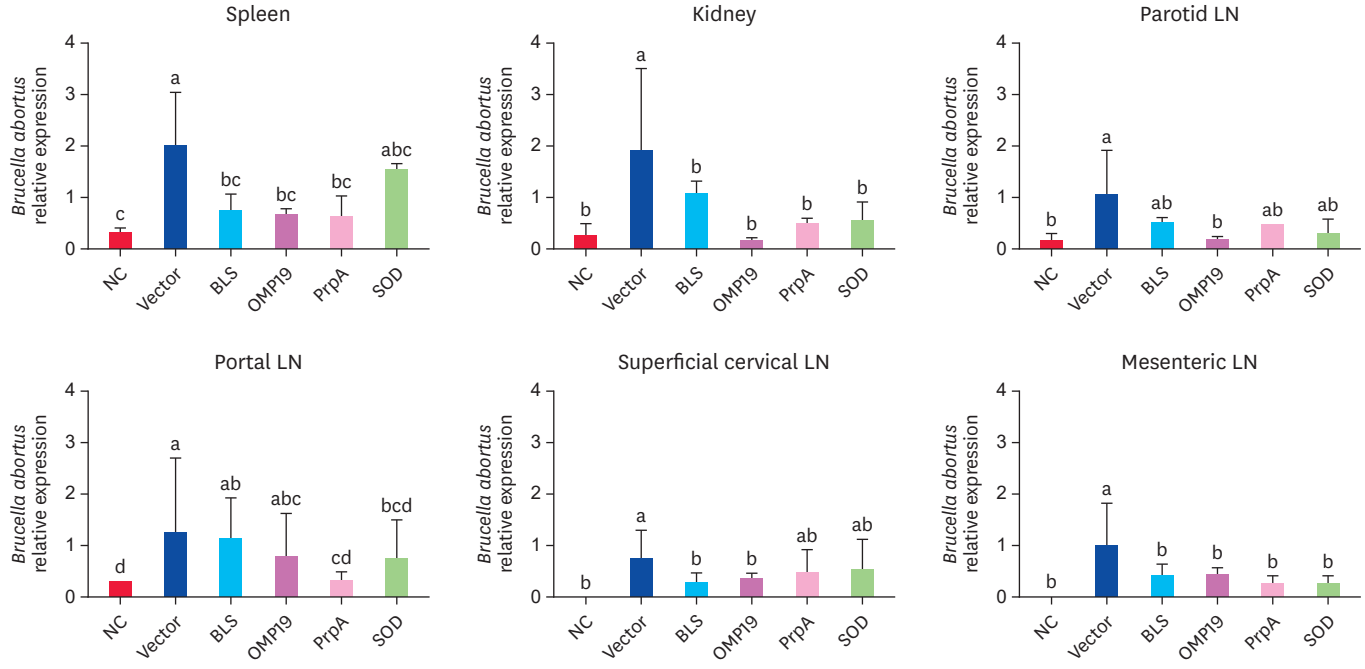

Fig. 4. Real-time PCR data showing amounts of Brucella antigens expressed in the tissue samples. Expression levels of *Brucella* antigens were decreased in the spleen, kidney, parotid LN, portal LN, superficial cervical LN, and mesenteric LN of BLS, Omp19, PrpA, and SOD, compared to those in vector control. Results represent all goat means in each group, and the error bars denote standard deviation. Dissimilar letters stipulate significant differences, as stated by Duncan's multiple range test. A p value of $p < 0.05$ was considered statistically significant.
NC, negative control group; 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, portal LN, parotid 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

https://doi.org/10.4142/jvs.2021.22.e15

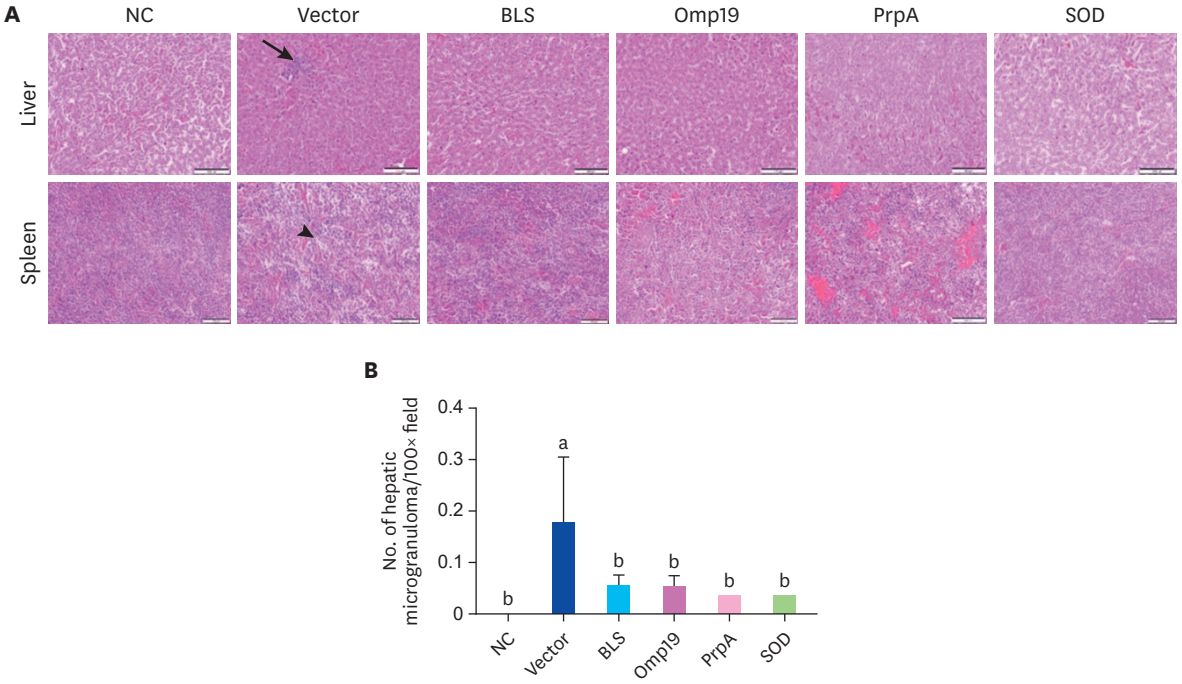

Fig. 5. Microphotograph of liver and spleen tissues. (A) Hepatic lesions observed were mainly microgranulomas (arrow). Splenic sections showed an expansion of trabeculae (arrowhead). (B) Comparison of microgranuloma was done by taking account of the number of foci in 10 microscopic fields/tissue samples. The microgranulomas/100 × field in the vector group was significantly higher than that in the vaccinated groups. Results represent all goat means in each group, and the error bars denote standard deviation. Dissimilar letters stipulate significant differences, as stated by Duncan's multiple range test. A p value of $p < 0.05$ was considered statistically significant (Hematoxylin and eosin staining; Scale bar, 100 μm).
NC, negative control group; BLS, *Brucella* lumazine synthase; Omp19, outer-membrane protein 19; PrpA, proline racemase subunit A; and SOD, superoxide dismutase; LN, lymph node.

(goat) [9,10]. 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 [26].

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 [27]. 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 [28]. 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.

REFERENCES

1. Poester FP, Samartino LE, Santos RL. Pathogenesis and pathobiology of brucellosis in livestock. Rev Sci Tech. 2013;32(1):105-115.
PUBMED | CROSSREF
2. Spink W. The Evolution of the Concept that Brucellosis is a Disease of Animals and Man. The Nature of Brucellosis Minneapolis. Lund: Lund Press Inc.; 1956, 3-27.
3. McDuff CR, Jones LM, Wilson JB. Characteristics of *Brucella* phage. J Bacteriol. 1962;83(2):324-329.
PUBMED | CROSSREF
4. Payne JM. The pathogenesis of experimental brucellosis in the pregnant cow. J Pathol Bacteriol. 1959;78(2):447-463.
PUBMED | CROSSREF
5. Nicoletti P. The epidemiology of bovine brucellosis. Adv Vet Sci Comp Med. 1980;24:69-98.
PUBMED
6. Oyewumi MO, Kumar A, Cui Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines. 2010;9(9):1095-1107.
PUBMED | CROSSREF
7. Stabel TJ, Mayfield JE, Morfitt DC, Wannemuehler MJ. Oral immunization of mice and swine with an attenuated *Salmonella* choleraesuis [delta cya-12 delta(crp-cdt)19] mutant containing a recombinant plasmid. Infect Immun. 1993;61(2):610-618.
PUBMED | CROSSREF
8. Hur J, Lee JH. Enhancement of immune responses by an attenuated *Salmonella* enterica serovar Typhimurium strain secreting an *Escherichia coli* heat-labile enterotoxin B subunit protein as an adjuvant for a live *Salmonella* vaccine candidate. Clin Vaccine Immunol. 2011;18(2):203-209.
PUBMED | CROSSREF
9. Lalsiamthara J, Lee JH. *Brucella* lipopolysaccharide reinforced *Salmonella* delivering *Brucella* immunogens protects mice against virulent challenge. Vet Microbiol. 2017;205:84-91.
PUBMED | CROSSREF
10. Kim WK, Moon JY, Kim S, Hur J. Comparison between immunization routes of live attenuated *Salmonella* Typhimurium strains expressing BCSP31, Omp3b, and SOD of *Brucella abortus* in murine model. Front Microbiol. 2016;7:550.
PUBMED | CROSSREF

11. Ackermann MR, Cheville NF, Deyoe BL. Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19. Vet Pathol. 1988;25(1):28-35.
PUBMED | CROSSREF
12. Paixão TA, Roux CM, den Hartigh AB, Sankaran-Walters S, Dandekar S, Santos RL, et al. Establishment of systemic *Brucella melitensis* infection through the digestive tract requires urease, the type IV secretion system, and lipopolysaccharide O antigen. Infect Immun. 2009;77(10):4197-4208.
PUBMED | CROSSREF
13. Hong CB, Donahue JM, Giles RC Jr, Poonacha KB, Tuttle PA, Cheville NF. *Brucella abortus*-associated meningitis in aborted bovine fetuses. Vet Pathol. 1991;28(6):492-496.
PUBMED | CROSSREF
14. Baldwin CL, Winter AJ. Macrophages and *Brucella*. Immunol Ser. 1994;60:363-380.
PUBMED
15. Liautard JP, Gross A, Dornand J, Köhler S. Interactions between professional phagocytes and *Brucella* spp. Microbiologia 1996;12(2):197-206.
PUBMED
16. Skendros P, Boura P. Immunity to brucellosis. Rev Sci Tech. 2013;32(1):137-147.
PUBMED | CROSSREF
17. Velikovsky CA, Cassataro J, Giambartolomei GH, Goldbaum FA, Estein S, Bowden RA, et al. A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. Infect Immun. 2002;70(5):2507-2511.
PUBMED | CROSSREF
18. Spera JM, Ugalde JE, Mucci J, Comerci DJ, Ugalde RA. A B lymphocyte mitogen is a *Brucella abortus* virulence factor required for persistent infection. Proc Natl Acad Sci U S A. 2006;103(44):16514-16519.
PUBMED | CROSSREF
19. Pasquevich KA, Estein SM, García Samartino C, Zwerdling A, Coria LM, Barrionuevo P, et al. Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection. Infect Immun. 2009;77(1):436-445.
PUBMED | CROSSREF
20. Cloeckaert A, Vizcaíno N, Paquet JY, Bowden RA, Elzer PH. Major outer membrane proteins of *Brucella* spp.: past, present and future. Vet Microbiol. 2002;90(1-4):229-247.
PUBMED | CROSSREF
21. Oñate AA, Vemulapalli R, Andrews E, Schurig GG, Boyle S, Folch H. Vaccination with live *Escherichia coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. Infect Immun. 1999;67(2):986-988.
PUBMED | CROSSREF
22. Tabatabai LB, Pugh GW Jr. Modulation of immune responses in Balb/c mice vaccinated with *Brucella abortus* Cu-Zn superoxide dismutase synthetic peptide vaccine. Vaccine. 1994;12(10):919-924.
PUBMED | CROSSREF
23. Lee JJ, Lim JJ, Kim DG, Simborio HL, Kim DH, Reyes AW, et al. Characterization of culture supernatant proteins from *Brucella abortus* and its protection effects against murine brucellosis. Comp Immunol Microbiol Infect Dis. 2014;37(4):221-228.
PUBMED | CROSSREF
24. Lalsiamthara J, Kamble NM, Lee JH. A live attenuated *Salmonella* Enteritidis secreting detoxified heat labile toxin enhances mucosal immunity and confers protection against wild-type challenge in chickens. Vet Res (Faisalabad). 2016;47(1):60.
PUBMED | CROSSREF
25. Neta AVC, Mol JP, Xavier MN, Paixão TA, Lage AP, Santos RL. Pathogenesis of bovine brucellosis. Vet J. 2010;184(2):146-155.
PUBMED | CROSSREF
26. Corbel MJ. Brucellosis in humans and animals: World Health Organization; 2006.
27. Dorneles EM, Sriranganathan N, Lage AP. Recent advances in *Brucella abortus* vaccines. Vet Res (Faisalabad). 2015;46(1):76.
PUBMED | CROSSREF
28. Leya M, Kim WK, Cho JS, Yu EC, Kim YJ, Yeo Y, et al. Vaccination of goats with a combination *Salmonella* vector expressing four *Brucella* antigens (BLS, PrpA, Omp19, and SOD) confers protection against *Brucella abortus* infection. J Vet Sci. 2018;19(5):643-652.
PUBMED | CROSSREF

29. Lalsiamthara J, Won G, Lee JH. Effect of immunization routes and protective efficacy of *Brucella* antigens delivered via *Salmonella* vector vaccine. J Vet Sci. 2018;19(3):416-425.
PUBMED | CROSSREF

30. Gomez G, Adams LG, Rice-Ficht A, Ficht TA. Host-*Brucella* interactions and the *Brucella* genome as tools for subunit antigen discovery and immunization against brucellosis. Front Cell Infect Microbiol. 2013;3:17.
PUBMED | CROSSREF

31. Skendros P, Pappas G, Boura P. Cell-mediated immunity in human brucellosis. Microbes Infect. 2011;13(2):134-142.
PUBMED | CROSSREF

32. Yingst S, Hoover DL. T cell immunity to brucellosis. Crit Rev Microbiol. 2003;29(4):313-331.
PUBMED | CROSSREF

33. Baldwin CL, Goenka R. Host immune responses to the intracellular bacteria Brucella: does the bacteria instruct the host to facilitate chronic infection? Crit Rev Immunol. 2006;26(5):407-442.
PUBMED | CROSSREF