



Protective efficacy of a canine brucellosis vaccine candidate based on live attenuated *Salmonella* expressing recombinant *Brucella* BCSP31, Omp3b and SOD proteins in Beagles

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ABSTRACT. The aim of this study was to establish a proof-of-concept of protective efficacy of *Salmonella*-based *B. abortus* vaccine candidate in Beagles. Group A Beagles (n=10) were subcutaneously (SC) inoculated with *S. Typhimurium* delivery strain containing pMMP65 (vector to deliver antigens) only as vector control. Group B Beagles (n=10) were SC vaccinated with the mixture of the three *Salmonella* delivery strains expressing the recombinant *B. abortus* BCSP31, Outer membrane protein 3b (Omp3b), and superoxide dismutase (SOD) proteins, respectively. No *Salmonella* delivery strains were isolated from all tissues tested. Serum IgG, interleukin-4, tumor necrosis factor- α , and interferon- γ concentrations were significantly higher in group B than in group A. Following intraconjunctival challenge with *B. abortus* 544, among 5 group B Beagles, the challenge strain was isolated from mandibular, and retropharyngeal lymph nodes of three Beagles, and no isolates were observed from all tissues of two Beagle. However, the challenge strains were detected from spleen, uterus (except two Beagles), and mandibular, prescapular, retropharyngeal, and superficial inguinal lymph nodes of all group A Beagles. These results suggest that the mixture of three *S. Typhimurium* delivery strains be a good vaccine candidate against brucellosis by *B. abortus* in dogs. Further investigations are needed to improve the protective efficacy of the *Salmonella*-based *B. abortus* vaccine candidate and explore its practical application in dogs.

KEY WORDS: attenuated *Salmonella* Typhimurium, *Brucella abortus*, dog, immunization, *S. Typhimurium*-based *B. abortus* vaccine

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Canine brucellosis is usually caused by *Brucella canis* (*B. canis*). In some countries, such as U.S.A. and Canada, brucellosis in dogs, kept in close association with cattle, can also be caused by *B. abortus* [6, 7, 10, 15, 29]. The most likely means of cattle to dog transmission may be by contact with aborted fetus or placental membranes infected with *B. abortus* [5, 15, 29]. In addition, dog to cattle transmission of *B. abortus* has been reported in experiments, using *B. abortus*-infected dogs imprisoned with *Brucella* free cattle and under field conditions [15, 29].

Cell-mediated immune (CMI) response is crucial to eradicate *Brucella* strains from the host organisms [33], because *Brucella* strains invade and multiply intracellularly in macrophages within the host immune system [4, 9]. In particular, the Th1-type immune cells mediated by interferon gamma (IFN- γ) is crucial to defend *Brucella* infection [39]. Many various types of vaccines have been developed to prevent brucellosis by *B. abortus*, but live, attenuated *Brucella* strains such as *B. abortus* strain 19 and strain RB51 have been used as commercially available vaccines in field. However, these strains can revert into pathogenic strains and some strains may interfere with the diagnosis [2, 28]. Therefore, there is necessary to develop better and safer vaccine.

Live, attenuated *Salmonella* strains have been known to be a useful delivery system for carrying protective antigens of other pathogens [16, 17]. A protein, BCSP31, purified from *B. abortus* strain 19 designated has been reported to serve as a protective subunit vaccine [34, 35]. Outer membrane protein 3b (Omp3b) has been known to be one of the most abundant and immunogenic *Brucella* proteins [14, 38]. Cu/Zn superoxide dismutase (SOD) has been also reported as one of virulence factors scavenging harmful oxygen radicals mediated during phagocytosis by macrophages within host immune system [36].

In a previous study [21], *S. Typhimurium* strains expressing the recombinant *B. abortus* BCSP31, Omp3b, and SOD proteins were evaluated a vaccine candidate in a murine model. Intraperitoneal immunization with the mixture of the *S. Typhimurium*-based

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B. abortus vaccine strains induced adequate humoral and CMI responses and effectively protected against *B. abortus* infection in mice. Although the most important agent for canine brucellosis is *B. canis*, *B. abortus* is known to infect dogs. Especially, the protective efficacy of the *S. Typhimurium*-based strains against *B. abortus* infections were mainly evaluated as *B. abortus* vaccine candidate [21, 24, 35, 36]. At the first time, the present study aimed to establish a proof-of-concept of the protective efficacy of *S. Typhimurium* delivery strains expressing recombinant *B. abortus* BCSP31, Omp3b, and SOD proteins as a *B. abortus* vaccine candidate in Beagles. Beagles were subcutaneously immunized with the mixture of the live, attenuated *S. Typhimurium* strains expressing recombinant *B. abortus* BCSP31, Omp3b, and SOD proteins. The vaccinated Beagles exhibited robust humoral and CMI responses, thereby vaccine Beagles were effectively protected from virulent *B. abortus* infection.

MATERIALS AND METHODS

Animals, and ethics statement

A total of 20 female Beagles aged 6 months were distributed into two equal groups. All Beagles were purchased from tested brucellosis-free flocks to use in this study. All Beagles used for this study were again confirmed seronegative for brucellosis in the laboratory by Rose Bengal test (RBT) for *B. abortus*, and 2-mercaptoethanol rapid slide agglutination test (2ME-RSAT) for *B. canis*. All animal experiments performed in this study received ethical approval (CBU 2015-052) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean council on Animal Care.

Bacterial strains, and growth conditions

Live, attenuated *S. Typhimurium* strains expressing the recombinant *B. abortus* BCSP31, Omp3b, and SOD proteins, which have been constructed and used as a vaccine candidate for mouse brucellosis in a previous study [21], were evaluated as a vaccine candidate for canine brucellosis in the present study. In addition, attenuated *S. Typhimurium* delivery strain containing pMMP65 vector only, was used as vector control strain [21]. *B. abortus* strain 544 was used as the virulent challenge strain [23]. The recombinant BCSP31, Omp3b, and SOD proteins were overexpressed and purified from HJL906, HJL904, and HJL908 strains, respectively [21]. Except the virulent challenge strain, all strains were grown in Luria–Bertani broth (LB; Becton, Dickinson and Co., Sparks, MD, U.S.A.) or on LB agar. Virulent challenge strain was grown on Brucella broth or Brucella agar. All strains were cultured at 37°C.

Preparation of individual recombinant proteins

The recombinant BCSP31, Omp3b, and SOD proteins purified from HJL906, HJL904, and HJL908, respectively [21], and each recombinant protein was used as coating antigens of ELISA to evaluate serum IgG titers and as re-stimulating protein for peripheral blood mononuclear cells (PBMCs) in the present study.

Preparation of S. Typhimurium-based B. abortus vaccine candidate

Live, attenuated *S. Typhimurium* strains expressing the recombinant *B. abortus* BCSP31, Omp3b, and SOD proteins of *B. abortus* were prepared as the method described in previous study [19]. The mixture of three *S. Typhimurium* strains was as *S. Typhimurium*-based *B. abortus* vaccine candidate in Beagles.

Immunization of Beagles and sample collection

Twenty 6 months of age female Beagles were divided into two equal groups (n=10 Beagles per group). All Beagles were subcutaneously (SC) primed at 6 months of age [0 weeks post prime immunization (WPPI)], and were SC boosted at 3 WPPI. Group A Beagles were inoculated with approximately 3.0×10^9 colony-forming units (CFU)/ml of *S. Typhimurium* delivery strain containing pMMP65 only in 1 ml as the vector control. Group B Beagles were immunized with approximately 3.0×10^9 CFU/ml of the mixture (equal CFU counts of each strain) of three *S. Typhimurium*-based *B. abortus* vaccine strains in 1.0 ml. Blood samples were collected at 0, 3 (before booster vaccination), and 6 (before challenge) WPPI to evaluate the serum IgG titers. All samples were stored at -70°C until use.

Assessment of vaccine safety

The safety of the live and attenuated *Salmonella*-based *B. abortus* vaccine candidate in Beagles was assessed. Daily clinical thermometry observation of the Beagles in the immunized group was performed during 6 WPPI. At 6 WPPI, 5 Beagles per group were euthanized aseptically, from which samples of the lymph nodes (mandibular, prescapular, retropharyngeal, and superficial inguinal), and parenchymal organs (spleen, kidney, and uterus) were collected. In total, 7 organs were sampled from each animal. The tissue homogenates were plated onto Brilliant Green Agar (Becton, Dickinson and Co.) and incubated under aerobic conditions for 48 hr at 37°C. Isolation of *Salmonella* delivery strains was confirmed by PCR using OMPC- and TYPH-specific primer set described in a previous study [18], and BCSP31-, Omp3b-, and SOD-specific primers described in a previous study [20, 21].

Immune response measurement by ELISA

Standard ELISA was performed to evaluate the immune responses against the recombinant BCSP31, Omp3b, and SOD proteins in serum samples obtained from Beagles according to slightly modified method described previously [21]. Briefly, Ninety-six well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with pretitrated recombinant BCSP31 (4 µg/ml), Omp3b (2

$\mu\text{g/ml}$), or SOD ($4 \mu\text{g/ml}$) proteins in phosphate-buffered saline (PBS), blocked for 1 hr using PBS containing 1% bovine serum albumin (diluent; $200 \mu\text{l/well}$), and washed with PBS containing 0.05% Tween-20. Serum samples were diluted as 1:100 in diluent. The plates were treated with horseradish peroxidase-conjugated goat anti-dog IgG antibody (Bethyl Lab Inc., Montgomery, TX, U.S.A.). Enzymatic reactions were carried out by adding substrate containing o-phenylenediamine (Sigma-Aldrich, St. Louis, MO, U.S.A.) and were measured through an automated ELISA spectrophotometer (Thermo Scientific Multiskan GO, Thermo Fisher Scientific Oy, Ratastie, Vantaa, Finland) at 492 nm. The ELISA results are expressed as mean \pm standard deviation.

Preparation of Peripheral blood mononuclear cells (PBMCs)

At 6 WPPI, blood was obtained from all Beagles and placed into an acid-citrate dextrose solution. PBMCs were enriched by density centrifugation using a Ficoll sodium diatrizoate gradient (Sigma Diagnostics, Inc., St. Louis, MO, U.S.A.). PBMCs were diluted in RPMI 1640 medium to 2.5×10^6 viable cells per ml as determined by trypan blue dye exclusion. The PBMCs (5×10^5 cells/well) were stimulated *in vitro* as previously described [21, 37] with media as unstimulated control, Concanavalin A ($0.5 \mu\text{g/well}$) as positive control, or with each recombinant BCSP31, *Omp3b*, and SODC protein ($8 \mu\text{g/well}$), and incubated at 37°C under 5% CO_2 . Culture supernatants were collected after 72 hr of re-stimulation and stored at -70°C till used for cytokine measurement.

Cytokines measurement by ELISA

ELISA was used to measure the concentration of IL-4, TNF- α , and IFN- γ in the culture supernatants using DuoSet canine cytokine reagent set according to the manufacturer's instructions (R & D system, Minneapolis, MN, U.S.A.). Results of the ELISA are expressed as the mean concentration \pm standard deviation.

Challenge experiments

For challenge experiments, the challenge strain, strain 544, was prepared. Briefly, the strain was grown in *Brucella* broth at 37°C for 24 hr and resuspended to approximately 2×10^8 CFU/ml. At 6 WPPI, the Beagles were fasted for 16 hr prior to being anesthetized with carfentanil (Wildnil; 0.007 to 0.008 mg/kg of body weight; Wildlife Pharmaceuticals, Ft. Collins, CO, U.S.A.) and xylazine (0.10 to 0.13 mg/kg; Mobay Corp., Shawnee, KS, U.S.A.) administered intramuscularly. At 8 weeks after intraconjunctival challenge with approximately 2×10^7 CFU of *B. abortus* strain 544/Beagle ($50 \mu\text{l}$ of inoculum per eye), all Beagles were euthanized by intravenous administration of sodium pentobarbital. Beagle samples obtained at necropsy included lymph nodes (mandibular, prescapular, retropharyngeal, and superficial inguinal), spleen, kidney, and uterus. Tissue samples were triturated in 0.15 M NaCl by using a tissue grinder and plated on Farrell's medium containing 5% bovine serum, antibiotics and ethyl-violet (*Brucella*-selected agar) [30]. Isolation of *Brucella* bacteria from lymph nodes and subsequent biochemical identification of isolates were performed by conventional methods [29]. Briefly, each node was trimmed of excess fat, dipped in 95% ethanol, and flamed to remove surface contaminants. The lymph nodes were trimmed into small pieces, were weighed and were homogenized with an equal volume of sterile PBS in a stomacher blender for ≥ 2 min. The resulting homogenate was then swabbed onto Farrell's medium with 5% bovine serum, antibiotics and ethyl-violet. All agar plates were incubated at 37°C and 10% CO_2 for ≥ 10 days. *B. abortus* bacteria were identified on the basis of colony morphology, growth characteristics, and were confirmed by PCR using the *B. abortus*-specific primer (5'-GACGAACGGAATTTTCCAATCCC-3') and IS711-specific primer (5'-TGCCGATCACTTAAGGGCCTTCAT-3') described in previously study [10, 11]. If no colony detected on the *Brucella*-selected agar, then the number of viable challenge strain from each tissue of the Beagles means $<10^2$ CFU.

Statistical analysis

To observe differences among different vaccinated groups, absorbance data values of ELISA were used for the analysis of variance with post-hoc turkey for pair-wise comparison using SPSS version 16.0 (SPSS Inc., Chicago, IL, U.S.A.). Kruskal-Wallis followed by Dunn's procedure for multiple comparisons between groups was carried out to compare the log transformed CFU values in organs obtained from each immunized group of Beagles with the respective control group for bacterial challenge experiments. Statistical significance was determined at ($P < 0.05$).

RESULTS

Safety of the vaccine

SC immunization of Beagles with the mixture of *S. Typhimurium*-based *B. abortus* vaccine strains did not cause any negative impact on the overall clinical status (behavior, appetite, etc.) during our study period (6 WPPI). The body temperature of all the Beagles remained within normal limits (38.0 – 39.0°C) (data not shown). Importantly, no side effects and residual numbers of strain were observed in the inoculation site of the immunized beagles. Furthermore, no *Salmonella* delivery strains were isolated from 7 tissues of Beagles in groups A and B tested at 6 WPPI.

Antibody response to recombinant BCSP31, *Omp3b* or SOD protein

The antibody titers against each antigen in the sera are presented in Fig. 1. In Group A, serum IgG titers against each recombinant BCSP31, *Omp3b* and SOD protein were 0.22 ± 0.035 , 0.10 ± 0.015 , and 0.19 ± 0.007 , respectively, at 0 WPPI. The serum IgG titers against each recombinant protein of group A Beagles at 2 and 4 WPPI were maintained similar to those of group A at 0 WPPI. However, the serum IgG titers against each recombinant BCSP31, *Omp3b* and SOD protein of group B Beagles were

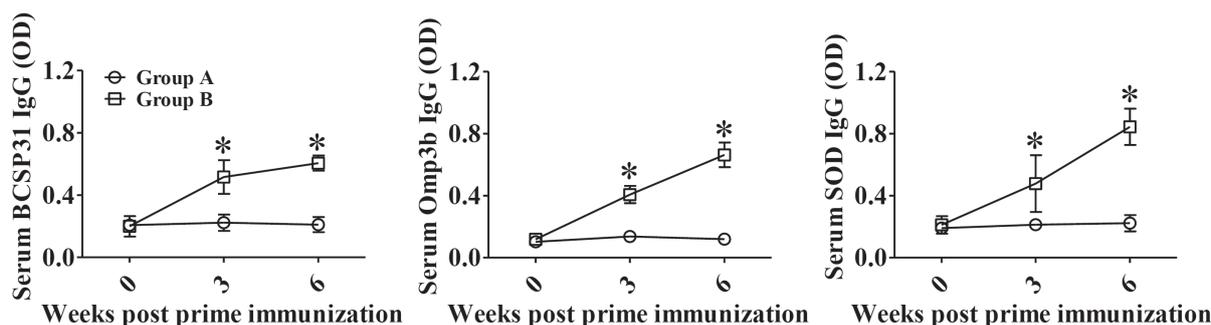


Fig. 1. Antibody titers (optical density) against each recombinant BCSP31, Omp3b, and SOD protein. Group A Beagles were inoculated with attenuated *Salmonella* Typhimurium containing pMMP65 only as a vector control. Group B Beagles were immunized with approximately 3.0×10^9 CFU/ml of the mixture of *S. Typhimurium* delivery strains expressing recombinant BCSP31, Omp3b, and SOD proteins in 1 ml. Data represent the means of all Beagles in each group, and error bars represent the standard deviations (SDs). Asterisks indicate a significant difference between the values obtained for the immunized group and those obtained for the vector control group (* $P < 0.05$).

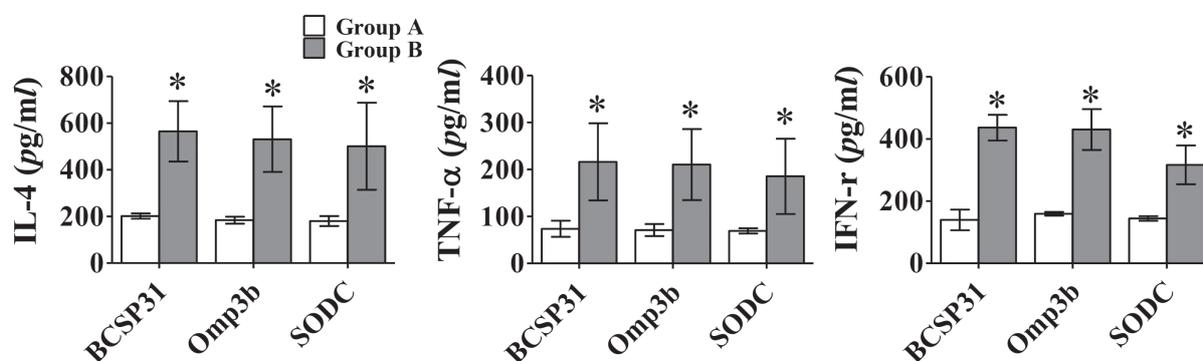


Fig. 2. IL-4, TNF- α , and IFN- γ concentrations (pg/ml) in peripheral blood mononuclear cells at 6 weeks post prime immunization. Groups A, and B are indicated as in Fig. 1. Data represent the means of all Beagles in each group, and error bars indicate the standard deviations (SDs). Asterisks indicate significant differences among the values obtained for each group (* $P < 0.05$).

0.20 ± 0.066 , 0.12 ± 0.010 , and 0.21 ± 0.056 , respectively, at 0 WPPI. The serum titers were increased from 2 WPPI till the end of this study. At 6 WPPI, serum IgG titers to each recombinant protein of group B were 0.61 ± 0.049 , 0.66 ± 0.080 , and 0.84 ± 0.117 , respectively.

Cytokine analysis

IL4, TNF- α , and IFN- γ concentrations in response to recombinant BCSP31, Omp3b, and SOD proteins after re-stimulation with the individual proteins were measured using ELISA kit at 6 WPPI. IL4, TNF- α , and IFN- γ concentrations to each recombinant BCSP31, Omp3b, and SOD protein in the culture supernatant from group B Beagles when PBMCs were re-stimulated with each recombinant protein *in vitro*, were significantly higher than those from group A Beagles (Fig. 2) ($P < 0.05$).

Protective efficacy against *B. abortus* infection

Protective efficacy of the *S. Typhimurium*-based *B. abortus* vaccine candidate against *B. abortus* strain 544 challenge was evaluated by assessing three basic parameters: the effectiveness of vaccination (or degree of protection against infection), the index of infection (the number of *B. abortus* strain 544-infected tissues), and the rates of *Brucella* colonization in tissues. Among 5 Beagles of group B, the *S. Typhimurium*-based *B. abortus* vaccine candidate provided complete protection (effectiveness of vaccination) against *B. abortus* strain 544 challenge in 40% of Beagles. The severity of *B. abortus* strain 544 challenge in group B Beagles as indicated by the index of infection (1.2 ± 1.10 , $P < 0.05$) (Table 1) (Fig. 3A) and rates of *B. abortus* colonization in tissues [0.0 ± 0.0 to 2.5 ± 216 log₁₀ CFU/g of tissue, $P < 0.05$ (Fig. 3B)] were significantly lower than that of the vector control group [index of infection 5.6 ± 0.55 (Table 1) (Fig. 3A); *B. abortus* colonization 1.45 ± 2.05 to 4.72 ± 0.76 log₁₀ CFU/g of tissue (Fig. 3B)].

DISCUSSION

In this study, we experimentally evaluated the protective efficacy of live, attenuated *S. Typhimurium* expressing recombinant

Table 1. Summary of *B. abortus* isolation from culture positive Beagles after challenge with *B. abortus* strain 544 in vector control and *S. Typhimurium*-based vaccinated groups

Group		Specimen							
		Kidney	Spleen	Uterus	Mammary	Mandibular LN ^{a)}	Prescapular LN	Retropharyngeal LN	Superficial inguinal LN
A	A1	- ^{b)}	+ ^{c)}	+	-	+	+	+	+
	A2	-	+	-	-	+	+	+	+
	A3	-	+	+	-	+	+	+	+
	A4	-	+	+	-	+	+	+	+
	A5	-	+	-	-	+	+	+	+
B	B1	-	-	-	-	+	-	+	-
	B2	-	-	-	-	-	-	-	-
	B3	-	-	-	-	+	-	+	-
	B4	-	-	-	-	+	-	+	-
	B5	-	-	-	-	-	-	-	-

a) LN means lymph node. b) “-” means that no colony detected on the *Brucella*-selected agar. c) “+” means that at least one colony of *B. abortus* strain 544 was isolated on the *Brucella*-selected agar.

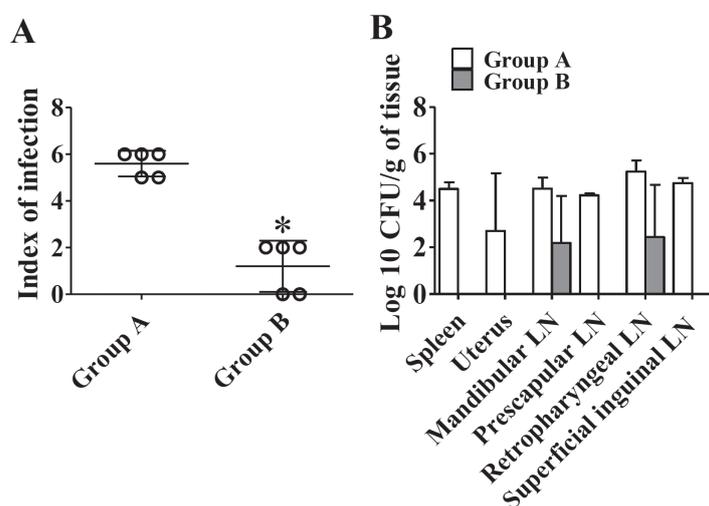


Fig. 3. Index of infection for Beagles challenged with *B. abortus* strain 544 at 6 weeks post prime immunization (A), colonization and incidence of recovery of *B. abortus* strain 544 in tissues (B). Groups A, and B are indicated as in Fig. 1. All Beagles in each group were conjunctively challenged with approximately 2×10^7 CFU of virulent *B. abortus* 544 at 6 weeks post prime immunization. The numbers of viable bacteria recovered from each tissues of Beagles at 8 weeks post challenge are shown. LN, lymph node.

BCSP31, *Omp3b*, and *SOD* proteins of *B. abortus* against *B. abortus* infection as a *B. abortus* vaccine candidate in a dog model. In a previous study [21], live, attenuated *S. Typhimurium* delivery strains expressing recombinant *B. abortus* BCSP31, *Omp3b*, and *SOD* proteins were constructed as a *B. abortus* vaccine candidate. Furthermore, effective protection of the *S. Typhimurium*-based *B. abortus* vaccine candidate against *B. abortus* infection were observed in murine model [21]. Accordingly, we thought that the *S. Typhimurium*-based *B. abortus* vaccine candidate could also help in control canine brucellosis caused by *B. abortus*, although brucellosis by *B. canis* in dogs is mainly caused. Therefore, in this study, our goal was to evaluate safety and protective efficacy of *S. Typhimurium*-based *Brucella* vaccine candidate against brucellosis by *B. abortus* but not *B. canis* in Beagles. As expected *S. Typhimurium*-based *B. abortus* vaccine candidate was found safe in clinical thermometry in Beagles. Notably, any inflammatory infiltrates were not observed in Beagles immunized SC with *S. Typhimurium*-based *B. abortus* vaccine candidate. Moreover, *Salmonella*-based *B. abortus* vaccine strains were not isolated from any tissues of all Beagles tested at 6 WPPI. These results revealed that our vaccine candidate is safe in Beagles.

We analyzed antigen-specific humoral and CMI responses in Beagles immunized SC with the *S. Typhimurium*-based *B. abortus* vaccine candidate. Strong serum IgG is necessary to protect hosts from intracellular pathogens infections [1, 12, 31], because serum IgG helps to eradicate the intracellular pathogens from the blood and to improve the phagocytosis efficacy within their immune system through opsonization [12, 25]. In present study, serum IgG titers were investigated in Beagles immunized SC with the *S. Typhimurium*-based *B. abortus* vaccine candidate. The serum IgG titers from the candidate-immunized Beagles were significantly increased compared to those from the un-immunized Beagles at 6 WPPI. The result shows that protective serum IgG

titers against each recombinant BCSP31, Omp3b, and SOD protein can be induced by each recombinant protein delivered from each *S. Typhimurium*-based *B. abortus* vaccine strain without antagonism between expressed-recombinant proteins. Furthermore, induction of cytokines to each recombinant protein from PBMCs collected from all Beagles immunized with the *S. Typhimurium*-based *B. abortus* vaccine candidate, and re-stimulated *in vitro* with each the recombinant protein indicated a powerful Th2 type immunity (IL-4 levels represent the Th2 bias of immunity). These results show that serum IgG, and IL-4, which are associated with enhancing serum IgG response, were significantly increased by the *S. Typhimurium*-based *B. abortus* vaccine candidate.

CMI cells play a major role in clearance of *B. abortus* species, because *B. abortus* is a facultative intracellular pathogen [4, 19, 27, 40]. Therefore, in present study, CMI using TNF- α , and IFN- γ cytokines ELISA kits were evaluated. After all, showed high TNF- α , and IFN- γ concentrations to each recombinant protein in the culture supernatant when PBMCs re-stimulated with each recombinant protein *in vitro*, giving an indication of CMI response, which was reflected in levels of protection induced by *S. Typhimurium*-based *B. abortus* vaccine candidate. These results show that SC immunization with the *S. Typhimurium*-based *B. abortus* vaccine candidate effectively produces the cytokines, which are related to CMI response.

Brucella canis cause abortions, stillbirths, epididymitis, orchitis, and sperm abnormalities in dogs [8]. However, criteria for clinical signs of brucellosis by *B. abortus* in dogs are not well-settled [3, 15, 29], uncommon, and variable [22, 29], but abortion, epididymitis, and arthritis have been described [7, 13, 26, 32]. A main difficulty that has interrupted the development of vaccines to protect host from *Brucella* infections, is the shortage of the precise proof of effective protection. Isolation of challenge strain, *B. abortus* strain 544, was attempted to in kidney, spleen uterus, and lymph nodes, such as mandibular, prescapular, retropharyngeal, and superficial inguinal lymph nodes, at 8 weeks after challenge. Among 5 Beagles of immunized group, challenge strain was isolated from only mandibular and superficial inguinal lymph nodes of 3 dogs, whereas the challenge strains were detected from spleen, uterus (except two Beagles) and mandibular, prescapular, retropharyngeal, and superficial inguinal lymph nodes of all unimmunized Beagles. Infection of these sites, such as spleen, and retropharyngeal lymph node, does not result in shedding of *B. abortus* strain in urine, feces or estrual discharges [7]. Furthermore, a number of the challenge strains were isolated from uterus of 3 dogs among unimmunized Beagles, whereas no isolates were observed from 5 immunized Beagles. The *Brucella* strain in this region can result in abortion when the Beagles become in pregnancy. Thankfully, *S. Typhimurium*-based *B. abortus* vaccine candidate provided complete protection against *B. abortus* strain 544 infection in 2 dogs among 5 Beagles. Furthermore, the severity of brucellosis even among diseased Beagles vaccinated with the *S. Typhimurium*-based *B. abortus* vaccine candidate was significantly reduced, indicated by infection index 4.7 times-lower in Beagles with *B. abortus* colonization in tissue at least 1.9 log₁₀ times lower in *S. Typhimurium*-based *B. abortus* vaccine candidate-immunized group Beagles ($P < 0.05$) compared to vector control group Beagles. This infection index in Beagles immunized with the *S. Typhimurium*-based *B. abortus* vaccine candidate showed slightly low levels of protective efficacy than that in mice immunized with same vaccine strains in a previous study [21]. Though the *S. Typhimurium*-based *B. abortus* vaccine candidate in Beagles provided lower levels of efficacy than in mice, considering its safety and ability to elicit enhanced protective humoral and CMI responses in Beagles, it might be useful to use in Beagles in high risk herds. Further investigations are in progress to improve the protective efficacy of the *Salmonella*-based *B. abortus* vaccine candidate in Beagles, such as use of higher dose of the vaccine, different inoculation route, and administration of the combination of the *Salmonella*-based *B. abortus* vaccine candidate with suitable potent adjuvants.

In conclusion, the results of this study demonstrated that SC immunization with approximately 3×10^9 CFU of the mixture of *S. Typhimurium*-based *B. abortus* vaccine strains induced robust serum IgG and CMI responses in Beagles. In addition, the Beagles immunized with the vaccine candidate displayed more efficient protection than unimmunized Beagles. Therefore, our *S. Typhimurium*-based *B. abortus* vaccine candidate elicits antibodies to *B. abortus* each recombinant BCSP31, Omp3b, and SOD protein, which are effective at protecting the Beagles from brucellosis. Thus, this study suggests that future study such as the combination of our vaccine strains with appropriate potent adjuvants is necessary for more powerful protection in Beagles.

CONFLICT OF INTEREST. The authors do not have competing interest.

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