Protective efficacy of an inactivated *Brucella abortus* vaccine candidate lysed by GI24 against brucellosis in Korean black goats

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Abstract

The efficacy of GI24-lysed *Brucella abortus* cells as a vaccine candidate against brucellosis in goats was evaluated on 2 groups of Korean black goats. Group A goats were immunized subcutaneously (SC) with sterile phosphate-buffered saline, whereas group B goats were immunized SC with approximately 3×10^9 lysed *B. abortus* cells. Subcutaneous immunization with the lysed cells did not cause any negative impact on the overall clinical status, such as behavior and appetite, throughout the study period. The enzyme-linked immunosorbent assay (ELISA) optical densities values for *B. abortus* lipopolysaccharide in serum were considerably higher in group B than those in group A. Also, the levels of the cytokines interleukin 4 (IL-4), tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ) were significantly elevated in group B compared with those in group A. Following intraconjunctival challenge with *B. abortus* strain 544, the severity of brucellosis in terms of infection index and colonization of *B. abortus* in tissues was significantly lower in group B than in group A. The present study concluded that 3 of 5 goats immunized with GI24-lysed bacteria were completely protected against challenge. Future investigations are required to improve the protective efficacy offered by lysed *B. abortus* cells for practical applications in small ruminants.

Résumé

L'efficacité de cellules lysées de Brucella abortus *GI24 comme vaccin candidat contre la brucellose chez les chèvres a été évaluée chez deux groupes de chèvres noires coréennes. Les chèvres du groupe A ont été immunisées par voie sous-cutanée (SC) avec de la saline tamponnée stérile, alors que les chèvres du groupe B ont été immunisées SC avec environ* 3×10^9 *cellules lysées de* B. abortus. *L'immunisation sous-cutanée avec les cellules lysées n'a pas eu d'impact négatif sur l'état clinique général, tel que le comportement et l'appétit, tout au long de la période d'étude. Les valeurs de densité optique obtenues lors d'épreuves immunoenzymatiques (ELISA) utilisant le lipopolysaccharide de* B. abortus *étaient considérablement plus élevées avec le sérum des animaux du groupe B que celui des animaux du groupe A. De plus, les niveaux des cytokines interleukine-4 (IL-4), du facteur-alpha nécrosant de tumeur (TNF-α), d'interféron-gamma (IFN-γ) étaient significativement plus élevés dans le groupe B comparativement au groupe A. Pour donner suite à l'infection-défi intra-conjonctivale avec la souche 544 de* B. abortus, *la sévérité de brucellose en termes d'index d'infection et de colonisation des tissus par* B. abortus *était significativement moindre dans le groupe B que dans le groupe A. La présente étude a permis de conclure que 3 des 5 chèvres immunisées avec les bactéries GI24 lysées étaient complètement protégées contre l'infection. Des études ultérieures sont requises pour améliorer l'efficacité protectrice offerte par les cellules lysées de* B. abortus *pour une application pratique chez les petits ruminants.*

(Traduit par Docteur Serge Messier)

Introduction

Brucellosis is a contagious disease that induces abortion and decreases milk yield in domestic animals; it often causes economic losses (1,2). *Brucella* species affect a wide range of hosts such as cattle, goats, sheep, pigs, and dogs (3–5). Four major *Brucella* species — *B. melitensis*, *B. suis*, *B. canis*, and *B. abortus* — can infect humans (6–8). Although *B. melitensis* commonly infects small ruminants such as goats (9,10), *B. abortus* is also infectious for these animals (2,11). Goat brucellosis, therefore, is considered a devastating zoonotic disease (2).

Live attenuated vaccines, particularly *B. abortus* strains S19 and RB51, are widely used to eliminate brucellosis from livestock (12,13). However, live vaccines may lead to bacterial shedding from vaccinated animals in milk, urine, semen, or fecal matter, thereby posing a risk for humans (12). In an effort to reduce brucellosis risk, many different approaches using killed vaccines, subunit vaccines using recombinant proteins, or vector vaccines, have been tested against brucellosis, with variable success (12,14).

In the past few years, lysed bacterial cells have emerged as an effective inactivated vaccine candidate against a broad variety of Gram-negative bacteria (15–18). Lysed bacterial cells are inactivated bacteria with intact bacterial surface structures without cytoplasmic contents (19,20). The action mechanism of antimicrobial peptides (AMPs), a part of the innate immune system (21,22), involves the disruption of membrane barrier function by forming pores or changing membrane permeabilization without altering membrane structures

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(23–25). Some AMPs function as adjuvants (26,27). Porcine myeloid antimicrobial peptide-36 (PMAP-36) has the highest positive charge among AMPs discovered from pigs (28). The positively charged peptide binds to the negatively charged surface of the bacterial cell membrane through electrostatic interactions (29). Among the 36 amino acids in PMAP-36, 24 amino acids (GI24) compose the N-terminal α-helical domain, and like PMAP-36, GI24 can form pores in the bacterial cell membrane (16).

In our previous study (16), lysed *B. abortus* cells were constructed using GI24 and were exploited as a vaccine candidate against brucellosis in mice. The study subjects were intraperitoneally immunized with the lysed *B. abortus* cells, leading to the production of considerable serum IgG and cytokines, such as tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ), associated with a Th1-type immune response. In addition, the immunized mice showed significant protection against brucellosis.

The aim of the present study was to investigate the protective efficacy of the GI24-lysed *B. abortus* cells as a vaccine candidate against *B. abortus* infection in goats. Goats were immunized subcutaneously (SC) with the GI24-lysed *B. abortus* cells and evaluated for an induced cell-mediated immune (CMI) response as well as a humoral immune response. Protective efficacy was estimated following intraconjunctival challenge with *B. abortus* strain 544.

Materials and methods

Animals and ethics statement

Ten nonpregnant female black goats (age = 6 mo) were divided into 2 equal groups: A and B. All goats were procured from tested brucellosis-free flocks. All goats were again confirmed to be seronegative for brucellosis in the laboratory by the Rose Bengal test. All animal experiments performed in this study received ethical approval (CBU 2016-98) from the Chonbuk National University Animal Ethics Committee, in accordance with the guidelines of the Korean Council on Animal Care.

Bacterial strains, peptide, and growth conditions

Brucella abortus bacteria isolated from Korean cattle, HJL900, was as a host strain to construct a vaccine using GI24 lysis (16). *Brucella abortus* strain 544 (ATCC 23448), a smooth, virulent *B. abortus* biovar 1, was chosen as the virulent challenge strain (30). The strain was grown in *Brucella* broth and on *Brucella* agar (Becton Dickinson, Sparks, Maryland, USA) at 37°C. The GI24 (GRFRRLRKKTRKRLKKIGKVLKWI-NH_2) peptide was chemically synthesized by Peptron (Daejeon, South Korea).

Preparation of lysed *B. abortus* cells

GI24-lysed *B. abortus* cells were prepared as a vaccine candidate in goats according to the method described in a previous study (16). One colony of *B. abortus* isolate was inoculated into 200 mL of *Brucella* broth and incubated it at 37°C until an optical density (OD) of 0.3 at 600 nm was reached. Then 40 μg/mL GI24 peptide was added to the cultured broth and incubated the mixture at 37°C to induce lysed cells. After 24 h, lysis induction was determined by counting the number of viable bacteria on *Brucella* agar incubated for 72 h at 37°C. The lysed cells were harvested by centrifugation at 4000 × *g* for 10 min and resuspended the lysate in sterile phosphate-buffered saline (PBS) to approximately 3×10^9 cells/mL and stored the samples at −20°C until use.

Transmission electron microscopy (TEM)

Lysed cells were prepared for TEM analyses using the method described and TEM was used to confirm the intracellular alteration in the *B. abortus* cells before and after treatment with GI24 peptide, according to the method described by Lv et al (31).

Immunization of goats and sample collection

Ten female black goats (age = 6 mo) were divided into 2 equal groups (A and B) (n = 5 each). The goats were primed SC [0 wk post prime immunization (WPPI)] and 3 wk later, the animals were boosted SC (3 WPPI). Group A goats (control) was inoculated with 1.0 mL sterile PBS solution and group B goats with 1 mL of the GI24-lysed *B. abortus* cell suspension (approximately 3.0×10^9 cells/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

To assess the safety of GI24-lysed *B. abortus* cells, the temperatures of group B goats were monitored daily for 6 WPPI.

Immune response measurement by an enzyme-linked immunosorbent assay (ELISA)

A modified ELISA was done to assess the *B. abortus* lipopolysaccharide (LPS)-specific IgG titers in serum samples using the Bovine *Brucella* Ab ELISA 2.0 Kit (BioNote, Hwaseongsi, Gyeonggi-do, Republic of Korea). Tthe plates were blocked using PBS containing 1% ovalbumin (PBS–OVA; 200 μL/well) for 1 h, then the plates were washed with PBS containing 0.05% Tween-20. Serum samples were diluted to 1:100 in PBS–OVA and the plates were treated with horseradish peroxidase-conjugated rabbit anti-goat IgG antibody (Bethyl Laboratories, Montgomery, Texas, USA). A substrate containing *o*-phenylenediamine (Sigma-Aldrich, St. Louis, Missouri, USA) was used to cause enzymatic reactions that were measured using an automated ELISA spectrophotometer (Thermo Scientific Multiskan GO; Thermo Fisher Scientific Oy, Vantaa, Finland) at 492 nm. The ELISA results are expressed as mean OD ± standard deviation (SD).

Preparation of peripheral blood mononuclear cells

At 6 WPPI, blood was collected from each goat and all samples were placed into an acid-citrate-dextrose solution. The peripheral blood mononuclear cells (PBMCs) were enriched by density centrifugation using a Ficoll sodium diatrizoate gradient (Sigma Diagnostics, St. Louis, Missouri, USA). The PBMCs were diluted in Roswell Park Memorial Institute (RPMI) 1640 medium to 2.5×10^6 viable cells/mL as determined by trypan blue dye exclusion. Then 50 μL of each cell suspension, containing 5×10^5 cells, were added to a flat-bottomed 96-well microtiter plate that contained 100 μL RPMI 1640 medium or RPMI 1640 medium plus 10^6 cells/well of heat-inactivated JHL900. Wells counting 0.5 μg/well of Concanavalin

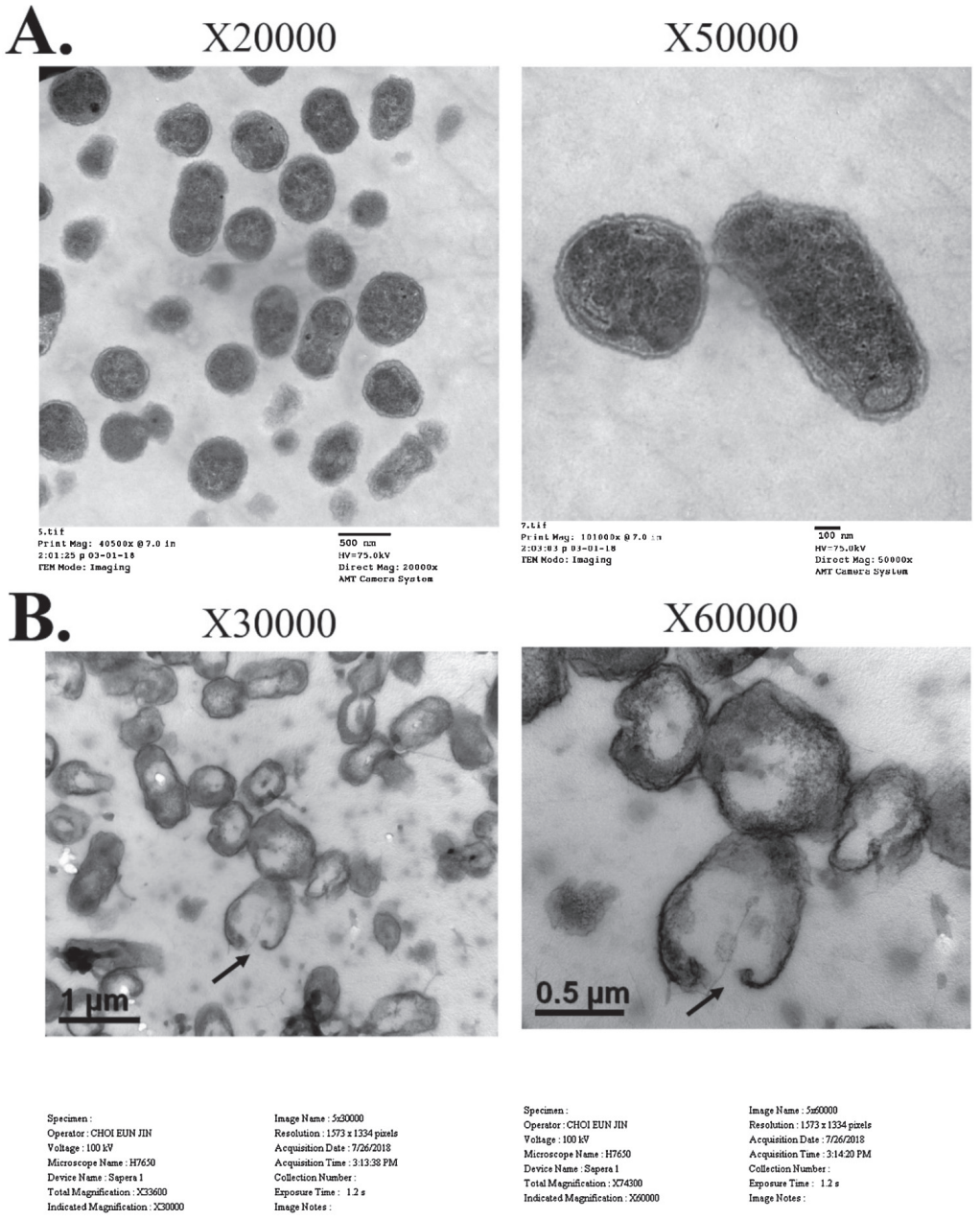

Figure 1. Transmission electron micrographs of *B. abortus* biotype 1 treated with GI24. A — The untreated *B. abortus* cells. B — *B. abortus* cells treated with GI24. The bacterial cells were incubated with 40 µg/mL of GI24 for 30 h at 37°C.

A were used as positive controls for cytokine induction. All plates were performed in triplicate. The microtiter plates were incubated for 72 h at 37°C under 5% CO_2 following an earlier standardized method (16,35). After incubation, the cell culture supernatants were moved to fresh microcentrifuge tubes and stored at −70°C until a commercially available kit was used to assay for cytokines.

Cytokine measurement by ELISA

The concentrations of IL-4, TNF-α, and IFN-γ in the culture supernatants were measured using goat cytokine kits according to the manufacturer's instructions (MyBioSource, San Diego, California, USA). Results of the ELISA are expressed as mean OD ± SD.

Challenge experiments

Brucella abortus strain 544 was used for the challenge experiments using the following methods. The bacterium was grown in *Brucella* broth at 37°C for 24 h, then the culture was resuspended to obtain a final concentration of approximately 1×10^8 CFU/mL. At 6 wk following primary immunization, all goats were fasted for 16 h and anesthetized with an intermuscular injection of Zoletil 50 [7 to 10 mg/kg of body weight (BW); Virbac, Carros, France] and xylazine (2.32 to 3.48 mg/kg BW; Bayer Korea, Ansan, South Korea) before instilling 50 µL *B. abortus* 544 into each eye, for a total dose of approximately 10^7 CFU.

At 12 wk, all goats were euthanized and necropsied. Samples of the lymph nodes (bronchial, mediastinal, mesenteric, parotid, portal, retropharyngeal, submandibular, superficial, and supramammary) and parenchymal organs (heart, kidney, liver, lung, spleen, and uterus) were taken for a total of 15 organs from each animal. The tissue homogenates were plated onto Farrell's medium containing 5% bovine serum, antibiotics, and ethyl violet (32). The *Brucella* bacteria were isolated from the lymph nodes and conventional methods were used for subsequent biochemical identification of isolates (33). Each node was trimmed into small pieces that were weighed and homogenized with an equal volume of sterile PBS. The resulting homogenate was then swabbed onto Farrell's medium with 5% bovine serum, antibiotics, and ethyl violet (34). All agar plates were incubated at 37°C and 10% CO_2 for ≥ 10 d. Polymerase chain reaction (PCR) was used to confirm colonies as the *B. abortus* challenge strain using the *B. abortus*-specific primer (5′-GACGAACGGAATTTTTCCAATCCC-3′) and the IS711-specific primer (5′-TGCCGATCACTTAAGGGCCTTCAT-3′). Sequencing was performed as described in previous studies (36,37). A tissue sample was considered *B. abortus*-infected even if only 1 colony from each tissue was positive for the *B. abortus* challenge strain by PCR and sequencing. The infection index was defined as the number of organs and lymph nodes infected with the *B. abortus* challenge strain in each animal.

Statistical analyses

The statistical analyses of data were performed using GraphPad Prism Software version 5.0 (La Jolla, California, USA). The significance of serum IgG titers, concentration of IFN-γ, infection index, and colonization of *B. abortus* strain 544 in tissues between groups were analyzed using 2-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. *P*-values < 0.05 were considered statistically significant.

Results

TEM

Intact cell membranes and complete intracellular contents of untreated *B. abortus* cells were observed (Figure 1A). *Brucella abortus* cells lysed with GI24 had visible pores in the bacterial membrane and lower amounts of cytoplasm (Figure 1B).

Safety of the vaccine

There was no negative impact on the overall clinical status (e.g., behavior, appetite) of goats that received SC immunization with the lysed *B. abortus* cells and no side effects at the vaccination sites were observed throughout the study period (6 WPPI). The body temperatures of all the goats remained within normal limits (38.5°C to 40.5°C) (data not shown).

Antibody response to *B. abortus* LPS

The ODs of ELISA values for *B. abortus* LPS in the sera are presented in Figure 2. Serum titers of IgG antibodies against *B. abortus* LPS in group A and B goats were 0.225 ± 0.072 and 0.246 ± 0.032, respectively, before immunization. Serum IgG titers in group A goats were 0.225 ± 0.058 at 3 WPPI; those in group B goats increased gradually to 0.613 ± 0.095 at 3 WPPI ($P < 0.05$). At 6 WPPI, serum IgG titers in group B goats were elevated to 1.338 ± 0.354 ($P < 0.05$); those in group A goats were 0.227 ± 0.061.

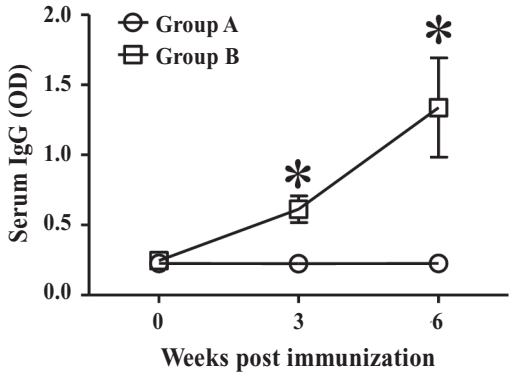

Figure 2. The optical density (OD) of ELISA against *B. abortus* LPS. Group A goats were inoculated with 1.0 mL of sterile PBS as a control. Group B goats were immunized subcutaneously with approximately 3×10^9 GI24-lysed *B. abortus* cells in 1 mL PBS. Data represent the means of all goats in each group and error bars represent the standard deviations (SD). Asterisks indicate a significant difference between the values obtained for the immunized group and those obtained for the control group (*$P < 0.05$).

Cytokine analysis

The IL-4, TNF-α, and IFN-γ concentrations in PBMCs in response to *B. abortus* after re-stimulation with HK *B. abortus* cells using ELISA kits at 6 WPPI were measured. The mean concentrations of IL-4 compared with those of heat-inactivated *B. abortus* cells from groups A and B PBMCs were 32.23 ± 18.11 pg/mL and 212.93 ± 135.16 pg/mL ($P < 0.05$), respectively. The TNF-α concentrations of group A and B goats were 14.72 ± 2.54 pg/mL and 49.48 ± 14.45 pg/mL ($P < 0.05$), respectively. Concentrations of IFN-γ in group A goats were 16.33 ± 2.38 ng/mL; however, IFN-γ concentrations in group B goats were 82.71 ± 29.21 pg/mL (Figure 3).

Protective efficacy against *B. abortus* infection

Protective efficacy of the GI24-lysed *B. abortus* cells against *B. abortus* strain 544 infection was evaluated *via* 3 basic parameters: the effectiveness of vaccination (i.e., degree of protection against *B. abortus* infection), the infection index (i.e., number of *B. abortus*-infected tissues in each animal), and the number of *B. abortus* challenge strain isolates in each tissue. Among the 5 goats in group B, the lysed *B. abortus* cells provided complete protection (i.e., vaccine is effective) against *B. abortus* strain 544 infection in 3 goats. The severity of *B. abortus* strain 544 infection in group B goats (Figure 4A) as indicated by the infection index (1.0 ± 1.4, $P < 0.05$) and number of *B. abortus* challenge strain isolates in tissues (0.0 ± 0.0 to 1.1 ± 1.5 $\log_{10}$ CFU/g of tissue, $P < 0.05$) were significantly lower than those in group A (infection index 13.0 ± 2.4; *Brucella* colonization 1.8 ± 1.7 to 4.0 ± 0.3 $\log_{10}$ CFU/g of tissue) (Figure 4B).

Discussion

In our previous study (16), inactivated *B. abortus* vaccine candidates were constructed using GI24 lysis. The lysed *B. abortus* cells

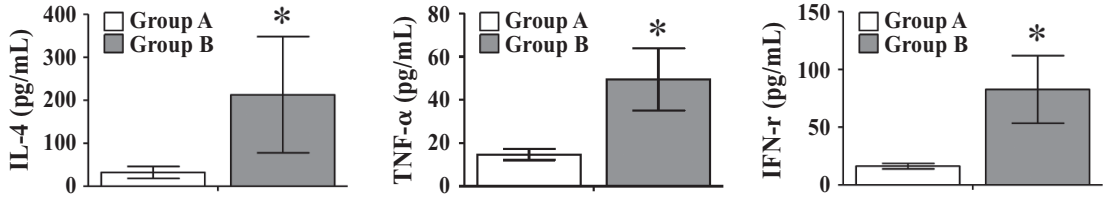

Figure 3. IL-4, TNF-α, and IFN-γ concentrations (pg/mL) in peripheral blood mononuclear cells at 6 wk post prime immunization. Group A goats were inoculated with 1.0 mL of sterile PBS as a control. Group B goats were immunized subcutaneously with approximately 3 × 10^9 GI24-lysed *B. abortus* cells in 1 mL PBS. Data represent the means of all goats in each group and error bars indicate the standard deviations (SD). Asterisks indicate significant differences among the values obtained for each group (*$P < 0.05$).

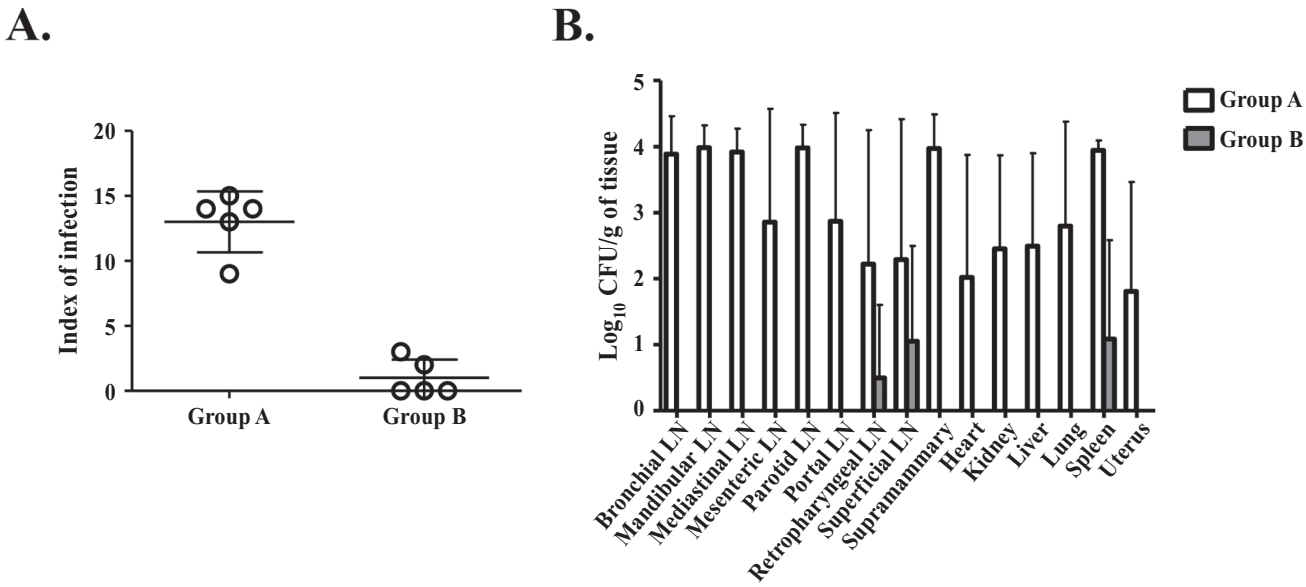

Figure 4. A — Index of infection for goats challenged with *B. abortus* strain 544 at 6 wk post prime immunization. B — Colonization and incidence of recovery of *B. abortus* strain 544 in tissues. Group A goats were inoculated with 1.0 mL of sterile PBS as a control. Group B goats were immunized subcutaneously with approximately 3 × 10^9 GI24-lysed *B. abortus* cells in 1 mL PBS. All goats in each group were intraconjunctivally challenged with 1 × 10^7 CFU of virulent wild-type *B. abortus* 544 at 6 wk post prime immunization. The numbers of viable bacteria recovered from each tissue of goats at 12 wk post challenge are shown (LN — lymph node).

showed an effective immunity against *B. abortus* colonization in a murine model (16). Therefore, it was speculated that the inactivated *B. abortus* vaccine candidate lysed with GI24 could also control brucellosis in small ruminants. The prime aim in this study was to evaluate safety and protective efficacy against *B. abortus* infection of inactivated *B. abortus* cells lysed with GI24 in goats. Using clinical thermometry, it was determined that injection of the inactivated *B. abortus* cells lysed with GI24 was safe for use in goats. Moreover, inflammatory infiltrations in goats vaccinated SC with the lysed *B. abortus* cells were not observed. It can, therefore, be concluded that the GI24-inactivated *B. abortus* vaccine candidate is safe for goats.

Potent serum IgG titers are essential for defending the host from systemic infection by intracellular pathogens (38–40). These serum IgGs eradicate pathogens from the blood by stimulating the phagocytosis efficacy of host phagocytes *via* opsonization (39,41). In the present study, serum IgG titers were investigated in goats immunized by SC administration of the GI24-inactivated *B. abortus* vaccine candidate. The serum IgG titers in the goats immunized with lysed *B. abortus* cells (group B) were significantly elevated compared with those of the non-immunized group of goats (group A). Furthermore, induction of cytokines from PBMCs of all goats immunized with the lysed *B. abortus* cells and re-stimulated *in vitro* with heat-inactivated *B. abortus* whole cells indicated a powerful Th2 type immunity (IL-4 represents the Th2 bias of immunity). These results confirmed that the production of serums IgG and IL-4, which are associated with enhancing IgG response, could be significantly increased by SC immunization with the inactivated lysed *B. abortus* cells (without combining the lysed cells with a suitable adjuvant). Based on reports that some AMPs can function as an adjuvant as well as an antimicrobial agent (26,27), we feel that GI24 might be working as an adjuvant.

The CMI responses, such as TNF-α and IFN-γ, as well as the humoral immune response, are essential for removal of facultative intracellular pathogens such as *B. abortus* (42–45). Notably, the CMI response mediated by IFN-γ plays a crucial role in the elimination of *Brucella* species bacteria from the host organs. Therefore, TNF-α and IFN-γ concentrations were analyzed as well as serum IgG titers to

B. abortus LPS in goats immunized with GI24-inactivated *B. abortus* cells. Significantly higher concentrations of TNF-α and IFN-γ were induced in goats immunized with the GI24-lysed *B. abortus* cells compared with those in the control group. This reflected the high levels of protection induced by GI24-inactivated *B. abortus* cells. These results revealed that the lysed cells might not act only as a potent vaccine candidate but also have effective adjuvant properties, leading to induction of both humoral and CMI responses (19,46,47). Subcutaneous immunization with the *B. abortus* vaccine candidate lysed with GI24 provided complete protection against *B. abortus* infection in 60% of goats. Moreover, the infection index was significantly lower in the group immunized with the GI24-lysed *B. abortus* cells than in the unimmunized control group. The *B. abortus* challenge strain was isolated in only 3 tissues [retropharyngeal (except one goat), superficial lymph nodes, and spleen] from 15 organs of 2 *B. abortus*-infected goats immunized with GI24-lysed *B. abortus* cells. Furthermore, the number of *B. abortus* colonies in the 3 tissues from goats vaccinated with the GI24-inactivated *B. abortus* vaccine candidate was at least 134 times lower ($P < 0.05$) compared with that in the non-immunized goats. Moreover, a considerable number of the challenge strain bacteria was isolated from the uteri of 3 (60%) of the 5 unvaccinated goats, whereas no isolates were observed in the uteri of the 5 immunized goats. *Brucella* infection in the reproductive organs could result in abortion of pregnant goats.

The protective efficacy of the *B. abortus* vaccine candidate lysed with GI24 in goats receiving SC immunization was similar to that observed in mice (16). Therefore, considering its safety and ability to elicit both protective humoral and cellular immune responses in goats, it might be valuable for use in small ruminants with infection risk. Further investigations are in progress, to improve the protective efficacy of lysed *B. abortus* cells as a vaccine candidate against goat brucellosis by immunization with the combination of lysed *B. abortus* cells and other potent adjuvants.

The present study concluded that SC immunization with GI24-lysed *B. abortus* cells induced robust humoral and CMI responses in goats. Moreover, future studies with the combination of the above vaccine strains and other potent adjuvants are essential for establishing better immune responses in small ruminants and in pregnant ruminants.

Acknowledgments

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