FULL PAPER *Immunology*

Safety and Efficacy Testing of a Novel Multivalent Bovine Bacterial Respiratory Vaccine Composed of Five Bacterins and Two Immunogens

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ABSTRACT. Bovine bacterial respiratory diseases have been one of the most serious problems due to their high mortality and economic loss in calves. The vaccinations of bovine bacterial respiratory vaccines have been complex because of no multivalent vaccine. In this study, novel multivalent bovine bacterial respiratory vaccine (BRV) was developed and tested for its safety and efficacy. BRV was composed of two immunogens and five bacterins. These were leukotoxoid and bacterin of *Mannheimia haemolytica* type A, outer membrane protein and bacterin of *Pasteurella multocida* type A, and bacterins of *Haemophilus somnus, Mycoplasma bovis*, and *Arcanobacterium pyogenes*. ELISA antibody titers to five bacterial antigens in vaccinated guinea pigs increased, compared with those in unvaccinated ones. BRV was safe for calves and pregnant cattle in this study. In calves challenged with *M. haemolytica* and *P. multocida*, the average daily weight gain and antibody titers of vaccinated calves increased, and respiratory symptoms ($P<0.05$) and treatment frequency ($P<0.01$) of vaccinated calves significantly decreased, compared with those of unvaccinated calves. Interestingly, the antibody titers of *M. haemolytica* leukotoxoid and *Mycoplasma bovis* were closely related with the reduction of respiratory symptoms. BRV would be an ecomonical measure for the protection against bovine bacterial respiratory diseases.

KEY WORDS: *Arcanobacterium pyogenes*, *Haemophilus somnus*, *Mannheimia haemolytica* leukotoxoid, *Mycoplasma bovis*, *Pasteurella multocida* outer membrane protein.

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There are major causative agents of bovine bacterial repiratory diseases, such as *Mannheimia haemolytica* type A, *Pasteurella multocida* type A, *Haemophilus somnus, Mycoplasma bovis*, and *Arcanobacterium pyogenes*. Shipping fever pneumonia is one of the main causes of death and economic loss in calves [14]. *Mannheimia haemolytica*, the most common causative agent of shipping fever pneumonia, colonizes normally in the nasopharynx of cattle. Although calves are innately resistant to pulmonary infection with *M. haemolytica*, this bacterium infects the lower respiratory tract and causes pneumonia due to stress, adverse climate conditions, and respiratory viral infections [1, 19]. *Pasteurella multocida* represents a heterogeneous group of Gram-negative bacteria that are commensals in the upper respiratory tract of many mammals and birds [8]. This microorganism is also an economically important cause of disease in domesticated animals worldwide, where it can act as both a primary or secondary pathogen [8]. *Haemophilus somnus* is associated with acute respiratory disease, meningoencephalitis, myocarditis, and reproductive tract infections in cattle [11]. While *Arcanobacterium pyogenes* is a common inhabitant of the upper respiratory tract in cattle [12], a physical or microbial attack to the host can cause pneumonia [7]. *Mycoplasma bovis* is a major causative bacterium of pneumonia and arthritis in calves, and of mastitis and genital infections in adult cows. Although the importance of *Mycoplasma bovis* in respiratory diseases has been often underestimated, it is responsible for high economic loss in feedlot cattle [16] and is widespread within the bovine population in enzootically infected areas [15].

M. haemolytica bacterin and leukotoxoid, *P. multocida* bacterin, and *H. somnus* bacterin as killed vaccines, and attenuated *P. multocida* and *M. haemolytica* as live vaccines are currently used worldwide in the cattle industry for the protection against bacterial respiratory diseases [5, 20]. Because commercial vaccines were monovalent or divalent vaccines, we developed and tested for the safety and efficacy of a novel multivalent vaccine to simplify the vaccination schedule and increase the protection range, which is composed of 2 immunogens and 5 bacterins—leukotoxoid of *M. haemolytica* type A, outer membrane protein of *P. multocida* type A, and bacterins of *M. haemolytica* type A, *P. multocida* type A, *H. somnus*, *M. bovis*, and *A. pyogenes*.

MATERIALS AND METHODS

Bacterial strains and cultures: *M. haemolytica* type A NS (National Veterinary Research & Quarantine Service), *P. multocida* type A NS, *H. somnus* NS, *Mycoplasma bovis* NS, and *A. pyogenes* NS were isolated from cattle at the abattoirs in the Republic of Korea and used as the seed strains of bovine respiratory vaccine (BRV). Each strain was confirmed by the characteristics of colony, the biochemical tests, and the species-specific gene detection. Each strain was cultured on the optimal medium for manufacturing the vaccine components. *M. haemolytica* type A NS and *P. multocida* type A NS were cultured in brain heart

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infusion (BHI) broth (BD, Franklin Lakes, NJ, U.S.A.) at 37°C, and *H. somnus* and *A. pyogenes* were cultured in tryptic soy broth (TSB) (BD) at 37°C. *Mycoplasma bovis* was cultured at 37°C for 1 week in Mycoplasma medium [10 × Hanks' balanced salts solution (HBSS; GIBCOBRL, Gaithersburg, MD, U.S.A.) 25 m*l*, BHI broth base 4.1 g, PPLO broth base (BD) 4.35 g, lactalbumin hydrolysate (Sigma Chemical, St. Louis, MO, U.S.A.) 1 g, yeast extract (Sigma Chemical) 2.25 g, 0.2% of phenol red (Sigma Chemical) 10 m*l*, distilled water 1,212.5 m*l*, 25 % of fresh yeast extract 30 m*l*, inactivated fetal bovine serum (GIBCOBRL, Gaithersburg, MD, U.S.A.) 100 m*l*, glucose (Sigma Chemical) 0.9375 g, thallium acetate (Sigma Chemical) 0.125 g, and deoxyribonucleic acid from calf thymus (Sigma Chemical) 0.01 g].

Preparation of 2 immunogens from M. haemolytica and P. multocida: *P. multocida* cultured on BHI agar (BD) was harvested, disrupted by sonication, and centrifuged at 7,000 rpm for 25 min at 4°C. After inactivation by treatment with 0.3% formalin for 3 days, the supernatant was used as immunogen I, which contained outer membrane protein (OMP) from *P. multocida*. *M. haemolytica* cultured in RPMI 1640 for 48 hr was inactivated by adding 0.3% formalin for 3 days and centrifuged at 7,000 rpm for 30 min. The supernatant was filtered by 0.2 μm filter and concentrated to 10% of its original volume by ultrafiltration. This concentrated supernatant was used as immunogen II and contained leukotoxoid (LK) from *M. haemolytica*. Immunogen I and II were used for the production of BRV.

Production of bovine respiratory vaccine: Each strain was cultured and inactivated by adding 0.3% formalin for 3 days. Each bacterin was centrifuged at 10,000 × g for 30 min, resuspended and washed with 0.01 M phosphate buffered saline (pH 7.2). Each bacterin was centrifuged again and resuspended in 0.01 M phosphate buffered saline (pH 7.2) with 1/50th of the original volume. The optical density (OD) at 410 nm of each bacterin was calculated using an UV-2 spectrophotometer (UNICAM, Co., Cambridge, England) and the concentration of each bacterin was adjusted appropriately to yield the desired formulation as follows: The OD at 410 nm of *M. haemolytica* type A (MH), *P. multocida* type A (PM), and *A. pyogenes* (AP) bacterins was 1.2, while that of *H. somnus* (HS) and *Mycoplasma bovis* (MB) bacterins was 0.6 and 0.12, respectively. The protein concentrations of immunogen I and II were 200 and 10 μg/m*l*, respectively, as determined using a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.). The concentrations of aluminum hydroxide gel and ISA25 were 10 mg/m*l* and 5% (v/v), respectively. Formalin and gentamicin were used as preservatives at 0.1% (v/v) and 75 μg/m*l*, respectively [2, 6, 10, 17].

Safety testing: Mice (ICR), guinea pigs (Hartley), and beef cattle (Hanwoo) were used for the safety testing of BRV. One group of mice (n=11) was injected with 0.5 m*l* of BRV intraperitoneally, while another group of mice (n=11) was injected with 0.5 m*l* of BRV subcutaneously. Four groups of guinea pigs were used (2 guinea pigs per group). Two groups of them were injected intramuscularly (IM) with 1.0 m*l* and 2.0 m*l* of BRV, respectively, and the other two groups of them subcutaneously with 1.0 m*l* and 2.0 m*l* of BRV, respectively. All vaccinated guinea pigs were examined every day for above 10 days. We also examined the safety of BRV in calves (n=3) and pregnant cows (n=3) for above 14 days. They were injected IM with 2 doses, that is, 4 m*l* for the calves and 6 m*l* for the pregnant cattle. Calves and pregnant cattle were examined for adverse reactions, such as inflammation, loss of appetite, fever, shock, and abortion.

Potency testing: Guinea pigs (Hartley) (n=11) were vaccinated at 2-week intervals, and the resultant antibody titers were examined by ELISA using sonicated antigens and leukotoxoid from each of vaccine components. Sonicated antigens for ELISA were prepared as described for the preparation of immunogen I.

Safety testing in the field and efficacy testing of challenged calves: Pregnant cattle were vaccinated IM with 3 m*l* of BRV at 6 to 7 weeks before parturition, and calves IM with 2 m*l* of BRV between 45 to 50-day old. The second vaccination was vaccinated 3 weeks after the first vaccination. Each cattle was examined the adverse effects due to BRV vaccination.

In the calf challenge study, 40 day-old beef cattle were vaccinated (n=4) and compared with an unvaccinated group (n=5) by examining antibody titers, clinical respiratory signs, treatment frequency and daily weight gain. The vaccinated calves were periodically tested for whole IgG of blood to each antigen, represented as antibody titers by ELISA [6]. Each antibody titer was calculated as a geometric mean titer (GMT) of individual antibody titers, using the OD-cut off method in which an antibody titer is defined as the reciprocal of the maximum dilution corresponding to a negative control OD. Clinical respiratory signs were represented by clinical index. Clinical index of each group was calculated by the following equation; Clinical index = [(clinical data of each calf/(number of calf in each group)] × 100. Clinical data were given to each calf as 0, 1, 2, and 3 according to severity of clinical signs, which was no, mild, middle, and severe sign, respectively. Clinical signs in this study were examined loss of appetite, claudication, depression, diarrhea, tremor, nasal discharge, cough, and other clinical signs related to respiratory disease. Calves of both groups were controlled medically, giving vitamin, antibiotics, anti-febrile, and eletrolytes according to the severity of clinical signs. Treatment frequency was recorded for examining the difference between vaccination and unvaccinated group.

The challenge strains were *Mycoplasma bovis* NS and ATCC 25025, *M. haemolytica* PHA-1, and *P. multocida* 2BPA-1, originated from National Veterinary Research & Quarantine Service in the Republic of Korea. *Mycoplasma bovis* NS and ATCC 25025 were cultured for 1 week at 37°C and mixed with 2 strains, of which the concentrations were adjusted to 10^{10} CFU/m*l*. The concentrations of *M. haemolytica* PHA-1 and *P. multocid*a 2BPA-1 were 1.5 ×

Table 1. The experimental design for calf groups, vaccination regimen, stress, and bacterial challenge exposures

Day of study	Procedures			
	BRV-MH[a)]	unvaccinated-MH[b)]	BRV-PM[c)]	unvaccinated-PM[d)]
1	2 m*l* of vaccine, IM	–	2 m*l* of vaccine, IM	–
21	2 m*l* of vaccine, IM	–	2 m*l* of vaccine, IM	–
46	MB, IN	MB, IN	MB, IN	MB, IN
47	Cold stress	Cold stress	Cold stress	Cold stress
57	MH, IT & IN	MH, IT & IN	PM, IT & IN	PM, IT & IN
61	MH, IT & IN	MH, IT & IN	PM, IT & IN	PM, IT & IN
69	necropsy	necropsy	necropsy	necropsy

a) BRV vaccination and MH challenge group, b) Unvaccinated and MH challenge group, c) BRV vaccination and PM challenge group, d) Unvaccinated and PM challenge group; IM, intramuscularly; IN, intranasally; IT, intratracheally; MB, *M. bovis*; MH, *M. haemolytica*; PM, *P. multocida*. Cold stress was induced by spraying the calves with water.

10^9 CFU/m*l* and 7.0×10^8 CFU/m*l*, respectively. At fifteen days after the second vaccination, calves were strained with cold stress by spraying of 200 m*l* water per head on their body surface, and then they challenged intranasally with 10 m*l* of *Mycoplasma bovis* NS and ATCC 25025 per head. Eleven and fifteen days after the challenge with *Mycoplasma bovis*, all calves were challenged intranasally and intratracheally with 20 m*l* of *M. haemolytica* and *P. multocida* (Table 1).

Statistical analysis: Data were analyzed by Student's *t*-test.

RESULTS

No adverse reactions were observed in vaccinated mice (*n*=10) and guinea pigs (*n*=4) for 10 days regardless of injection route. When calves and pregnant cattle were injected IM with 2 doses, the rectal temperatures (RTs) of calves and pregnant cows were 39.0 and 38.1°C on pre-injection, 39.2 and 38.2°C on 1 hr post-injection and 38.8 and 38.0°C on 14 days after injection, respectively. Vaccinated calves and pregnant cows exhibited no local or systemic adverse reactions, such as the swelling or inflammation of injection site, loss of appetite, fever, cough, nasal discharge, shock, dyspnea, depression, vomit, and abortion. In addition, none of the vaccinated calves (*n*=771) and pregnant cattle (*n*=400) observed in the field showed any adverse reaction.

Antibody titers of vaccinated guinea pigs (*n*=11) for *M. haemolytica* type A (MH), *M. haemolytica* type A leukotoxoid (LK), *P. multocida* type A (PM), *H. somnus* (HS), *Mycoplasma bovis* (MB), and *A. pyogenes* (AP) were 219, 160, 118, 300, 234, and 282, respectively, whereas those from the unvaccinated ones (n=4) 34, 16, 28, 57, 40, and 48, respectively (Fig. 1). Antibody titers from the vaccinated guinea pigs to MH, PM, MB, and LK were significantly (P<0.05) higher than those of the unvaccinated ones.

In the challenge study, antibody titers and average daily weight gain of vaccinated calves significantly increased (P<0.05) (Figs. 2, 4). Respiratory symptoms of vaccinated calves after challenge were significantly decreased, compared with unvaccinated ones (P<0.05). Clinical index of vaccinated and unvaccinated groups at 3 days after challenge was 0 and 200, respectively. We injected electrolytes, anti-febrile, vitamins, and antibiotics to the calves showing the respiratory symptoms before challenge. The treatment frequency of vaccinated calves was 61, whereas that of unvaccinated ones was 87 during the experiment period. Treatment frequency in vaccination calves was significantly decreased (P<0.01), compared with unvaccinated ones. The

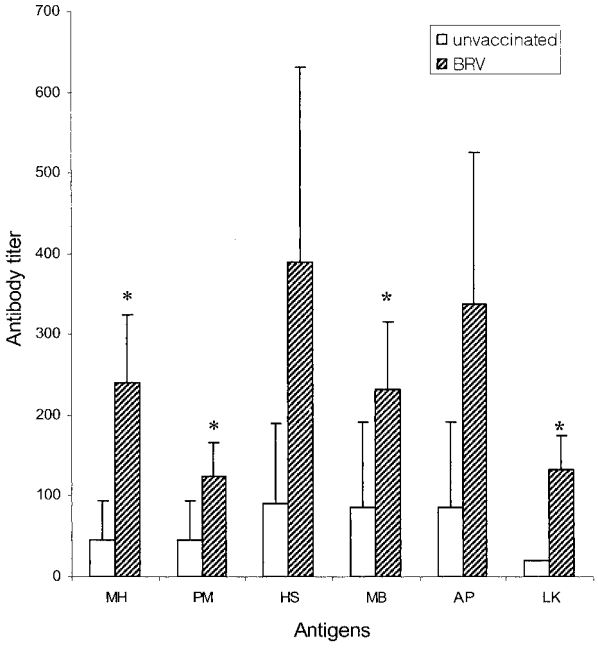

Fig. 1. Comparison of antibody titers in vaccinated (*n*=10) and unvaccinated (*n*=5) guinea pigs. Each antibody titer was examined by ELISA and expressed as geometric mean titer (GMT). ELISA antigens were the sonicated antigens of *M. haemolytica* type A (MH), *P. multocida* type A (PM), *H. somnus* (HS), *Mycoplasma bovis* (MB) and *A. pyogenes* (AP), and leukotoxoid of *M. haemolytica* type A (LK). The mean ± standard deviation (SD) of the antibody titers for each antigen was presented. * P<0.05.

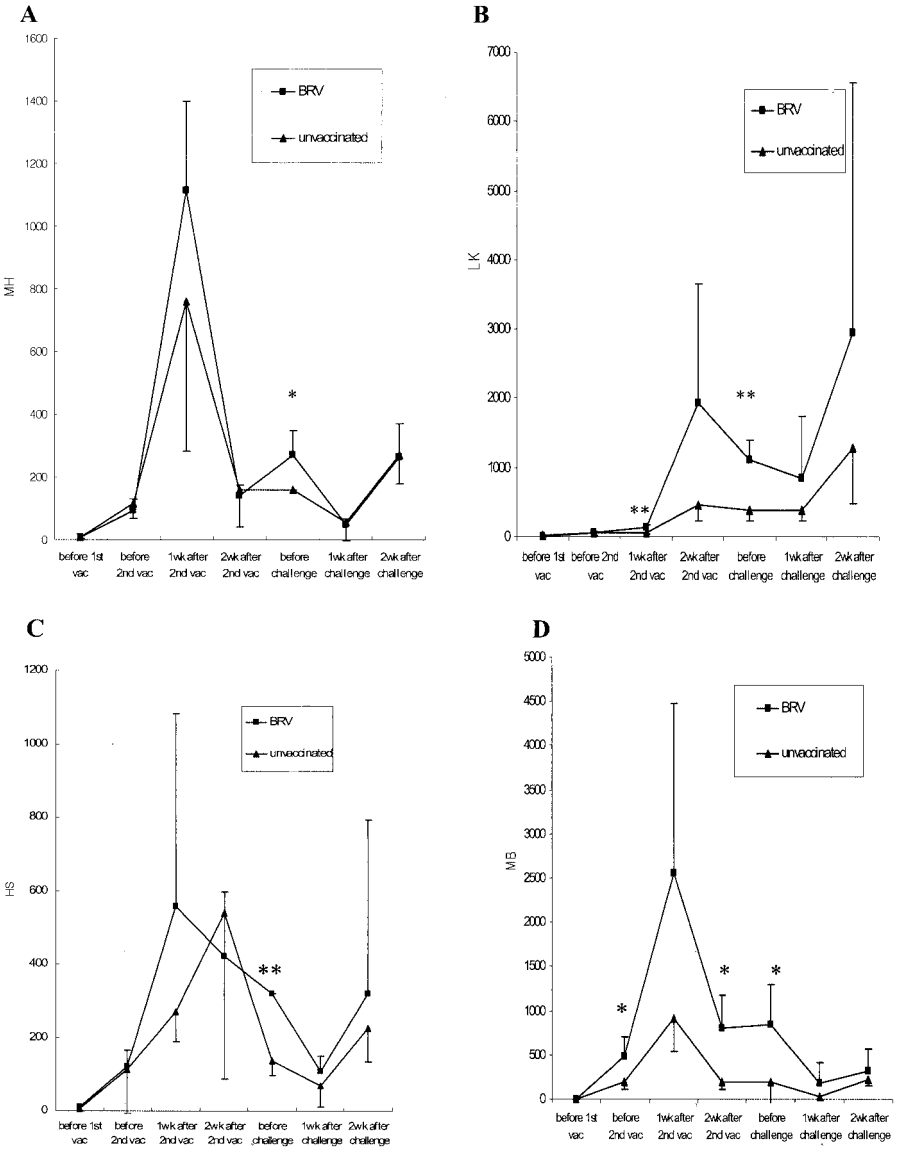

Fig. 2. Comparison of antibody titers in vaccinated and unvaccinated calves. * BRV, vaccinated calves (n=5); unvaccinated, unvaccinated calves (n=4). Each antibody titer was examined by ELISA and expressed as geometric mean titer (GMT). ELISA antigens were the sonicated antigens of *M. haemolytica* type A [panel A], leukotoxoid of *M. haemolytica* type A [panel B], *H. somnus* [panel C], and *Mycoplasma bovis* [panel D]. The mean ± SD of the antibody titers for each antigen was presented. * P<0.05, ** P<0.01. MH: *M. haemolytica* type A, LK: leukotoxoid of *M. haemolytica* type A, HS: *H. somnus*, MB: *Mycoplasma bovis*.

antibody titer to MH (P<0.05), LK (P<0.01), HS (P<0.01), and MB (P<0.05) of vaccinated calves (n=5) was significantly higher than those of unvaccinated ones (n=4) before challenge (Fig. 2). The mean ± standard deviation of rectal temperature (RT) after challenge in vaccinated calves was 39.0 ± 0.1°C, and that in unvaccinated ones 39.2 ± 0.4°C. The range of RT after challenge in vaccinated calves was from 38.5 to 39.4°C, and that in unvaccinated ones from 38.7 to 41.1°C. RT of unvaccinated calves was not significantly higher than vaccinated ones, but that of an unvaccinated calf was increased till 41.1°C after challenge. Compared with unvaccinated calves, the treatment frequency of vaccinated ones was significantly decreased (P<0.01). The difference of average daily weight gain (DWG) per day between vaccinated and unvaccinated calves was 0.24 kg for 72 days. Daily weight gain (DWG) in vaccinated calves was significantly (P<0.05) higher than that in unvaccinated ones (Fig. 4).

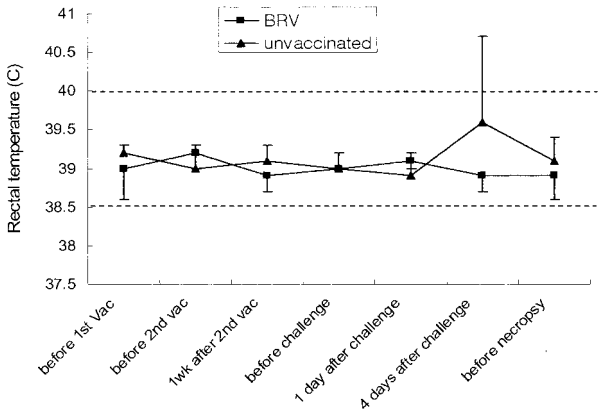

Fig. 3. Comparison of rectal temperature in vaccinated (BRV) and unvaccinated calves. Dot line was represented the normal range of calf's rectal temperature. Normal range of below 1 year old calf is from 38.5 to 40.0°C.

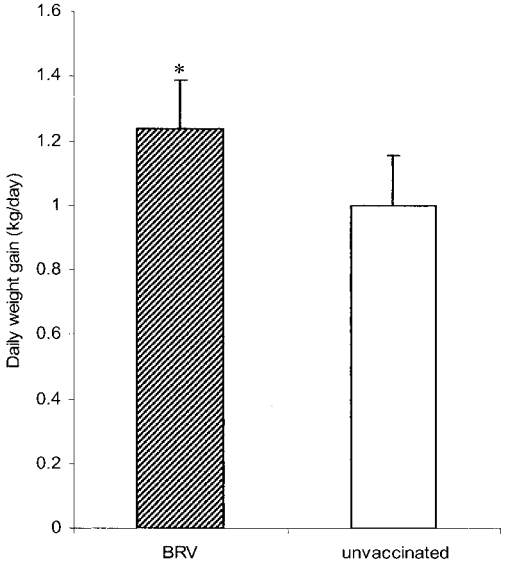

Fig. 4. Comparison of daily weight gain in vaccinated (BRV) and unvaccinated calves. Daily weight gain was differed significantly ($P<0.05$) between vaccinated and unvaccinated calves.

DISCUSSION

BRV was composed of two immunogens and five bacterins based on the bacteria isolation from the cattle showing respiratory symptoms in the Republic of Korea. To simplify the vaccination program and increase the protection range against bovine bacterial respiratory diseases, we developed novel vaccine which was composed of 2 immunogens and 5 bacterins. Major causative bacteria in respiratory diseases were *M. haemolytica* type A, *P. multocida* type A, *H. somnus*, *Mycoplasma bovis*, and *A. pyogenes*. The vaccine was composed of two immunogens which were leukotoxoid of *M. haemolytica* type A and outer membrane protein of *P. multocida* type A, and five bacterins which were *M. haemolytica* type A, *P. multocida* type A, *H. somnus*, *Mycoplasma bovis*, and *A. pyogenes* [6, 10, 16, 17]. Shipping fever caused by *M. haemolytica* type A and *P. multocida* type A is one of the most important and frequent respiratory diseases of cattle [2, 4, 8, 9]. Thus, the *M. haemolytica* type A and *P. multocida* type A bacterins have been used as vaccine components to protect against shipping fever. With regard to *M. haemolytic*a type A, both leukotoxoid and capsule have been added to standard vaccines to improve their immunogenicity [2]. Thrombotic meningoencephalitis caused by *H. somnus* is prevented by the vaccination of *H. somnus* bacterin worldwide. Interestingly, the incidence of pneumonia caused by *Mycoplasma bovis* in abattoir was equivalent to *M. haemolytica* and *P. multocid*a in the Republic of Korea. *A. pyogenes* has been also isolated in Korean abattoir from nasal discharges and pneumonic lungs of cattle with respiratory symptoms.

We used aluminum hydroxide and ISA25 (oil-in-water type) as vaccine adjuvant. Even though most vaccines were used aluminum hydroxide as the adjuvant because of its acceptable safety and efficacy, oil adjuvants have been used increasingly to improve the efficacy of vaccines [3]. After manufacturing BRV with aluminum hydroxide and ISA25 oil adjuvants, BRV had a good appearance, compared with the vaccines having aluminum hydroxide only as the adjuvant.

BRV has been tested for its safety to mice, guinea pigs, calves, and pregnant cattle. Notably, the vaccinated groups did not show any adverse effects, and BRV will be safe for calves and pregnant cattle if it is injected according to general vaccination precautions, which are storage at 2–8°C, equilibration of room temperature before injection, and vaccination only to healthy animals.

The efficacy of BRV was tested in guinea pigs, calves, and pregnant cattle. The antibody titers of vaccinated guinea pigs were significantly higher than those of the unvaccinated ones (Fig. 1). Because ELISA antigens of this study were the sonicated antigens, that is, crude proteins, there was cross-reactivity of ELISA in the unvaccinated group. The cross-reactivity of cattle was higher than that of guinea pigs. ELISA antigens should be purified for the specific antigen of ELISA.

In the challenge study, vaccinated calves showed the vaccine efficacy, such as daily weight gain, antibody titer, and improvements in clinical respiratory signs, with reduced treatment frequency, compared with unvaccinated ones. Especially, the antibody titers to *Mycoplasma bovis* (MB) and leukotoxoid of *M. haemolytica* type A (LK) were highly increased in vaccinated calves. The antibody titers to *Mycoplasma bovis* and *M. haemolytica* were highly related with the protection against bovine bacterial respiratory diseases.

In evaluating bovine bacterial respiratory vaccines, clinical data—including clinical signs, the frequency of treatment, and daily weight gain—are as important as the

antibody titers [18]. In the challenge study, one unvaccinated calf showed an increase in rectal temperature on the first day after the challenge (Fig. 3). Clinical signs were used as good indicators of the vaccine efficacy against bovine bacterial respiratory diseases [13, 16]. Conclusively, our results suggest that BRV might improve animal welfare as a result of improvement in daily weight gain and decreased treatment frequency, result in economic advantages, and provide the effective protection against bovine bacterial respiratory diseases.

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