Vol. 18, No. 10

A Multivalent *Mannheimia-Bibersteinia* Vaccine Protects Bighorn Sheep against *Mannheimia haemolytica* Challenge[▽]

Renuka Subramaniam,[1] Sudarvili Shanthalingam,[1] Jegarubee Bavananthasivam,[1] Abirami Kugadas,[1] Kathleen A. Potter,[1,2] William J. Foreyt,[1] Douglas C. Hodgins,[3] Patricia E. Shewen,[3] George M. Barrington,[4] Donald P. Knowles,[1,5] and Subramaniam Srikumaran[1]*

Department of Veterinary Microbiology and Pathology[1] and Washington Animal Disease Diagnostic Laboratory,[2] College of Veterinary Medicine, Washington State University, Pullman, Washington 99164-7040; Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada[3]; Department of Veterinary Clinical Medicine and Surgery, College of Veterinary Medicine, Washington State University, Pullman, Washington 99164-7040[4]; and Animal Disease Research Unit, USDA Agricultural Research Service, Pullman, Washington 99164-6630[5]

Bighorn sheep (BHS) are more susceptible than domestic sheep (DS) to *Mannheimia haemolytica* pneumonia. Although both species carry *M. haemolytica* as a commensal bacterium in the nasopharynx, DS carry mostly leukotoxin (Lkt)-positive strains while BHS carry Lkt-negative strains. Consequently, antibodies to surface antigens and Lkt are present at much higher titers in DS than in BHS. The objective of this study was to determine whether repeated immunization of BHS with multivalent *Mannheimia-Bibersteinia* vaccine will protect them upon *M. haemolytica* challenge. Four BHS were vaccinated with a culture supernatant vaccine prepared from *M. haemolytica* serotypes A1 and A2 and *Bibersteinia trehalosi* serotype T10 on days 0, 21, 35, 49, and 77. Four other BHS were used as nonvaccinated controls. On the day of challenge, 12 days after the last immunization, the mean serum titers of Lkt-neutralizing antibodies and antibodies to surface antigens against *M. haemolytica* were 1:160 and 1:4,000, respectively. Following intranasal challenge with *M. haemolytica* A2 (1×10^5 CFU), all four control BHS died within 48 h. Necropsy revealed acute fibrinonecrotic pneumonia characteristic of *M. haemolytica* infection. None of the vaccinated BHS died during the 8 weeks postchallenge observation period. Radiography at 3 weeks postchallenge revealed no lung lesions in two vaccinated BHS and mild lesions in the other two, which resolved by 8 weeks postchallenge. These results indicate that if BHS can be induced to develop high titers of Lkt-neutralizing antibodies and antibodies to surface antigens, they are likely to survive *M. haemolytica* challenge which is likely to reduce the BHS population decline due to pneumonia.

The bighorn sheep (BHS; *Ovis canadensis*) population in North America has declined drastically during the last century due to a combination of factors, including loss of habitat, competition for forage with domestic livestock, predation, and disease. Pneumonia is the primary disease that causes significant mortality in BHS (2, 26). Although *Mannheimia haemolytica*, *Bibersteinia trehalosi*, and *Pasteurella multocida* have been isolated from several pneumonia outbreaks, only *M. haemolytica* has been shown to consistently cause fatal pneumonia in BHS under experimental conditions (4, 7, 9, 22). Virulence factors of *M. haemolytica* include capsule, outer membrane proteins, neuraminidase, lipopolysaccharide, and a potent exotoxin called leukotoxin (Lkt), which is cytolytic to all subsets of ruminant leukocytes (3, 14). Based on the fact that Lkt deletion mutants cause no mortality in BHS (4) and lower mortality and mild lung lesions in cattle (12, 19, 24), Lkt has been accepted as the major virulence factor of this organism. Although *M. haemolytica* causes pneumonia in all ruminants,

BHS are highly susceptible to this disease (4, 7, 9, 22). There are a number of potential factors contributing to the apparent difference in susceptibility to pneumonia. Enhanced susceptibility of polymorphonuclear leukocytes (PMNs) from BHS to Lkt-induced cytolysis is one of the factors responsible for the higher susceptibility of BHS (20, 22), which underscores the importance of Lkt-neutralizing antibodies (Lkt-nAb) for protection of BHS against *M. haemolytica* pneumonia.

Vaccination studies in cattle have shown that antibodies against Lkt and surface antigens of *M. haemolytica* confer protection against experimental challenge (13, 21) and that such antibodies can be induced by vaccination of cattle or BHS with log-phase culture supernatant fluid (1, 13, 16, 27). However, to date systematic analysis of the response of BHS to vaccination and subsequent challenge with *M. haemolytica* has not been performed. Thus, unambiguous data on the role of antibodies in protection of BHS against pneumonia caused by *M. haemolytica* are not available. Several pneumonia outbreaks have occurred in the recent past, resulting in the death of large numbers of BHS (17). Thus, there is a critical need for development of efficacious vaccines to protect BHS against pneumonia. This study represents an initial step in addressing this need.

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-4572. Fax: (509) 335-8529. E-mail: ssrikumaran@vetmed.wsu.edu.

BHS are more susceptible to *M. haemolytica* pneumonia than the related species of domestic sheep ([DS] *Ovis aries*) (4, 9, 18, 22). Although both species carry *M. haemolytica* and *B. trehalosi* as commensal bacteria in the nasopharynx, DS carry mostly Lkt-positive strains while BHS carry Lkt-negative strains (23; S. Shanthalingam et al., unpublished data). It is very likely that because of the difference in Lkt expression levels by the *M. haemolytica* strains inhabiting the nasopharynx of DS and BHS and the consequent difference in exposure to the immune system, Lkt-nAb are present at much higher titers in DS than in BHS, where Lkt-nAb are present at negligibly low titers (11). We hypothesized that if BHS can be induced to develop high titers of antibodies to surface antigens and Lkt, they will withstand subsequent challenge with *M. haemolytica*. *B. trehalosi* also was used in this vaccine because Lkt-positive *B. trehalosi* has been shown to cause fatal pneumonia in BHS (R. P. Dassanayake et al., unpublished data). Therefore, the objective of this study was to determine whether repeated immunization with a multivalent *Mannheimia-Bibersteinia* culture supernatant vaccine would protect BHS against experimental *M. haemolytica* challenge.

MATERIALS AND METHODS

Experimental animals. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Washington State University (WSU) before the onset of the study. Eight (2-year-old) BHS from the hand-raised captive herd at WSU were divided into two groups of four each. At the onset of the experiment, all the animals were healthy and did not have any history of respiratory disease. Nasal and pharyngeal swabs were collected to determine the presence of *M. haemolytica* and *B. trehalosi* in the nasopharynx before the onset of the study. Blood samples were collected to determine serum antibody titers against Lkt and surface antigens of *M. haemolytica* and *B. trehalosi*.

Detection of *M. haemolytica* and *B. trehalosi* in nasopharynx. Nasal and pharyngeal swabs were submitted to the Washington Animal Disease Diagnostic Laboratory (WADDL) at WSU for detection of *M. haemolytica* and *B. trehalosi* using cultural and biochemical tests. Culture results were further confirmed by genus-specific PCR as previously described (5). All *M. haemolytica* and *B. trehalosi* cultures were tested for the presence of the *lktA* gene by *lktA* gene-specific PCR (6).

Determination of antibody titers. Antibodies against surface antigens of *M. haemolytica* serotypes A1 and A2 and *B. trehalosi* serotype T10 and Lkt-containing culture supernatant fluids were measured by enzyme-linked immunosorbent assay (ELISA) (11). Lkt-neutralizing antibodies were determined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) dye reduction cytotoxicity assay (10).

Antibodies against bacterial surface antigens. Serum antibodies specific for surface antigens of *M. haemolytica* and *B. trehalosi* were measured by indirect ELISA. Briefly, bacteria were immobilized onto the wells of an ELISA plate (Becton Dickinson, Franklin Lakes, NJ) at 2×10^6 CFU/50 μl/well in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C. The plate was rinsed three times with 1:20 nonanimal protein (NAP) blocking agent (NAP-Blocker; G-Biosciences St. Louis, MO) in $1\times$ phosphate-buffered saline (PBS). Serially 2-fold diluted serum samples (50 μl) were added to the wells and incubated for 45 min at 37°C. After the plate was washed three times, rabbit anti-sheep IgG(H+L) conjugated to horseradish peroxidase (50 μl of 1:5,000 dilution; Pierce, Rockford, IL) was added. After 45 min of incubation, the substrate (100 μl of ABTS [2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate)]; KPL, Gaithersburg, MD) was added, and the plate was incubated at room temperature for 30 min. The optical density at 405 nm (OD_{405}) of the final product was measured using an ELISA plate reader (Becton Dickinson, Franklin Lakes, NJ). The highest dilution of serum yielding an OD_{405} value equal to or greater than twice the OD_{405} value given by a comparable dilution of negative-control serum was considered the antibody titer of the serum.

Antibodies against Lkt and other soluble antigens in the culture supernatant fluid. Lkt in the form of culture supernatant fluid from *M. haemolytica* A1 (50 μl per well) was immobilized onto a 96-well ELISA plate overnight at 4°C. The

plate was blocked with 1:4 NAP-Blocker (G-Biosciences) in $1\times$ PBS for 1 h at 37°C. The remaining steps were performed as described above for determination of surface antigen titers.

Neutralizing antibodies against Lkt. Lkt-nAb in serum were determined using an MTT (Sigma Chemical Co., St. Louis, MO) dye reduction cytotoxicity assay, as previously described (10). Fifty microliters of each serially diluted serum sample was incubated with 50 μl of log-phase culture supernatant fluid containing Lkt at a dilution which caused 50% cytotoxicity to bovine lymphoma cells (BL-3 cells) in the wells of a U-bottom microtiter plate for 1 h at 4°C. BL-3 cells (50 μl of 5×10^6 cells/ml) were then added to the serum-Lkt mixture and incubated at 37°C for another 1 h. The plate was centrifuged at $600 \times g$ for 5 min, and supernatant fluid was removed. The cells were resuspended in 100 μl of colorless RPMI 1640 medium (Invitrogen, Carlsbad, CA) and 20 μl of MTT dye (5 mg/ml) and incubated at 37°C for 1 h. The plate was centrifuged again at $600 \times g$ for 5 min, the fluid was decanted, and 100 μl of acid isopropanol was added to dissolve the precipitate. The OD_{540} of the final product was measured using an ELISA plate reader (Becton Dickinson). Percent inhibition was calculated as described previously (11) according to the following formula: $(\text{cytotoxicity}_{\text{Lkt+ medium}} - \text{cytotoxicity}_{\text{Lkt+ serum}})/(\text{cytotoxicity}_{\text{Lkt+ medium}}) \times 100$.

The titer was the highest dilution of the serum which yielded a minimum of 50% inhibition of Lkt-induced cytotoxicity.

Vaccines. The commercial vaccine Presponse SQ which consists of logarithmic-phase culture supernatant from *M. haemolytica* serotype A1 (ATCC 43270) as the immunogen was purchased from Boehringer Ingelheim, Ridgefield, CT (lot 084331A). An experimental vaccine against *M. haemolytica* serotype A2 and *B. trehalosi* serotype T10 was prepared as described below.

Preparation of experimental vaccine. The experimental vaccine was prepared using the logarithmic-phase culture supernatant fluids from *M. haemolytica* serotype A2 (strain WSU-1) and *B. trehalosi* serotype T10 (ATCC 33374) as the immunogen. Formulation of the vaccine was similar to that described previously (13, 16). Briefly, the culture supernatant fluid was prepared using separate logarithmic cultures of each strain in RPMI 1640 medium, filtered using a 0.22-μm-pore-size filter (Millipore, Billerica, MS), and then dialyzed across a 3,000-molecular-weight-cutoff (MWC) membrane (Spectrum, Rancho Dominiguez, CA) against distilled water. Culture supernatant fluids were frozen (−80°C) overnight and lyophilized. Lyophilized powder was reconstituted in RPMI 1640 medium to obtain a $20\times$ concentration. The vaccine consisted of equal amounts of culture supernatant fluid and adjuvant complex. The adjuvant complex consisted of $Al(OH)_3$ (23% Rehydragel HPA; General Chemical, Parsippany, NJ) and Quil-A (0.005%; Brenntag Biosector, Germany).

Immunization protocol. Four BHS were inoculated subcutaneously with 2 ml each of Presponse SQ and experimental vaccine at two different sites on day 0. Vaccination was repeated on days 21, 35, 49, and 77. Serum samples were collected from all vaccinated and control animals immediately before each administration of vaccine and before the challenge on day 89.

Challenge of BHS. All animals were challenged intranasally with *M. haemolytica* A2 (WSU-1), cultured overnight at 37°C on brain heart infusion (BHI) agar supplemented with 5% sheep blood (Remel, Lenexa, KS). The BHI agar plate was washed with colorless RPMI 1640 medium, and the bacteria were scraped from the plate and resuspended in RPMI 1640 medium to obtain an OD_{600} of 0.4. The suspension was cultured for 1.5 to 2 h until the OD_{600} reached 0.6. Following centrifugation at 4,000 rpm for 20 min, the bacterial pellet was resuspended in colorless RPMI 1640 medium to obtain a concentration of approximately equivalent to 1×10^5 CFU/5 ml. The bacterial count was extrapolated based on the standard curve plotted with number of CFU against OD. One BHS in the control group was challenged with 1×10^6 CFU on day 57. The remaining three BHS in the control group and all four BHS in the vaccinated group were challenged intranasally with 1×10^5 CFU on day 89. Animals in each group were monitored daily for clinical signs of pneumonia, including anorexia, lethargy, cough, nasal discharge, and dyspnea. Necropsy was performed on all dead animals. Lung tissue was collected aseptically from the lesional areas for histological examination and bacterial isolation. The degree of involvement of the lung lobes was scored as percent pneumonic tissue (percentage of lung that was consolidated on gross examination). Animals that survived the challenge were not euthanized but were radiographed to assess the lung consolidation at 3 and 8 weeks postchallenge.

Statistical analysis. Antibody titers against surface antigens and Lkt were logarithmically transformed to satisfy the assumption of normal distribution, and geometric mean titers (GMT) were calculated. Repeated-measures analysis of variance (ANOVA) was used to analyze the titers, and a Bonferroni test was used to make pairwise comparisons between days of vaccination. Antibody

TABLE 1. Prevaccination microbial profile of the nasopharynx of experimental BHS[a]

Group	Animal no.	M. haemolytica detected in:		B. trehalosi detected in:		lktA detection by PCR
		Nasal swab	Pharyngeal swab	Nasal swab	Pharyngeal swab	
Control	16	−	+	−	−	−
	24	−	−	−	+	−
	39	−	−	−	+	−
	45	−	−	−	+	−
Vaccinated	20	−	−	−	+	−
	22	−	−	−	+	−
	23	−	−	−	+	−
	50	−	−	−	+	−

[a] Isolates were tested by cultural and biochemical tests and confirmed by genus-specific PCR. Presence of the *lktA* gene was tested by *lktA* gene-specific PCR.

titers against surface antigens of *M. haemolytica* A1 were analyzed using Student's *t* test. GMT were transformed back to the corresponding reciprocal values to present the titers in the results. Results were analyzed using Graph-Pad Prism, version 5.0, software. A *P* value of ≤ 0.05 was considered significant.

RESULTS

Prevaccination microbial profile of the nasopharynx of BHS. No *Pasteurellaceae* of interest (*M. haemolytica* and *B. trehalosi*) were detected by standard aerobic culture or biochemical analyses in samples obtained prior to vaccination from the nasal cavities of the control or vaccinated BHS, but three controls and all four vaccinates carried *B. trehalosi* in the pharynx. The other BHS in the control group had *M. haemolytica* in the pharynx. However, neither the *B. trehalosi* nor the *M. haemolytica* isolates possessed the *lktA* gene, as determined by the *lktA* gene-specific PCR (Table 1).

Vaccinated BHS exhibited high serum titers of antibodies against surface antigens of *M. haemolytica* A1 and A2 and *B. trehalosi* T10. An indirect ELISA revealed that, prevaccination, all the BHS had low titers (<1:150) of serum antibodies against surface antigens of *M. haemolytica* A1 and A2. Following vaccination, serum antibody titers against surface antigens of *M. haemolytica* A2 increased significantly in all four BHS in comparison to the control group ($P \leq 0.0001$) (Fig. 1A). A significant increase in antibodies was also observed between priming and the subsequent two injections ($P < 0.05$). On the day of challenge, the mean serum antibody titer of the vaccinated BHS against surface antigens on *M. haemolytica* A2 was 1:4,000, whereas that of the control group was 1:125. Similarly, the vaccinated group had significantly higher serum antibodies against surface antigens of *M. haemolytica* A1 (1:2,000) than the control group (1:150; $P \leq 0.001$) (Fig. 1B) on the day of challenge.

Prevaccination, BHS in the control and the vaccinated groups had serum titers of 1:375 and 1:700, respectively, against the surface antigens of *B. trehalosi*. BHS in the vaccinated group responded to vaccination, which resulted in significantly higher antibody titers against surface antigens of *B. trehalosi* than those of the control group (Fig. 2) ($P \leq 0.0001$). The mean serum antibody titer of the vaccinated group was 1:3,200, whereas that of the control group remained at 1:375 on the day of challenge.

Vaccination of BHS resulted in high titers of serum antibodies against Lkt. The ELISA revealed that BHS in the vaccinated group developed significantly higher titers of anti-Lkt antibodies than the control group at all time points following vaccination (Fig. 3A) ($P \leq 0.0001$). The mean titer of these antibodies in the vaccinated group was 1:6,400, whereas that of the control group was 1:100 on the day of challenge. Obviously, this ELISA measures antibodies to the spectrum of soluble antigens in culture supernatant fluids, including both neutralizing and nonneutralizing antibodies to Lkt. Determination of the Lkt-nAb titers using the MTT dye reduction cytotoxicity assay revealed significantly higher Lkt-nAb titers in the vaccinated group following vaccination than in the controls ($P \leq 0.007$) (Fig. 3B). The mean serum Lkt-nAb titer in the vaccinated group on the day of challenge was 1:160, whereas that of the control group was <1:10.
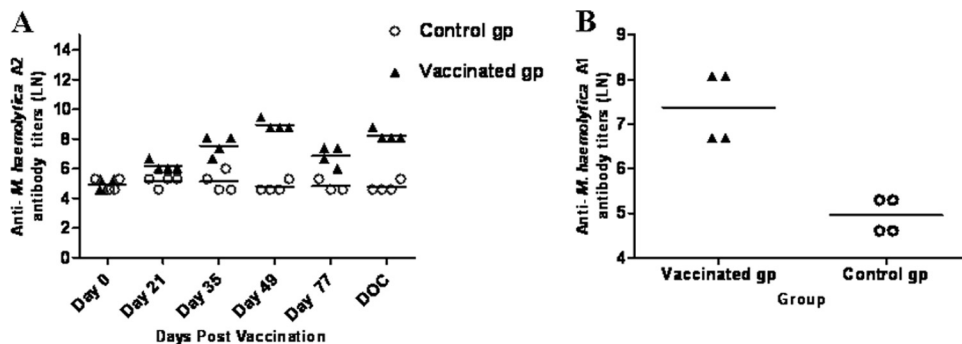

FIG. 1. Antibody titers against surface antigens of *M. haemolytica* in vaccinated and control groups of BHS. Serum antibody titers against *M. haemolytica* surface antigens were determined by indirect ELISA. Three independent assays were performed using serum samples from all the animals. Each data point represents the geometric mean titer of one animal in the respective group. The horizontal bar represents the geometric mean titer of the group at each time point. (A)Vaccinated group had significantly higher mean titers against surface antigens of *M. haemolytica* A2 than the control group after vaccination ($P < 0.0001$). (B) Vaccinated group had significantly higher mean titers against surface antigens of *M. haemolytica* A1 than the control group ($P < 0.001$) on the day of challenge (day 89). Titers are shown as the natural logarithm (LN) of the reciprocal dilution. Open circles represent control animals; closed triangles indicate vaccinated animals. DOC, day of challenge; gp, group. Titers of <100 were considered 100 for the purpose of analysis.
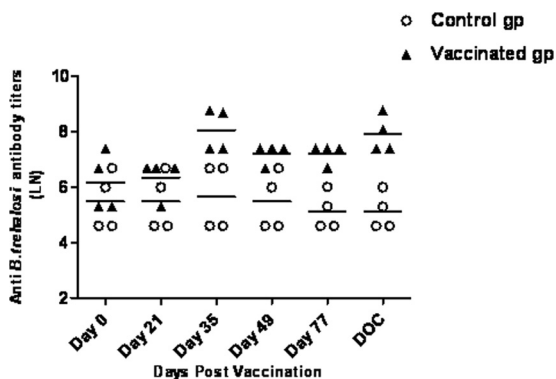
FIG. 2. Antibody titers against surface antigens of *B. trehalosi* in vaccinated and control groups of BHS. Serum antibody titers against *B. trehalosi* T10 surface antigens were determined by indirect ELISA. Three independent assays were performed using serum samples from all the animals. Each data point represents the geometric mean titer of one animal in the respective group. The horizontal bar represents the geometric mean titer of the group at each time point. The vaccinated group had significantly higher mean titers than the control group ($P \leq$ 0.0001) following vaccination. Titers are shown as the natural logarithm (LN) of the reciprocal dilution. Open circles represent control animals; closed triangles indicate vaccinated animals. DOC, day of challenge; gp, group. Titers of <100 were considered 100 for the purpose of analysis.

Vaccination protects BHS against *M. haemolytica* A2 challenge. Following challenge with *M. haemolytica* A2 (1×10^5 CFU), the BHS in the control group rapidly developed clinical signs of pneumonia. Nasal discharge, anorexia, and lethargy were apparent at 20 h postchallenge (hpc). All controls were recumbent at 30 hpc and died at 40 to 45 hpc. At necropsy, gross pathological examination revealed lesions characteristic of acute pneumonia including severe lung consolidation, fibrin deposition, and hemorrhage. Lesions were present in the entire cranial and middle lobes of the right lung (30 to 60%) and the cranial lobe of the left lung (0 to 25%) (Fig. 4A). Large numbers of bacteria were recovered from lung tissue (1×10^8 CFU/g of lung tissue), which were confirmed as *M. haemolytica*
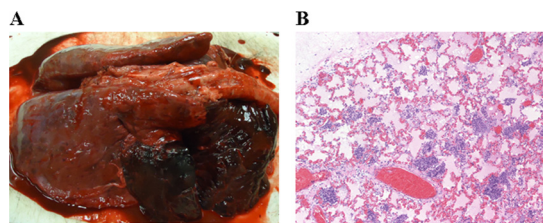

FIG. 4. Representative gross lesions and histopathological appearance of the lungs of unvaccinated control BHS. (A) Gross pathology showing severe consolidation in the right cranial, middle, and left cranial lobes of the lung. (B) Histopathology showing extremely congested pulmonary vasculature, expanded and edematous interlobular septa and alveoli, and clusters of necrotic neutrophils, intermixed with bacterial colonies. Sections were stained with hematoxylin and eosin (magnification, ×100).

by culture and biochemical method and by PCR. Histological sections of lesional lung tissue revealed extremely congested pulmonary vasculature, expanded and edematous interlobular septa and alveoli, and clusters of necrotic neutrophils, intermixed with bacterial colonies (Fig. 4B). Two BHS in the vaccinated group were slightly lethargic during the first 2 days postchallenge but recovered quickly. All of them were alert, active, and exhibited good appetite throughout the study period. Radiography at 3 weeks postchallenge revealed no lesions in two BHS (Fig. 5A). The other two BHS had moderately increased interstitial density within the cranial and/or caudal lung field and increased soft tissue opacity within the cranial lung field, suggestive of mild pneumonia (Fig. 5B). However, lungs of these two BHS did not exhibit any abnormalities when they were radiographed again at 8 weeks postchallenge (Fig. 5C). All four vaccinated BHS remain healthy up to the date of preparation of the manuscript (16 weeks postchallenge).

DISCUSSION

Two previous attempts to immunize BHS against *M. haemolytica* have not succeeded. In the first study, 100% of BHS ($n =$
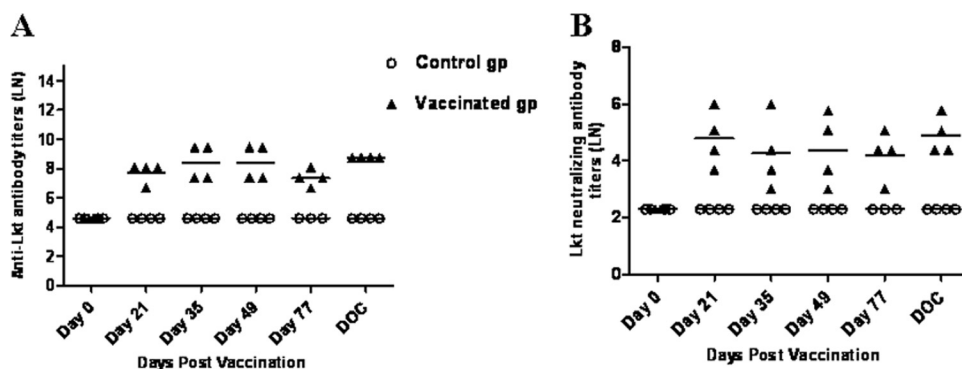

FIG. 3. Lkt antibody titers of vaccinated and control groups of BHS. (A) Antibodies against soluble antigens including Lkt in the culture supernatant fluid were measured by an indirect ELISA. The vaccinated group had significantly higher geometric mean titers than the control group ($P \leq$ 0.0001) following vaccination. Titers of <100 were considered 100 for the purpose of analysis. (B) Lkt neutralizing antibodies were measured by a MTT dye reduction cytotoxicity assay. Vaccinated BHS had significantly higher Lkt-nAb than controls ($P \leq$ 0.007) following vaccination. Titers of <10 were considered 10 for the purpose of analysis. Each data point represents the geometric mean titer of one animal in the respective group. The horizontal bar represents the geometric mean titer of the group at each time point. Three independent assays were performed using serum samples from all the animals. Titers are shown as the natural logarithm (LN) of the reciprocal dilution. Open circles represent control animals; closed triangles indicate vaccinated animals. DOC, day of challenge; gp, group.
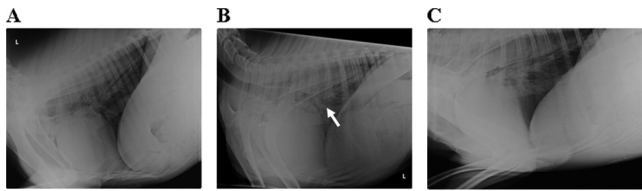
FIG. 5. Radiographs of vaccinated BHS at 3 weeks and 8 weeks postchallenge. (A) Radiograph of the thorax of one of the two healthy BHS at 3 weeks postchallenge. (B) Radiograph of the thorax of one of the two BHS, suggestive of mild pneumonic lesions, at 3 weeks postchallenge. The arrow indicates interstitial density and increased soft tissue opacity in the cranial field of the lungs. (C) Radiograph of the thorax of one of the two BHS (that had evidence of mild pneumonic lesions at 3 weeks postchallenge) at 8 weeks postchallenge.

3) vaccinated with an experimental bacterin-toxoid vaccine comprised of *Pasteurella haemolytica* serotypes A2, T3, and T10 (currently classified as *M. haemolytica* A2 and *B. trehalosi* T3 and T10, respectively) died of pneumonia following commingling with healthy DS known to carry *P. haemolytica* A1 and A2 in their nasal cavities (7). In the second study, 100% of BHS in two groups ($n = 2$ in each group) vaccinated by intratracheal inoculation of live cultures of cytotoxic or noncytotoxic strains of *M. haemolytica* A11 (currently classified as *Mannheimia glucosida*) succumbed after challenge with *M. haemolytica* A2 (8). In these two studies induction of antibodies in response to the vaccination was not evaluated; hence, the reason(s) for vaccine failure could not be determined. In the only study in which the development of antibodies in response to the vaccine (serotype A1, A2, and T10 culture supernatant fluids) was measured, the BHS were not challenged with *M. haemolytica*. Instead, *P. haemolytica* T10 (now *B. trehalosi* T10) was used for challenge. Even with *B. trehalosi* challenge, the percent protection was only 60 to 80% in the vaccinated BHS. Twenty percent of the controls also survived. Furthermore, cumulative postmortem scores based on gross lesions and bacteriology did not differ between vaccinated and control animals (15). Thus, unambiguous data on the role of vaccination in protection of BHS against pneumonia caused by *M. haemolytica* are not currently available. Therefore, we designed this study to test the hypothesis that vaccination to induce high titers of antibodies to *M. haemolytica* surface and soluble antigens and of Lkt-neutralizing antibodies will protect BHS against subsequent challenge with *M. haemolytica*. As a proof-of-concept experiment, we determined whether immunization of BHS with a multivalent (serotypes A1, A2, and T10) vaccine would protect against *M. haemolytica* A2 challenge. Our hypothesis was generated by the observation that DS, which are relatively resistant to *M. haemolytica* pneumonia, carry mostly Lkt-positive *M. haemolytica* in the nasopharynx and consequently have higher serum Lkt-nAb and antibodies against surface antigens of this organism (11, 22).

The prevaccination microbial profile of the BHS used in this study indicated that the *B. trehalosi* and *M. haemolytica* isolates from these animals did not possess the *lkt* gene (Table 1), which is in agreement with previous reports (18, 23) and supports our notion that the lack of Lkt-nAb in BHS is primarily due to the absence of Lkt-positive *M. haemolytica* and *B. trehalosi* as commensal bacteria in the nasopharynx. The single

study (25) that suggested that there was no difference between the *M. haemolytica* strains isolated from BHS and DS utilized a beta-hemolysis assay, which, unlike the cytotoxicity assay, is not absolutely confirmative of leukotoxicity (Shanthalingam et. al., unpublished). *M. haemolytica* has been shown to consistently cause pneumonia in BHS under experimental conditions (4, 9, 22). This organism has also been identified in pneumonic BHS lungs (4, 9, 22; also Shanthalingam et. al, unpublished). Although *B. trehalosi* also has been isolated from pneumonic lungs, the great majority of these isolates are Lkt-negative and hence cannot be the pathogens that cause fatal pneumonia in BHS (Shanthalingam et. al., unpublished). However, since the small number of Lkt-positive strains of *B. trehalosi* can cause fatal pneumonia in BHS (Dassanayake et al., unpublished), we included the culture supernatant from both species in the experimental vaccine. Although *M. haemolytica* A2 is the serotype that is commonly found in DS and BHS, serotype A1 also can cause fatal pneumonia in BHS (4). Hence, we also included the commercial vaccine Presponse SQ which contains A1 culture supernatant as the immunogen. Culture supernatant fluid that we used as the immunogen in our experimental vaccine has Lkt and other soluble antigens, including outer membrane proteins which can contribute to efficacy, for example, by inducing opsonizing antibodies. Earlier studies in BHS used challenge doses ranging from 1×10^7 to 6×10^9 CFU per animal intratracheally or oropharyngeally. However, in this study we administered 1×10^6 CFU intranasally to one control animal and 1×10^5 CFU by the same route to the other three control animals and all the four vaccinated animals. All control animals died of pneumonia within 48 h postchallenge, suggesting that intranasal administration of 1×10^5 CFU of the *M. haemolytica* A2 strain that we used (WSU-1) is adequate for causing fatal pneumonia.

The presence of significantly higher titers of Lkt-nAb and anti-surface antigen antibodies in all four vaccinated BHS, but not in nonvaccinated control BHS (Fig. 1 to 3), is consistent with the hypothesis that the vaccinated BHS survived the challenge because of the protection conferred by these antibodies. Even the two BHS that had mild lesions at 3 weeks postchallenge recovered, and their lungs were normal in the radiographs taken at 8 weeks postchallenge (Fig. 5C). More importantly, all four vaccinated BHS remained healthy up to the last observation, 16 weeks postchallenge.

Although we included *M. haemolytica* serotypes A1 and A2 and *B. trehalosi* serotype T10 in the vaccine, we challenged the vaccinated group only with *M. haemolytica* A2 in this phase of the study for two reasons. First, it is A2 that predominantly infects BHS, and, second, inclusion of all three organisms in the challenge inoculum would preclude us from knowing which one of them killed the BHS if they did not survive the challenge. In the experiment reported here, the vaccine was administered five times. However, anti-surface antigen antibodies and Lkt-nAb did not increase further after the third injection, suggesting that three injections might have given the same outcome as five if BHS were challenged after 12 days of last immunization. Nevertheless, one might question the practicality of giving even three injections to wild sheep. It should be emphasized here that this was a proof-of-concept study, designed to answer the question whether BHS would withstand challenge if they carried sufficiently high titers of Lkt-nAb and

anti-surface antigen antibodies. All controls died while 100% of vaccinated BHS survived the challenge with *M. haemolytica* A2, which, to our knowledge, has not been reported previously. With this new knowledge we will now focus on developing a vaccine that does not require repeated injections. A live vector-based vaccine may satisfy this requirement. A vaccine deliverable noninvasively, for example, through the feed or water, would represent the ideal vaccine for wild BHS.

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