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Comparison of Passively Transferred Antibodies in Bighorn and Domestic Lambs Reveals One Factor in Differential Susceptibility of These Species to *Mannheimia haemolytica*-Induced Pneumonia▽

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***Mannheimia haemolytica* consistently causes fatal bronchopneumonia in bighorn sheep (BHS; *Ovis canadensis*) under natural and experimental conditions. Leukotoxin is the primary virulence factor of this organism. BHS are more susceptible to developing fatal pneumonia than the related species *Ovis aries* (domestic sheep [DS]). In BHS herds affected by pneumonia, lamb recruitment is severely impaired for years subsequent to an outbreak. We hypothesized that a lack of maternally derived antibodies (Abs) against *M. haemolytica* provides an immunologic basis for enhanced susceptibility of BH lambs to population-limiting pneumonia. Therefore, the objective of this study was to determine the titers of Abs directed against *M. haemolytica* in the sera of BH and domestic lambs at birth through 12 weeks of age. Results revealed that BH lambs had approximately 18-fold lower titers of Ab against surface antigens of *M. haemolytica* and approximately 20-fold lower titers of leukotoxin-neutralizing Abs than domestic lambs. The titers of leukotoxin-neutralizing Abs in the serum and colostrum samples of BH ewes were approximately 157- and 50-fold lower than those for domestic ewes, respectively. Comparatively, the higher titers of parainfluenza 3 virus-neutralizing Abs in the BH lambs ruled out the possibility that these BHS had an impaired ability to passively transfer Abs to their lambs. These results suggest that lower levels of leukotoxin-neutralizing Abs in the sera of BH ewes, and resultant low Ab titers in their lambs, may be a critical factor in the poor lamb recruitment in herds affected by pneumonia.**

The bighorn sheep (BHS; *Ovis canadensis*) population of North America has declined from an estimated 2 million animals at the beginning of the 19th century to less than 70,000 at this time (2, 32). Factors contributing to this population decline include predation, loss of habitat, competition for forage with livestock, and respiratory disease. Outbreaks of bronchopneumonia in previously healthy populations of BHS often result in high death rates among all age groups initially, followed by years of impaired recruitment due to pneumonia in lambs (5, 21, 24, 28). During these outbreaks, members of the genera *Mannheimia*, *Bibersteinia*, and *Pasteurella*, including *Mannheimia* (*Pasteurella*) *haemolytica*, *Bibersteinia* (*Pasteurella*) *trehalosi*, and *Pasteurella multocida*, have commonly been isolated from pneumonic lungs (16, 26). Of these, *M. haemolytica* has consistently been shown to cause fatal bronchopneumonia in BHS under experimental conditions (9, 12, 20). While *M. haemolytica* can cause pneumonia in multiple ruminant species, including cattle and domestic sheep (DS), BHS are particularly susceptible to this disease. A number of studies have shown that BHS demonstrate greater susceptibility to *M. haemolytica*, and exhibit more severe pathology when infected, than DS (9, 11, 12, 24).

There is evidence for a protective role for antibodies (Abs) against surface antigens of *M. haemolytica* and its virulence factors, as vaccines containing these antigens and the exotoxin (leukotoxin [Lkt]) produced by this organism have conferred protection against experimental challenge (3, 19, 22, 30). High titers of Lkt-specific Abs also reduced the risk of pasteurellosis in cattle infected either experimentally or naturally (7). Furthermore, administration of immunoglobulins (Ig) from immune sheep to lambs resulted in protection against experimental *M. haemolytica* challenge (18).

In ruminants, due to the syndesmochorial nature of the placenta, maternal Abs are not transferred to the offspring *in utero* (25). In neonatal ruminants, the transfer of maternal Abs occurs via colostrum during the first 24 h of life. This transfer of maternal Abs via colostrum is critically important for immunologic protection of neonatal ruminants as their immune systems become fully competent. Failure of passive transfer may occur at multiple levels, including insufficient concentration of Ig in the colostrum due to lack of specific pathogen exposure or an inability to respond, insufficient intake of colostrum by the neonate, or inefficient transfer of Ig from the neonatal intestine to blood. Failure of passive transfer has been associated with multiple diseases of ruminant neonates, including respiratory disease, diarrhea, septicemia, and omphalophlebitis (13, 14). The poor lamb recruitment observed in BHS herds following outbreaks of pneumonia, and the importance of Abs in protection against *M. haemolytica*-caused pneumonia, prompted us to hypothesize that BH lambs receive lower levels of *M. haemolytica*-related Abs from their dams than domestic lambs. Therefore, the objective of this study was to determine the titers of Ab against *M. haemolytica*

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surface antigens and Lkt in the sera of lambs from these two species at birth and weekly for 12 weeks.

MATERIALS AND METHODS

Materials. Rabbit anti-sheep IgG conjugated to horseradish peroxidase and the ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate system were purchased from KPL (Gaithersburg, MD). PRO-BIND 96-well enzyme-linked immunosorbent assay (ELISA) plates were purchased from Becton Dickinson (Franklin Lakes, NJ). The MTT (3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyl tetrazolium bromide) dye and *o*-phenylenediamine were purchased from Sigma Aldrich (St. Louis, MO). Colorless RPMI 1640 was purchased from Invitrogen (Carlsbad, CA). Immulon IB plates were purchased from Dynatech Laboratories (Alexandria, VA).

Sample collection and processing. Serum samples were collected via venipuncture from 12 domestic ewes and 12 BH ewes (DS herd 1 and BHS herd 1) approximately 2 weeks prior to parturition. Colostrum samples were collected from these animals within 24 h postpartum. Serum samples were collected via venipuncture from 12 domestic lambs and 12 BH lambs on the first day of birth and at 1, 2, 3, 4, 6, 8, 10, and 12 weeks after birth. Serum samples were frozen at −80°C until used. Colostrum samples collected from each ewe within 24 h postpartum were diluted 1:7 in 1× phosphate-buffered saline (PBS) and centrifuged at 500 × *g* for 10 min at 4°C. The samples were frozen at −80°C after removal of the fat layer. Archived serum samples from wild BHS in Hells Canyon and domestic ewes from Dubois, ID, were also used in this study. All lambs received a commercially available 8-way clostridium vaccine (Covexin; Schering-Plough). All BH lambs also received a vaccine against Rota-Corona virus (Calf Guard; Pfizer) and *Escherichia coli* antibodies (Ecolizer; Novartis). None of the animals used in this study had a history of respiratory disease.

Measurement of Lkt-neutralizing antibodies. Lkt-neutralizing Abs from serum and colostrum samples of BHS and DS were measured with an inhibition-of-MTT-dye-reduction-cytotoxicity assay. Leukotoxin from *M. haemolytica* serotype A1 was prepared according to the method of Gentry and Srikumaran (15). Serial dilutions of serum samples (25 μl) were incubated with Lkt (25 μl) for 1 h at room temperature. The Lkt was used at a concentration determined to cause 50% cytotoxicity to bovine lymphoma 3 (BL-3) cells after the above-mentioned incubations. BL-3 cells were added at 2.5×10^6 cells/ml in a volume of 50 μl/well. Plates were then incubated at 37°C for 1 h, after which they were centrifuged at 600 × *g* for 5 min. Supernatant was discarded, and cells were resuspended in 100-μl/well colorless RPMI 1640. MTT dye was added at 5 mg/ml in colorless RPMI 1640 in a volume of 20 μl/well, and plates were incubated for 2 h at 37°C. Plates were centrifuged as described above, and precipitate was dissolved in 100-μl/well acid isopropanol. Absorbance of wells was measured with an automated plate reader at an optical density of 540 nm (OD_{540}). Percent inhibition of cytotoxicity was calculated as previously described (8): percent inhibition = [(cytotoxicity given by Lkt plus medium) − (cytotoxicity given by Lkt plus serum)]/(cytotoxicity given by Lkt plus medium) × 100.

The Lkt-neutralizing Ab titer was calculated as the reciprocal of the highest dilution yielding 50% inhibition of cytotoxicity.

Measurement of antibodies against *Mannheimia haemolytica* surface antigens. Titers of antibody against the surface antigens of *M. haemolytica* were determined by a standard indirect ELISA (33). *M. haemolytica* serotype A2 from a 24-h culture was added to the wells of an ELISA plate at 2×10^6 CFU/50 μl/well in a coating buffer consisting of 15 mM Na_2C0_3 and 35 mM $NaHCO_3$ overnight at 4°C. Serum or colostrum samples were serially diluted, added to plates, and incubated at 37°C for 45 min, followed by incubation with rabbit anti-sheep IgG Abs conjugated to horseradish peroxidase. All incubations were carried out at 37°C for 45 min, and ABTS was used as a substrate. Absorbance was read at 405 nm with an automated ELISA plate reader. Antibody titers were calculated as the reciprocal of the highest dilution yielding greater than 2× the optical density of serially diluted negative-control serum.

Measurement of antibodies against PI-3. Parainfluenza 3 virus (PI-3)-neutralizing Ab titers were measured by the Washington Animal Disease Diagnostic Laboratory in Pullman, WA. Briefly, serum samples were heat inactivated for 30 min at 57°C. Serum and colostrum samples were diluted 2-fold and added to wells of a 96-well plate in a volume of 50 μl/well, and the same volume of PI-3 virus diluted 1:500 was then added, followed by an incubation of 1 h at room temperature. Bovine turbinate cells (2×10^3 cells/50 μl/well) were added, and plates were incubated for 4 to 7 days. The virus-neutralizing Ab titer was calculated as the highest dilution yielding less than 50% cytopathic effect. Controls consisted of positive-control serum samples and cells without virus.

Data analysis. Titers of antibody against *M. haemolytica* surface antigens, Lkt-neutralizing Abs, and PI-3-neutralizing Abs in the serum and colostrum samples of BH and domestic ewes were logarithmically transformed to allow for normal distribution and analyzed by one-way analysis of variance (ANOVA), followed by a Bonferroni correction. Titers of antibody against surface antigens of *M. haemolytica* and Lkt-neutralizing Abs in the serum samples of BH and domestic lambs were logarithmically transformed to allow for normal distribution and analyzed by repeated measures ANOVA, where time was treated as a nested variable.

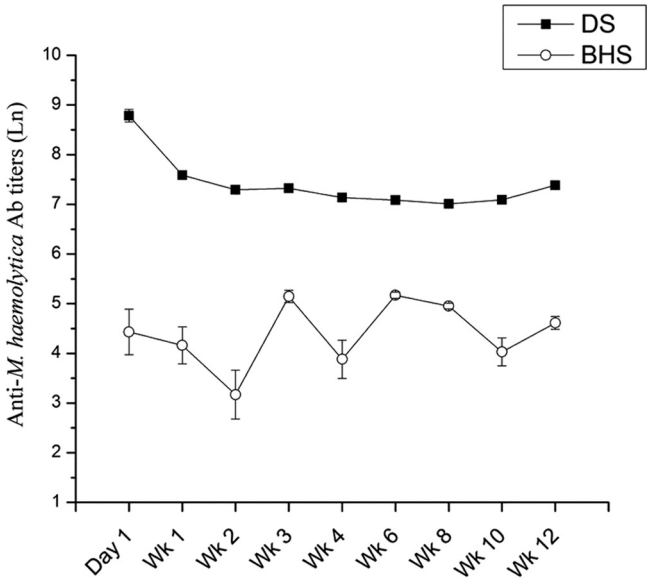

FIG. 1. Titers of antibody against *M. haemolytica* surface antigens in serum samples of BH and domestic lambs ($n = 12$ each). Antibody titers were measured by an indirect ELISA. Samples from each animal were tested in two independent experiments, and each data point represents the mean titer for each species at the indicated time point. Values for BH lambs are significantly lower ($P < 0.0005$) than those for domestic lambs by repeated-measures ANOVA. Error bars represent standard errors of the means (SEM).

RESULTS

Titers of antibody against *M. haemolytica* surface antigens and leukotoxin-neutralizing antibody titers in the sera of BH lambs were significantly lower than those for domestic lambs. Results of the ELISA evaluating sera from the BH and domestic lambs revealed that BH lambs had significantly lower Ab titers to surface antigens of *M. haemolytica* ($P < 0.0005$) (Fig. 1). The mean titer of Ab against *M. haemolytica* surface antigens in BH lambs 24 h after birth was 1:500, whereas that for domestic lambs was 1:9,000, an approximately 18-fold difference. Over the next 12 weeks, the mean titer of Abs against *M. haemolytica* surface antigens gradually decreased in domestic lamb sera but still remained high at the end of 12 weeks, at >1:1,000 compared to the level for BH lambs. The mean titer of Ab against surface antigens of *M. haemolytica* in BH lambs decreased from the already comparatively low titer of 1:500 on day 1 to 1:100 at 12 weeks after birth.

Results of the inhibition of cytotoxicity assays performed on lamb serum samples revealed extremely low levels of Lkt-neutralizing Abs in BH lambs in comparison to the level for domestic lambs ($P < 0.0005$) (see Fig. 3). The mean Lkt-neutralizing Ab titer for BH lambs 24 h after birth was 1:200, whereas that for domestic lambs was 1:4,000, an approximately

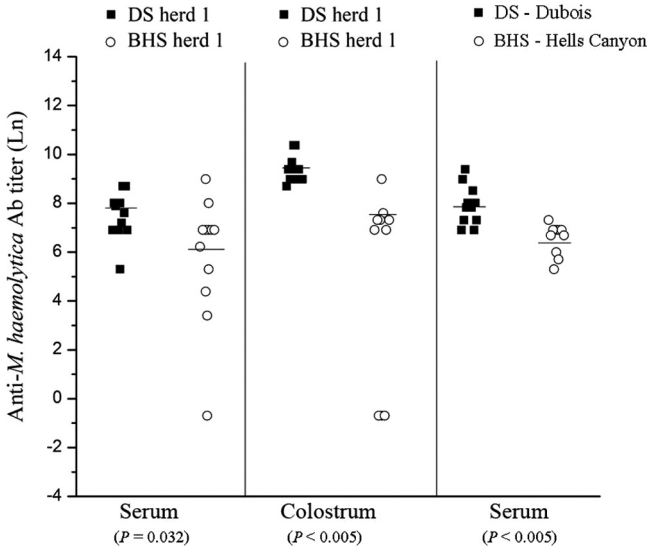

FIG. 2. Titers of antibody against *M. haemolytica* surface antigens in the serum and colostrum samples of BH and domestic ewes ($n = 12$ for each group). Antibody titers were measured by an indirect ELISA. Samples from each animal were tested in two independent experiments, and each data point represents the mean titer for an individual animal. Species means are indicated by horizontal lines.

20-fold difference. Over the subsequent 12 weeks, the mean Lkt-neutralizing Ab titer for domestic lambs gradually decreased, but still remained high at >1:2,500 at the end of 10 weeks, and dropped to 1:500 at 12 weeks after birth. In contrast, the mean Lkt-neutralizing Ab titer for BH lambs decreased from a titer of 1:200 on day 1 to approximately 1:10 by 12 weeks after birth.

Titers of antibody against *M. haemolytica* surface antigens and leukotoxin-neutralizing antibody titers in the sera of BH ewes were lower than those for domestic ewes. The comparatively low levels of Abs against *M. haemolytica* surface antigens and Lkt-neutralizing Abs in BH lambs in comparison to those in domestic lambs prompted us to determine the levels of these Abs in the archived serum and colostrum samples from the dams of these lambs (Fig. 2, BHS herd 1 and DS herd 1). The mean serum titer of Abs against surface antigens of *M. haemolytica* for BH ewes 2 weeks prepartum was 1:1,400, whereas that for domestic ewes was 1:2,500, an approximately 1.8-fold difference ($P = 0.032$).

In contrast to the titers of Abs against *M. haemolytica* surface antigens, the Lkt-neutralizing Ab titers in the sera of BH ewes were extremely low, compared to those for domestic ewes ($P < 0.0005$) (see Fig. 4). The mean Lkt-neutralizing Ab titer for BH ewes 2 weeks prepartum was 1:70, whereas that for domestic ewes was 1:11,000, an approximately 157-fold difference.

Titers of antibody against *M. haemolytica* surface antigens and leukotoxin-neutralizing antibody titers in the colostrum samples of BH ewes were significantly lower than those for domestic ewes. Because there were lower titers of Ab against surface antigens of *M. haemolytica* and Lkt-neutralizing Abs in the sera of BH ewes than in those of domestic ewes, we wanted to determine whether this difference was reflected in the colostrum samples of these two species as well. As expected, the Ab titers specific for *M. haemolytica* surface antigens in the BH colostrum were lower than those for DS ($P < 0.005$) (Fig. 2). The mean titer of colostral Ab against *M. haemolytica* surface antigens on the day of parturition was 1:1,700, whereas that for DS was 1:13,500, an approximately 8-fold difference.

The Lkt-neutralizing Ab titers in the colostrum samples of BH ewes were also lower than those for domestic ewes ($P < 0.0005$) (see Fig. 4). The mean Lkt-neutralizing Ab titer in the colostrum samples of BH ewes was 1:50 on the day of parturition, whereas that for domestic ewes was 1:2,500, an approximately 50-fold difference.

Wild BH ewes and domestic ewes from different herds exhibited differences in titers of antibody against surface antigens of *M. haemolytica* and leukotoxin-neutralizing antibodies that were similar to the differences observed with the BHS and DS from our herds. To rule out the possibility that the remarkably lower titers of Ab against surface antigens of *M. haemolytica* and Lkt-neutralizing Abs in BHS were unique to our captive BHS herd, we tested serum samples from 12 randomly selected wild BH ewes. We also tested serum samples from 12 domestic ewes from a herd located more than 100 km from our herd. The BHS from Hells Canyon exhibited significantly lower levels of Abs against surface antigens of *M. haemolytica* ($P < 0.005$) and Lkt-neutralizing Abs ($P < 0.0005$) than the DS from Dubois, ID (Fig. 2; see also Fig. 4). The mean serum titer of Ab against *M. haemolytica* for the Hells Canyon BHS was 1:800, whereas that for the Dubois DS was 1:3,500, an approximately 4-fold difference. The mean Lkt-neutralizing Ab titer for BH ewes was 1:200, whereas that for domestic ewes was 1:16,000, an approximately 280-fold difference.

The BH ewes exhibited high titers of PI-3-neutralizing antibodies. To determine whether the BHS are deficient in Ab production against other pathogens as well, we tested serum samples from BHS herd 1 and DS herd 1 for neutralizing Abs against PI-3 (see Fig. 5). We chose to determine PI-3-neutralizing titers because several BHS herds have been reported to test positively for anti-PI-3 Abs (26, 29). Results from the virus neutralization assays revealed that the BHS serum and colostrum samples had high levels of PI-3-neutralizing Abs. The mean PI-3-neutralizing serum Ab titer for BH ewes was 1:400, whereas that for domestic ewes was 1:50 ($P < 0.0005$). The mean PI-3-neutralizing colostrum Ab titer for BH ewes was 1:100, while the PI-3-neutralizing Abs in the colostrum samples of the domestic ewes were below detection ($P < 0.0005$).

The BH lambs acquired high titers of PI-3-neutralizing antibodies from their dams. All the BH lambs had significantly higher titers of PI-3-neutralizing Abs in their sera than the domestic lambs ($P < 0.0005$). The mean titer for the BH lambs was 1:400, while the domestic lambs had a low titer of 1:50 (see Fig. 5).

DISCUSSION

Outbreaks of bronchopneumonia often result in high mortality among all age groups of BHS (5, 21, 24, 28), and lamb recruitment is extremely poor in the years subsequent to an outbreak. The major cause of impaired lamb recruitment is pneumonia (5, 6, 10, 27). Why are these lambs unable to resist the infection? Do they fail to acquire passive immunity from their mothers via colostrum? In an effort to answer these ques-

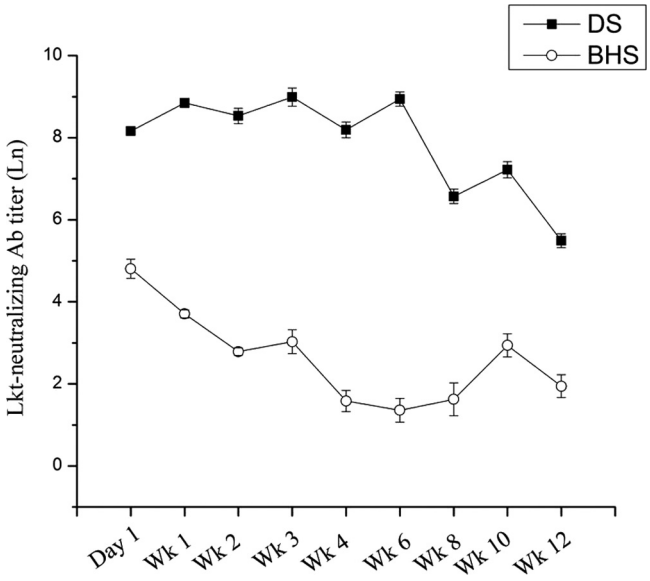

FIG. 3. Lkt-neutralizing antibody titers in serum samples of BH and domestic lambs ($n = 12$ each). Antibody titers were measured by an MTT dye reduction cytotoxicity assay. Each sample was tested in two independent experiments, and each data point represents the mean titer for each species at the indicated time point. Values for BH lambs are significantly lower ($P < 0.0005$) than those for domestic lambs by repeated-measures ANOVA. Error bars represent SEM.

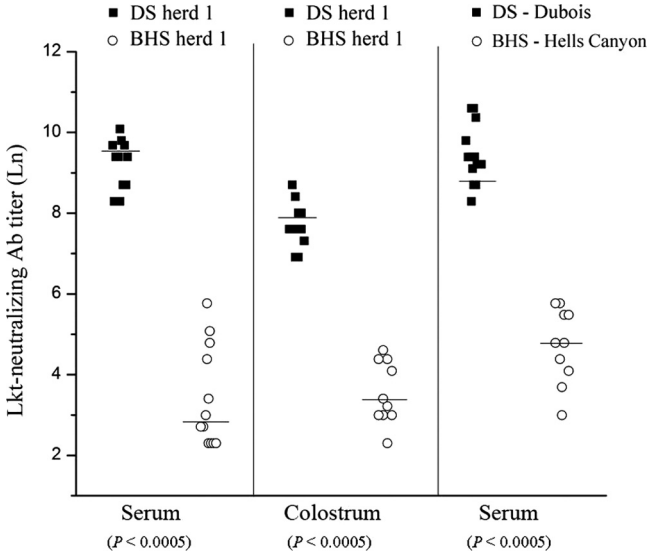

FIG. 4. Lkt-neutralizing antibody titers in the serum and colostrum samples of BH and domestic ewes ($n = 12$ per group). Antibody titers were measured by an MTT dye reduction cytotoxicity assay. Each sample was tested in two independent experiments, and each data point represents the mean titer for one individual animal. Species means are represented by horizontal lines.

tions, we chose to determine the titers of Ab against *M. haemolytica* in the sera of BH lambs. Although *M. haemolytica*, *B. trehalosi*, and *P. multocida* are isolated from the pneumonic lungs of BHS, *M. haemolytica* has consistently been shown to cause fatal pneumonia in BHS under experimental conditions (9, 17, 23). Furthermore, we have previously shown that Lkt-positive, but not Lkt-negative, *M. haemolytica* causes fatal pneumonia in BHS. Therefore, in this study, we compared the levels of Abs against *M. haemolytica* surface antigens and Lkt in the serum samples from BH and domestic lambs. Our ELISA results clearly showed that the BH lambs had lower serum titers of Abs against surface antigens of *M. haemolytica* than the domestic lambs (Fig. 1). This is not due to higher reactivity of the secondary Ab (rabbit anti-sheep Ig Abs) with DS Abs, because this Ab reacted equally with IgG purified from BHS and DS serum samples (data not shown). More importantly, the Lkt-neutralizing serum Ab titers of BH lambs were even lower than the serum titers of Ab against *M. haemolytica* surface antigens (Fig. 3).

The lower titers of Abs against *M. haemolytica* surface antigens and Lkt-neutralizing Abs in the sera of BH lambs could be due to (i) lower titers of these Abs in the sera of the dams, (ii) lower titers of these Abs in the colostrum samples of their mothers, (iii) reduced consumption of colostrum by the lambs, or (iv) impaired transfer of these Abs from the intestines into the blood of the lambs. The titers of Abs against *M. haemolytica* surface antigens and the Lkt-neutralizing Abs in the serum and colostrum samples of BHS were remarkably lower than those of DS (Fig. 2 and 4). Therefore, it is very likely that the lower titers of these Abs in the sera of BH lambs is not due to reduced consumption of colostrum or impaired transfer of these Abs from the intestines into the blood. The fact that the titers of these Abs were also lower in the serum samples from wild BHS than in those from DS from another herd suggests that the observed lower titers of these Abs are not unique to our captive herd of BHS but are representative of BHS in general. The difference in Abs against *M. haemolytica* surface antigens between BHS herd 1 and DS herd 1 was not statistically significant, although this difference was found to be significant when BH and domestic ewes from other herds were compared. This most likely reflects the large variation in the BH ewes from herd 1.

These observations raise the question as to whether BHS have an impaired ability to mount a robust Ab response against all pathogens. The results from the virus neutralization assays revealed that BHS could mount a strong Ab response against PI-3 virus (Fig. 5). Furthermore, the serum titers of Ab against *M. haemolytica* surface antigens were not as low as their Lkt-neutralizing Ab titers, which suggests that the ability of BHS to mount an Ab response is not impaired. Bighorn sheep are capable of producing levels of Lkt-neutralizing Abs comparable to those produced by DS (34). So why then are the Lkt-neutralizing serum Ab titers extremely low in BH ewes, in comparison with those for domestic ewes? This is very likely due to the fact that most of the *M. haemolytica* organisms occurring as commensal bacteria in the nasopharynx of BHS are Lkt negative, whereas most of those in the DS are Lkt positive (31). Continuous exposure of the immune system of DS to Lkt-positive organisms likely results in the development of high titers of Lkt-neutralizing Abs, whereas the lack of exposure to Lkt-positive organisms results in no or low titers of Lkt-neutralizing Abs. It is also possible that DS carry *M. haemolytica* serotypes that stimulate stronger cross-reacting Abs than those carried by BHS.

The question still remains as to why the mortality rate

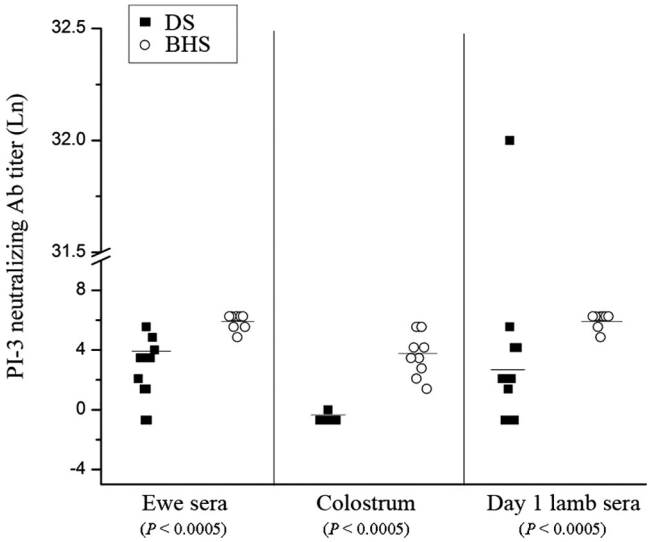

FIG. 5. Parainfluenza 3 virus-neutralizing antibody titers in the serum and colostrum samples of BH and domestic ewes and in the serum samples of BH and domestic lambs on day 1 (n = 12 each group). Neutralizing antibody titers were measured by inhibition of cytopathic effect on bovine turbinate cells. Each data point represents the antibody titer for one individual animal from a representative experiment. Species means are indicated by horizontal lines.

among the lambs is much higher than that among the adult BHS in the period following an outbreak of pneumonia. It is possible that some of the adult BHS surviving an outbreak shed low doses of Lkt-positive organisms that are adequate to cause fatal pneumonia in the lambs with low levels of maternally derived protective Abs but not in the adults. It is also possible that the immune system of BH lambs, in comparison to that of adult BHS, is not fully developed at the time of infection and hence is unable to resist the infection. Further studies are necessary to elucidate this prospect.

We should note that in this study, it was not possible to ensure that colostrum samples from all the ewes were collected before they were suckled by the lambs. It has been reported that Ab concentration in the sera of lambs increases rapidly after suckling, and it is therefore likely that the Ab concentration in the colostrum declines rapidly after suckling (1). Therefore, in this study, the Ab titers in the colostrum samples of different ewes may not accurately reflect the actual titers. Nevertheless, this does not affect the observations made regarding the serum Ab concentrations.

In summary, results of this study suggest that lower levels of Lkt-neutralizing Abs in BH ewes, and subsequently low Ab levels in the lambs, may be a crucial contributing factor to the poor lamb recruitment seen in the years following a pneumonia outbreak.

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