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TRANSMISSION OF *MANNHEIMIA HAEMOLYTICA* FROM DOMESTIC SHEEP (*OVIS ARIES*) TO BIGHORN SHEEP (*OVIS CANADENSIS*): UNEQUIVOCAL DEMONSTRATION WITH GREEN FLUORESCENT PROTEIN-TAGGED ORGANISMS

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ABSTRACT: Previous studies demonstrated that bighorn sheep (*Ovis canadensis*) died of pneumonia when commingled with domestic sheep (*Ovis aries*) but did not conclusively prove that the responsible pathogens were transmitted from domestic to bighorn sheep. The objective of this study was to determine, unambiguously, whether *Mannheimia haemolytica* can be transmitted from domestic to bighorn sheep when they commingle. Four isolates of *M. haemolytica* were obtained from the pharynx of two of four domestic sheep and tagged with a plasmid carrying the genes for green fluorescent protein (GFP) and ampicillin resistance (AP^R). Four domestic sheep, colonized with the tagged bacteria, were kept about 10 m apart from four bighorn sheep for 1 mo with no clinical signs of pneumonia observed in the bighorn sheep during that period. The domestic and bighorn sheep were then allowed to have fence-line contact for 2 mo. During that period, three bighorn sheep acquired the tagged bacteria from the domestic sheep. At the end of the 2 mo of fence-line contact, the animals were allowed to commingle. All four bighorn sheep died 2 days to 9 days following commingling. The lungs from all four bighorn sheep showed gross and histopathologic lesions characteristic of *M. haemolytica* pneumonia. Tagged *M. haemolytica* were isolated from all four bighorn sheep, as confirmed by growth in ampicillin-containing culture medium, PCR-amplification of genes encoding GFP and Ap^R, and immunofluorescent staining of GFP. These results unequivocally demonstrate transmission of *M. haemolytica* from domestic to bighorn sheep, resulting in pneumonia and death of bighorn sheep.

Key words: Bighorn sheep, domestic sheep, green fluorescent protein, *Mannheimia haemolytica*, *Ovis canadensis*, pneumonia, transmission.

INTRODUCTION

The large decline in the bighorn sheep (*Ovis canadensis*) population in North America, from an estimated two million at the beginning of the 19th century to fewer than 70,000 now (2009) (Buechner, 1960; Valdez and Krausman, 1999), has been attributed in part to diseases, particularly pneumonia caused by bacteria of the genera *Mannheimia*, *Bibersteinia*, and *Pasteurella* (Coggins, 1988; Miller, 2001). Bighorn sheep are much-more susceptible to pneumonia than are domestic sheep (*Ovis aries*; Foreyt, 1994). Since the early 1980s, there have been anecdotal field reports of bighorn deaths due to pneumonia following contact with domestic sheep (Foreyt and Jessup, 1982; Coggins, 1988; George et al., 2008).

Bacteria of the genera *Mannheimia, Bibersteinia*, and *Pasteurella* are commensal bacteria in the pharynx and nasal cavities of domestic and bighorn sheep (Ward et al., 1990). Experimental inoculation of some of the isolates from domestic sheep—isolates which do not readily cause disease in the domestic sheep—have resulted in fatal pneumonia in bighorn sheep (Onderka et al., 1988; Foreyt et al., 1994). In five experimental

commingling studies conducted by three investigators, 41 of 43 bighorn sheep died following contact with domestic sheep (Onderka and Wishart, 1988; Foreyt, 1989, 1990; Callan et al., 1991). These findings appeared to confirm earlier reports of the death of bighorn sheep after contact with domestic sheep, thus incriminating domestic sheep in the induction of fatal pneumonia in bighorn sheep. Although *Mannheimia* (*Pasteurella*) *haemolytica*, *Bibersteinia* (*Pasteurella*) *trehalosi*, and *Pasteurella multocida* were isolated from the dead bighorn sheep, these studies did not demonstrate that these organisms were transmitted from the domestic sheep to the bighorn sheep. In some of these studies, the bacteria that were isolated from the dead bighorn sheep were not shown to be present in the domestic sheep. It is possible that the bacteria responsible for the death of the bighorn sheep were not carried by the domestic sheep. It is also conceivable that these bacteria were present in the domestic sheep, but were not isolated, because nasal swabs rather than pharyngeal swabs were obtained or because adequate numbers of bacterial colonies from the initial isolation were not picked up for further characterization. Even the isolation of bacteria belonging to the same species, serotype, or biotype, from the domestic sheep and bighorn sheep did not demonstrate that the organism was transmitted from domestic sheep.

Our objective was to determine, unambiguously, whether a respiratory pathogen can be transmitted from domestic sheep to bighorn sheep. Multiple genera, species, and serotypes of bacteria can colonize the nasal cavities and the pharynx of a single animal (Ward et al., 1997). *Mannheimia haemolytica*, *B. trehalosi*, and *P. multocida* are commonly isolated from pneumonic lungs of bighorn sheep, (Jaworski et al., 1998; Kelley et al., 2007; George et al., 2008). *Mannheimia haemolytica* consistently causes severe bronchopneumonia and the rapid death of bighorn sheep under experimental conditions (Onderka et al., 1988; Foreyt et al., 1994; Dassanayake et al., 2009). Therefore, we selected *M. haemolytica* for this study. We obtained four *M. haemolytica* isolates from the nasopharynx of domestic sheep and tagged them with a plasmid encoding genes for green fluorescent protein (GFP), and for beta-lactamase (Bla), which confers ampicillin resistance (Ap^R). The four domestic sheep were colonized with the tagged bacteria and allowed to commingle with bighorn sheep to determine whether there was transmission of the GFP-tagged bacteria.

MATERIALS AND METHODS

Screening of animals for respiratory pathogens

Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Washington State University.

Four, clinically normal domestic sheep from the same flock were selected for the study. Nasal and pharyngeal swabs, from two groups of four domestic sheep and four bighorn sheep, were collected twice at 1- to 2-wk intervals. The swabs were collected from the domestic sheep at the beginning of the study (61 wk and 63 wk prior to the beginning of the transmission study) to obtain *M. haemolytica* isolates for tagging with GFP and Ap^R. The bighorn sheep were sampled 42 days and 35 days prior to the beginning of the transmission study. The swabs were analyzed for the presence of ovine respiratory disease (ORD) pathogens by protocols routinely used at Washington Animal Disease Diagnostic Laboratory (WADDL; Pullman, Washington, USA). The pathogens screened for included the bacteria *M. haemolytica*, *B. trehalosi*, and *Mycoplasma ovipneumoniae* and the viruses respiratory syncytial virus (RSV), parainfluenza 3 virus (PI-3), bovine herpesvirus1 (BHV-1), and bovine viral diarrhea virus (BVDV).

Isolation of viruses from nasopharyngeal swabs and lungs

The bovine turbinate (BT) cell line was used for viral propagation because these cells were known to support the growth of all the above viruses. Swabs in universal viral transport medium (BD Biosciences, Sparks, Maryland, USA) were vortexed, and the medium was plated onto BT cells in minimal essential

medium (MEM) supplemented with 10% fetal bovine serum (FBS; free of antibodies to known respiratory viruses) and antibiotics (penicillin-streptomycin 100 IU/ml; gentamicin 50 μg/ml; and fungizone 25 μg/ml). Inoculated cell cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2. The BT cells were observed daily for cytopathic effect.

Isolation of *M. ovipneumoniae* and *M. haemolytica* from nasopharyngeal swabs and lungs

Swabs from each animal were streaked onto blood agar plates and kept at 37 C overnight under aerobic and anaerobic growth conditions. The bacterial colony morphology on brain-heart infusion (BHI) sheep blood agar and triple sugar iron (TSI) medium; Gram staining; the ability to hydrolyze arabinose, trehalose, indole, nitrate, xylose, and catalase; and oxidase activity were used to differentiate *M. haemolytica* from *B. trehalosi* and *P. multocida* isolates. *Mycoplasma ovipneumoniae* was isolated by growth on pleuropneumonia-like organism broth and selective agar plates according to a previously described protocol (Besser et al., 2008).

Serotyping of *M. haemolytica* isolates

Mannheimia haemolytica strains were serotyped using serotype-specific rabbit antisera obtained from Glynn Frank (National Animal Disease Center, Ames, Iowa, USA). Cells from a single colony of overnight growth on a sheep blood agar plate were swirled for 30 sec in 30 μl of serum on a glass microscope slide. Agglutination was observed under a dissecting microscope. Serotype-specific antisera for the following serotypes were tested: A1, A2, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, and A16.

Polymerase chain reaction (PCR) detection of *M. haemolytica*

The PCR assay specific for *M. haemolytica* has been described (Dassanayake et al., 2010). A portion of the gene encoding *M. haemolytica* O-sialoglycoprotein endopeptidase (*gcp*; Genbank accession number AY83967) was amplified by PCR using primers MhgcpF: 5′-AGA GGC CAA TCT GCA AAC CTC G-3′ and reverse primer MhgcpR: 5′-GTT CGT ATT GCC CAA CGC CG-3′. PCRs were carried out in a final, 50-μl volume with GoTaq® PCR SuperMix (Promega Inc., Madison, Wisconsin, USA) with 0.2 μM each primer and 2 μl bacterial culture. The PCR cycling conditions consisted of an initial denaturation at 95 C for 5 min followed by 35 cycles of denaturation at 95 C for 30 sec, annealing at 55 C for 30 sec, and extension at 72 C for 40 sec, and a final elongation at 72 C for 5 min. The PCR products were visualized after electrophoresis in 1.0% agarose gels run at 7.0 V/cm and staining with ethidium bromide.

PCR detection of *M. ovipneumoniae*

Both standard PCR and real-time PCR (RT-PCR) were used. Standard PCR amplification conditions were essentially the same as previously described (Besser et al., 2008). Real-time PCR was developed in-house at WADDL using the following primers: Movip F: 5′-GGG GTG CGC AAC ATT AGT TA-3′; Movip R: 5′-CTT ACT GCT GCC TCC CGT AG-3′; and Movip (Probe): 5′-6-FAM-TTA GCG GGG CCA AGA GGC TGT A-BHQ-1-3′ derived from GenBank sequences EU290066 and NR_025989 of *M. ovipneumoniae*. The RT-PCR was run in an ABI 7500 Fast Thermocycler (Applied Biosystems, Carlsbad, California, USA) with the following cycling parameters: Stage 1: 1 hold at 50 C for 2 min (optics off) 95 C for 600 sec (optics off); Stage 2: 45 repeat cycles of 95 C for 15 sec (optics off) to denature and 61 C for 60 sec for annealing and extension (optics on). Test samples were read on the FAM wavelength. Those with a cycle threshold below 40.0 on the FAM channel were classed as positive for *M. ovipneumoniae*.

Tagging of *M. haemolytica* isolates with a plasmid carrying the genes encoding GFP and Ap^R

Plasmid pAM2425 was constructed by cloning the *gfp* gene from plasmid pAG408 into an *M. haemolytica* shuttle vector, pAM2355 (Marciel, 2001). Briefly, the *Cla*I/*Eco*RI fragment of pAG408 was cloned into a pBluescript KS II+ plasmid carrying the leukotoxin C promoter, then the P_{lktC}::*gfp* fusion was amplified using M13 universal forward (5′-GTA AAA CGA CGG CCA GT-3′) and modified reverse (5′-GGG ATA TCT AG*A AGC TT*A ACA GCT ATG ACC ATG ATT ACG-3′, *Hin*dIII site italicized) primers, and then cloned as a *Hin*dIII/*XbaI* fragment into the Bla-resistant vector pAM2355 to create pAM2425 (Fig. 1). All constructions were performed in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, California, USA) as described (Fedorova and Highlander, 1997). Plasmid DNA was purified using the Qiagen miniprep kit (Qiagen, Valencia, California, USA), and the four *M. haemolytica* isolates from the domestic sheep were transformed with plasmid pAM2425, by electroporation, as described by Craig et al. (1989). One-hundred

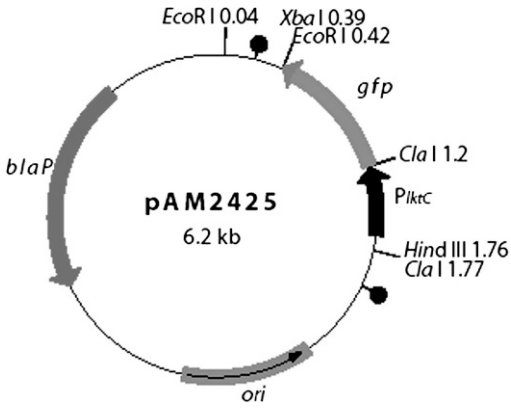

FIGURE 1. Schematic representation of the plasmid pAM2425 carrying *gfp* and *bla* genes. Plasmid pAM2425 was constructed by cloning the *gfp* gene from plasmid pAG408 into a *Mannheimia haemolytica* shuttle vector pAM2355, as described in materials and methods.

nanograms of plasmid DNA were added to each cuvette, which contained 100 µl electrocompetent cells. An electrical pulse of 15–20 kilovolt, 400 ohm, 25 µfarad was applied and, immediately, 1 ml BHI/SOC medium (BHI broth; 2.5 mM KCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; 20 mM glucose) was added and the mixture was incubated at 37 C for 3–4 hr to allow expression of markers. One-hundred-microliter aliquots were spread onto sheep blood agar plates containing 20 µg/ml ampicillin (Bioline, Randolph, Massachusetts, USA) and plates were incubated overnight at 37 C. Ampicillin-resistant colonies containing pAM2425 were identified by colony PCR using *gfp* and *bla* gene-specific primers, respectively (*gfp* forward 5′-ATG AGT AAA GGA GAA GAA CT-3′ and reverse 5′-GTA TAG TTC ATC CAT GCC ATG-3′ and *bla* forward 5′-ATG TTA AAT AAG TTA AAA ATC-3′ and reverse 5′-TTA GTT GAG CTG TAA AGT ATG AAA TAC-3′), in a 25-µl mastermix reaction containing GoTaq, as directed by the manufacturer (Promega Corp.) with slight modification. The PCR cycling conditions consisted of an initial denaturation at 95 C for 5 min, followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec, extension at 72 C for 1 min, and a final elongation at 72 C for 10 min.

Leukotoxin production by *M. haemolytica* isolates before and after tagging with GFP and Ap^R

Leukotoxin production by the *M. haemolytica* isolates was confirmed by subjecting culture supernatant fluid to MTT dye reduction cytotoxicity assay as described by Gentry and Srikumaran (1991). The percent cytotoxicity was calculated as follows: % cytotoxicity = [1−(OD of toxin-treated cells/OD of toxin-untreated cells)]×100.

Colonization of domestic sheep with tagged *M. haemolytica*

Bacteria were cultured overnight at 37 C in BHI agar supplemented with 5% sheep blood (Remel, Lenexa, Kansas, USA). Tagged *M. haemolytica* was cultured on plates containing BHI supplemented with 20 µg/ml ampicillin (Bioline). To prepare the inoculum, the bacteria were cultured in BHI broth at 37 C for 2–3 hr followed by growth in Roswell Park Memorial Institute (RPMI) 1640 medium, without phenol red (GIBCO), under the same conditions. The bacterial suspension was diluted in RPMI 1640 to obtain the desired concentration (colony-forming units [CFU]/ml; Petras et al., 1995). Using an atomizer, about 10^9 CFU of tagged *M. haemolytica* in 5 ml of phosphate-buffered saline (PBS) were sprayed intranasally into all four domestic sheep from which they were originally isolated. Nasal and pharyngeal swabs were collected 2 wk following inoculation to confirm the presence of tagged bacteria by colony PCR, as described above. A serotype-2 strain of *M. haemolytica*, isolated several years ago from a domestic sheep (Foreyt et al., 1994), also was tagged with the plasmid carrying the *gfp* and *bla* genes. This strain failed to colonize the pharynx of the four domestic sheep and was not used further.

Domestic sheep-bighorn sheep contact experiments

On day 0, the four domestic sheep and the four bighorn sheep were placed in two identical pens (about 20×3 m) separated by another pen (20×10 m), and animals were monitored for clinical signs. After 1 mo, the bighorn sheep were moved into the middle pen so that they had fence-line contact with domestic sheep. For the next 2 mo, the animals were observed for clinical signs of pneumonia, and nasal and pharyngeal swabs were collected twice (days 51 and 60) for detection of the presence of tagged *M. haemolytica*. After 2 mo in fence-line contact, the domestic sheep and bighorn sheep were allowed to commingle in the middle pen (20×10 m).

Clinical assessment and necropsy

The bighorn sheep were observed once a day for clinical signs including anorexia,

TABLE 1. Microbial profile of the nasopharynx of domestic sheep before commingling.

Animal no.	Sample site[a]	Bacteria recovered, sample 1/sample 2[b]			
		Mh[c]	Bt[d]	Past[e]	Movi[f]
1	P	+/+[g]	+/−	−/−	+/+
	N	−/−	−/−	−/−	+/−
2	P	−/+	−/−	+/−	+/+
	N	+/+	−/−	−/−	−/−
3	P	−/+	−/−	+/−	+/−
	N	−/+	−/−	−/−	−/−
5	P	+/−	+/−	−/−	+/−
	N	−/−	−/−	−/−	+/−

[a] Site of sample collection: P = pharynx; N = nasal cavity.

[b] Sample 1/sample 2 = Swabs collected at two different dates.

[c] Mh = *Mannheimia haemolytica*.

[d] Bt = *Bibersteinia trehalosi*.

[e] Past = *Pasteurella* species.

[f] Movi = *Mycoplasma ovipneumoniae*.

[g] (−) = Absent or not detected; (+) = present.

lethargy, cough, dyspnea, and nasal discharge. When the animals began to show clinical signs of pneumonia, they were observed more frequently. Animals that died during the experiment were necropsied within 6 hr. Lungs were removed from each animal and carefully examined for lesions of pneumonia. The degree of involvement of the lung lobes was estimated as percent pneumonic scores (percent of lung that appeared pneumonic on visual examination). Pleuritis was noted as present or absent. Representative samples of pneumonic and normal lung tissue were prepared for both bacteriologic and histopathologic examination (Odugbo et al., 2004). Animals that showed severe signs of pneumonia were euthanized by intravenous administration of pentobarbital and then necropsied in the same manner as those found dead.

Detection of tagged *M. haemolytica*

Colony PCR: Swabs were directly streaked onto sheep blood agar plates containing 20 μg/ml ampicillin and the plates were incubated overnight at 37 C. The following day, 5–10 representative colonies from each plate were picked and subjected to colony PCR assay, performed as described above, to confirm the presence of *gfp* and *bla* genes.

Imunoflurorescence labeling of GFP-tagged M. haemolytica: To detect GFP by immunofluorescence, bacterial cells were fixed in 2% paraformaldehyde for 10 min, washed with PBS, and incubated with 100 μl of FITC-conjugated rabbit polyclonal antibodies specific for GFP (Abcam, Cambridge, Massachusetts, USA) for 30 min at 4 C. The cells were washed with PBS and mounted onto microscopic slides and visualized using a fluorescence microscope.

RESULTS

Microbial flora of the upper respiratory tract before commingling

Microbial isolation revealed that all four domestic sheep carried *Pasteurellaceae* in the nasopharynx (Table 1). All four also yielded *M. haemolytica* from nasopharyngeal samples, at least once, prior to commingling (Table 1). All four domestic sheep were culture-positive for *M. ovipneumoniae* but were negative for the respiratory viruses RSV, PI-3, BVDV, and BHV-1.

Prior to beginning the study, the four bighorn sheep were negative for viruses and for *M. ovipneumoniae* by culture (Table 2). However, three of the bighorn sheep yielded *M. haemolytica* from nasopharyngeal swabs and all four had *B. trehalosi* in their pharynx (Table 2).

Characteristics of the *M. haemolytica* isolates from domestic sheep selected for tagging

Four *M. haemolytica* isolates obtained from two of the domestic sheep were designated as numbers 7, 10, 15, and 16. These isolates were determined to be *M. haemolytica* by cultural and biochemical characteristics and were confirmed by *M. haemolytica*-specific PCR assays. Serotype analysis with antisera specific for all known serotypes (A1, A2, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, and A16) revealed that isolate 7 belonged to serotype 9, while the other three were untypable. All of these isolates produced leukotoxin in culture (Fig. 2).

Mannheimia haemolytica isolates from domestic sheep get tagged with the plasmid carrying the *gfp* and *bla* genes

Growth of tagged *M. haemolytica* isolates on ampicillin plates suggested that

TABLE 2. Microbial profile of the nasopharynx of bighorn sheep before and after their commingling with domestic sheep. Bacteria were recovered via culture, except that *Mycoplasma ovipneumoniae* was also detected postmortem using polymerase chain reaction (PCR) assay.

Animal	Sample site[a]	Bacteria recovered before commingling (sample 1/sample 2)[b]			Sample site	Bacteria recovered after commingling (postmortem)			
		Mh[c]	Bt[d]	Movi[c]		Mh	Bt	Movi (culture)	Movi (PCR)
Y13	P	−/−[f]	+/+	−/−	P	+	−	−	−
	N	−/−	−/−	−/−	N	+	+	−	−
					L	+	+	−	−
Y15	P	−/+	+/−	−/−	P	−	+	+	+
	N	+/+	−/−	−/−	N	+	−	−	−
					L	+	−	−	−
Y16	P	+/−	+/+	−/−	P	−	+	−	−
	N	−/−	−/−	−/−	N	+	+	−	−
					L	+	+	−	−
Y47	P	−/−	+/+	−/−	P	nd[g]	nd	nd	−
	N	−/+	−/−	−/−	N	nd	nd	nd	−
					L	+	−	−	+

[a] Site of sample collection: P = pharynx; N = nasal cavity; L = lung.

[b] Sample 1/sample 2 = Swabs collected on two different dates.

[c] Mh = *Mannheimia haemolytica*.

[d] Bt = *Bibersteinia trehalosi*.

[e] Movi = *Mycoplasma ovipneumoniae*.

[f] (−) = Absent or not detected; (+) = present.

[g] nd = not done.

the bacteria were successfully tagged with GFP and Ap^R. PCR using *gfp*- and *bla*-specific primers confirmed the presence of *gfp* (Fig. 3A) and *bla* (Fig. 3C) in all four isolates. Immunofluorescence assays using FITC-labeled anti-GFP antibodies further confirmed the expression of GFP in these isolates (Fig. 4A). Cytotoxicity assays of the culture supernatant fluid, before and after the tagging, revealed that the leukotoxin production was not affected by the presence of extrachromosomal plasmid (Fig. 2). In a separate experiment, two bighorn sheep inoculated intratracheally with 5×10^9 CFU of the *M. haemolytica* isolates tagged with GFP/Ap^R plasmid developed pneumonia and died within 2 days postinoculation, indicating that organisms tagged with the GFP/Ap^R plasmid were pathogenic.

GFP- and Ap^R-tagged *M. haemolytica* effectively colonize the nasopharynx of domestic sheep

Three inoculations using a cocktail of all four, tagged *M. haemolytica* isolates resulted in colonization of the nasopharynx of three of the four domestic sheep. The colonization was detected by analyzing nasal and pharyngeal swabs for two consecutive weeks postinoculation (data not shown). The PCR amplification of *gfp* and *bla* genes confirmed the presence of the plasmid-tagged *M. haemolytica* in all of the three domestic sheep. All of the four domestic sheep continued to remain clinically normal after inoculation with tagged *M. haemolytica*.

Domestic sheep transmit GFP- and Ap^R -tagged *M. haemolytica* to bighorn sheep

The domestic sheep and bighorn sheep were separated by about 10 m in individual pens during the first month. During that time, no symptoms of respiratory disease were observed in either domestic sheep or bighorn sheep. Three bighorn sheep (Y13, Y15, and Y47) yielded tagged *M. haemolytica* from samples collected on days 51, 60, or both (21 days, 30 days, or both after fence-line contact began), as

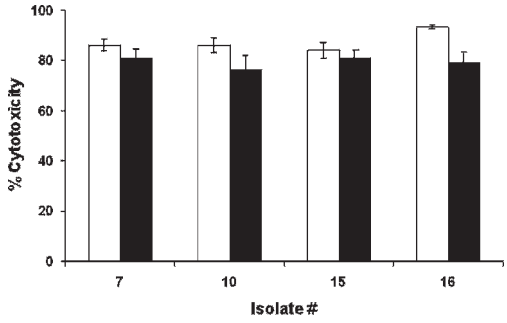

FIGURE 2. Leukotoxin production by *Mannheimia haemolytica* isolates before and after tagging with the plasmid carrying *gfp* and *bla*. Culture supernatant fluids from the *M. haemolytica* isolates numbers 7, 10, 15, and 16, before and after tagging with the plasmid carrying *gfp* and *bla*, were subjected to the MTT-dye reduction cytotoxicity assay. The percent cytotoxicity was calculated as follows: % cytotoxicity = [1−(OD of toxin-treated cells/OD of toxin-untreated cells)]×100. The open and shaded bars represent % cytotoxicity of culture supernatant fluids from the respective isolates, before and after tagging, respectively. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

revealed by *gfp* and *bla* gene-specific PCR. One of these bighorn sheep (Y15) developed coughing on day 83, 32 days following the first evidence of tagged *M. haemolytica* infection, but none of the animals died. On day 92 (2 days post-commingling), one bighorn sheep (Y15) died. The remaining animals at this time were lethargic and showed intermittent coughing. On day 95 (5 days postcommingling), two more bighorn sheep (Y13 and Y16) died, and on day 99 (9 days post-commingling), the remaining bighorn sheep (Y47) exhibited severe clinical signs of pneumonia and was euthanized.

Induction of pneumonia in, and death of, bighorn sheep are caused by *M. haemolytica* transmitted by the domestic sheep

Postmortem examinations revealed that all four bighorn sheep had acute, bilateral, fibrinohemorrhagic pneumonia that was equally distributed on both sides (Fig. 5A).

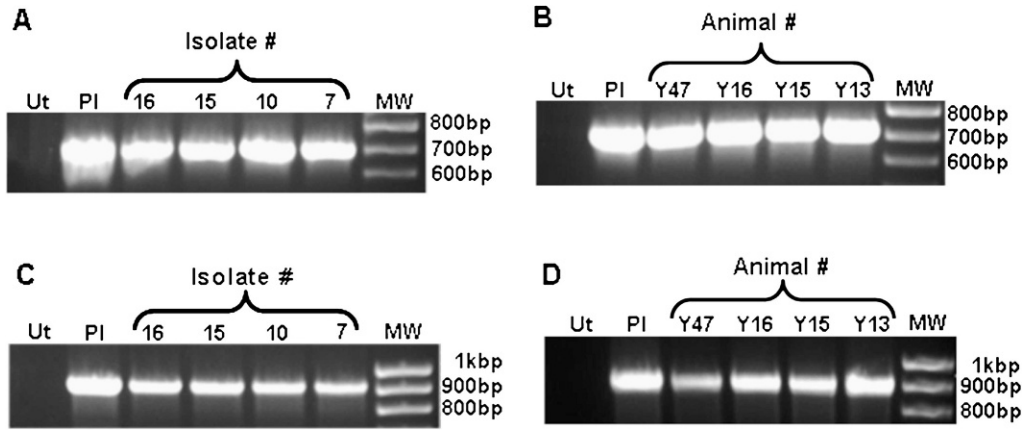

FIGURE 3. Detection of *gfp* and *bla* in *Mannheimia haemolytica* isolates by polymerase chain reaction (PCR) amplification. The *M. haemolytica* isolates tagged with the plasmid carrying *gfp* and *bla*, and the *M. haemolytica* isolates recovered from the lungs of the four dead bighorn sheep, were tested for the presence of *gfp* and *bla by* PCR analysis using primers described under materials and methods. Panels A and B represent PCR amplification of *gfp*. Panels C and D represent PCR amplification of *bla*. Ut=the untagged *M. haemolytica* (pool of all 4 isolates); Pl=plasmid pAM2425 used as positive control in PCR to indicate the presence of *gfp* and *bla*; numbers 16, 15, 10, and 7 represent the tagged isolates and the numbers Y47, Y16, Y15, and Y13 represent *M. haemolytica* isolated from the lungs of bighorn sheep numbers Y47, Y16, Y15, and Y13 at necropsy. MW=molecular weight markers. Results of one representative experiment out of three are shown.

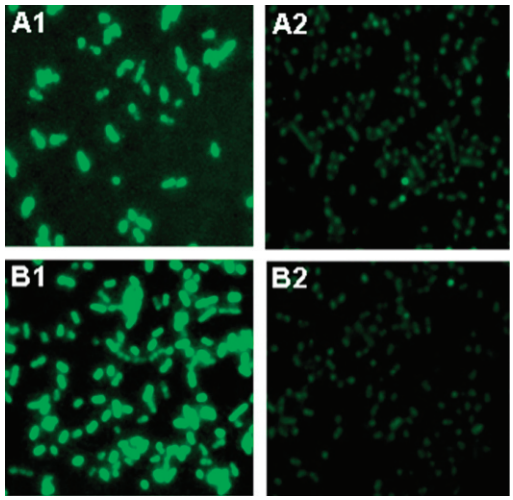

FIGURE 4. Detection of expression of GFP by immunofluorescence staining. The *Mannheimia haemolytica* isolates tagged with the plasmid carrying *gfp* and *bla*, and the *M. haemolytica* isolates recovered from the lungs of the four dead bighorn sheep, were tested for the expression of GFP by immunofluorescence staining with FITC-conjugated rabbit anti-GFP antibodies. All four tagged isolates (7, 10, 15, and 16), and isolates recovered from the lungs of all four dead bighorn sheep (Y13, Y15, Y16, and Y47), were positive for fluorescence expression. Fluorescence exhibited by one representative tagged isolate (Panel A1), and one representative isolate recovered from the lungs of the dead bighorn sheep (Panel B1), are shown. Panel A2 and B2 represent untagged *M. haemolytica* used as the negative control.

Estimated percent pneumonic involvement ranged from 70–95% in both the lungs. Fibrinous pleuritis was present in all four bighorn sheep. Although the lungs from the different bighorn sheep varied in severity in gross lesions, they were histologically very similar. In affected areas of the lungs, alveolar spaces and bronchioles were filled with edema, fibrin, red blood cells, and dense collections of primarily macrophages and neutrophils (Fig. 5B). The inflammatory cells showed degenerative changes and often had streaming nuclei ('oat cells'). Many alveolar walls, and occasional bronchiolar walls, were disrupted by necrosis and hemorrhage. When present, pleuritis was fibrinous.

Re-isolation of tagged *M. haemolytica* from pneumonic lungs of bighorn sheep

The swabs taken from lungs during necropsy were plated on BHI-agar plates which, upon incubation, showed the presence of colonies resistant to 20 μg/ml ampicillin. Further *gfp* gene- and *bla* gene-specific PCR confirmed the presence of tagged bacteria in the lungs (Fig. 3B, D). Immunofluorescence assays using FITC-labeled antiGFP antibodies further confirmed the expression of GFP in these isolates (Fig. 4B). None of the tagged isolates recovered from the lungs were typable with the antisera specific for the known serotypes of *M. haemolytica* (A1, A2, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, and A16).

DISCUSSION

Several anecdotal reports suggest that bighorn sheep die from pneumonia following contact with domestic sheep (Foreyt and Jessup, 1982; Coggins, 1988; George et al., 2008). Fatal pneumonia in bighorn sheep following experimental inoculation of *M. haemolytica* isolates from domestic sheep, isolates which did not cause disease in the domestic sheep, prompted researchers to perform commingling experiments to determine whether there was transmission of respiratory pathogens from domestic sheep to bighorn sheep (Onderka and Wishart, 1988; Foreyt, 1989, 1990; Callan et al., 1991). Although over 95% of the bighorn sheep in these studies died following contact with domestic sheep, there was not clear documentation of transmission of *M. haemolytica*, or of any other pathogen, from domestic sheep to bighorn sheep.

Whole genome sequencing, pulsed field gel electrophoresis, or amplified fragment length polymorphism, ribotyping, multilocus enzyme electrophoresis, and multilocus sequence typing are molecular tools that are available to compare bacterial pathogens isolated from domestic sheep and bighorn sheep. Whole genome se-

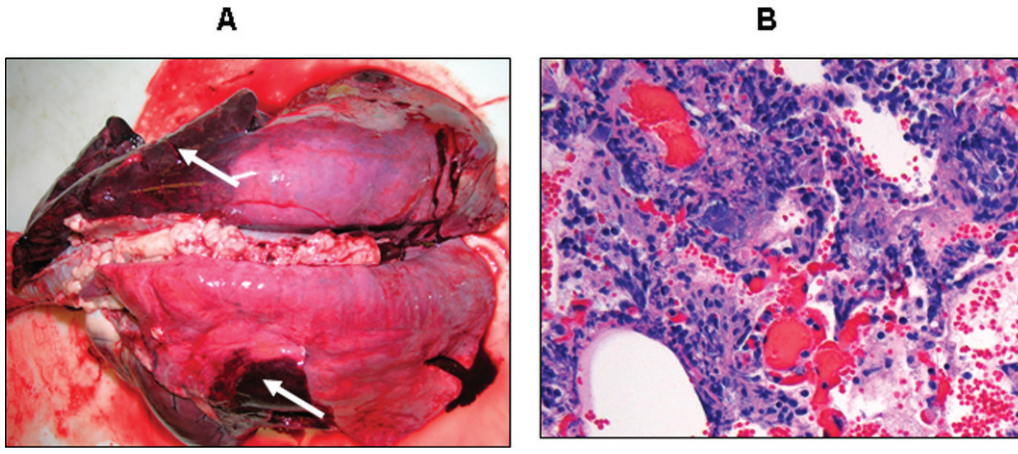

FIGURE 5. Representative gross lesions and histopathology of the lungs of the dead bighorn sheep. (A) Typical gross appearance of the lungs of the dead bighorn sheep. The lungs were removed from the carcass for examination, and the total area of gross lung consolidation was discerned by visual inspection and by palpation. In this case, the right cranial and middle, and the left middle lung lobes, are dark red and consolidated, and additional consolidation was evident from palpation; darkened areas in the photograph were subsequently determined to be areas of severe hemorrhage. Fibrin strands on the lung surface indicate pleuritis. (B) The typical histopathologic appearance of the lungs of the dead bighorn sheep. Lung tissue samples of bighorn sheep were aseptically removed and processed for histopathology. Alveolar septa are necrotic and replaced by fibrin and debris. Bronchioles and alveoli are filled with streaming mononuclear cells. H&E stain. 100×.

quencing is an elaborate and expensive procedure. The other molecular methods are time-consuming and cannot identify bacterial isolates with 100% certainty (Pitt, 1999; Yakubu et al., 1999). We reasoned that tagging the bacterial isolates obtained from domestic sheep, recolonizing the nasopharynx of these animals with the tagged bacteria, and commingling them with bighorn sheep would circumvent these problems and provide an irrefutable method of determining whether bacterial pathogens can be transmitted from domestic sheep to bighorn sheep. We selected *M. haemolytica* for this study because of its documented ability to consistently induce pneumonia in, and death of, bighorn sheep (Onderka et al., 1988; Foreyt et al., 1994; Dassanayake et al., 2009). We employed two markers, the GFP and Ap^R, to enhance the validity of our findings. We also utilized two tests to detect each marker (PCR and immunofluorescence for GFP and growth on ampicillin-containing medium and PCR for Ap^R). The growth of the tagged *M. haemolytica* in the presence of ampicillin, the PCR amplification of the genes *gfp* and *bla*, and the immunofluorescence staining with anti-GFP antibodies clearly indicated that the four isolates of *M. haemolytica* obtained from the domestic sheep were tagged with the markers (Fig. 3A, C, 4A). These three parameters were used to clearly document the successful colonization of the pharynx of domestic sheep by the tagged *M. haemolytica* and, more importantly, to identify the tagged organisms isolated from the dead bighorn sheep (Fig. 3B, D, 4B).

Tagged-isolate 7 typed as serotype 9 while the other three (numbers 10, 15, and 16) were untypable. However, none of the isolates recovered from the lungs of the four dead bighorn sheep typed as serotype 9. This could be because the tagged-isolate 7 did not colonize the nasopharynx of domestic sheep; because it colonized the domestic sheep but was not shed in adequate amounts to be

acquired by the bighorn sheep; or because it was acquired by the bighorn sheep but not recovered by us because it was present in the lungs in lower numbers than the other isolates at the time of sampling. Nevertheless, transmission from domestic sheep to bighorn sheep clearly occurred because other tagged isolates of *M. haemolytica* were recovered from the lungs of every bighorn sheep.

Our finding that three out of the four bighorn sheep acquired the tagged *M. haemolytica* within 1 mo of fence-line contact indicates that such contact was adequate for transmission of these organisms to occur. Death of the first bighorn sheep occurred about 1 mo after tagged *M. haemolytica* was first detected in that animal. This lag period may have been necessary for the transmitted *M. haemolytica* to colonize and proliferate to the threshold number of organisms required to induce pneumonia and death in bighorn sheep. It is conceivable that the bighorn sheep that acquired the tagged *M. haemolytica* during the fence-line contact would have died even without commingling with the domestic sheep. This notion is supported by the fact that one bighorn died only 2 days after commingling with the domestic sheep. However, in order to determine with certainty whether fence-line contact is adequate for induction of pneumonia and death of bighorn sheep, the experiment would need to be performed with a longer period of fence-line contact.

It is also possible that another pathogen(s) was necessary to predispose the bighorn sheep to pneumonia by *M. haemolytica* infection. The bighorn sheep were not positive for *M. ovipneumoniae* before commingling with the domestic sheep. Lung tissue from one of the dead bighorn sheep was positive for *M. ovipneumoniae* by standard and RT-PCR (Table 2), and *M. ovipneumoniae* was detected in the nasopharynx of a second dead bighorn sheep by culture and PCR, which raises the possibility that these organisms, along with the tagged *M. haemolytica*, were transmitted from the domestic sheep to the bighorn sheep. It is possible that during the lag period, *M. ovipneumoniae* colonized the upper respiratory tract of at least two bighorn sheep and predisposed them to the tagged *M. haemolytica*, but whether *M. ovipneumoniae* played any role in the other two bighorn sheep seems even less certain, based on available data (Table 2). In domestic sheep, *M. ovipneumoniae* has been shown to render the cilia on the epithelial cells of the upper respiratory tract dysfunctional (Jones et al., 1985; Niang et al., 1998). Previous studies have shown that *M. ovipneumoniae* does not kill bighorn sheep (Besser et al., 2008) but can predispose them to *M. haemolytica* infection (Dassanayake et al., 2010). However, it is not likely that *M. ovipneumoniae* is a necessary predisposing factor for fatal infection of bighorn sheep by every strain of *M. haemolytica* because, in an earlier study, intranasal inoculation with *M. haemolytica* resulted in the death of 75% of inoculated bighorn sheep ($n=4$) within 48 hr (unpubl. data). The *M. haemolytica* used in that study was a serotype 2 strain, which is known to be virulent in bighorn sheep (Foreyt et al., 1994). Therefore, we believe that only less-virulent strains of *M. haemolytica* may require *M. ovipneumoniae* or another predisposing agent. Studies are currently underway to elucidate the role of *M. ovipneumoniae* in the development of pneumonia in bighorn sheep following contact with domestic sheep. In summary, this study irrefutably demonstrated the transmission of *M. haemolytica* from domestic sheep to bighorn sheep and the resulting pneumonia and death of bighorn sheep.

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LITERATURE CITED

BESSER, T. E., E. F. CASSIRER, K. A. POTTER, J. VANDERSCHALIE, A. FISCHER, D. P. KNOWLES, D. R. HERNDON, F. R. RURANGIRWA, G. C. WEISER, AND S. SRIKUMARAN. 2008. Association of *Mycoplasma ovipneumoniae* infection with population-limiting respiratory disease in free-ranging Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). Journal of Clinical Microbiology 46: 423–430.

BUECHNER, H. K. 1960. The bighorn sheep in the United States, its past, present, and future. Wildlife Monograph 4: 3–174.

CALLAN, R. J., T. D. BUNCH, G. W. WORKMAN, AND R. E. MOCK. 1991. Development of pneumonia in desert bighorn sheep after exposure to a flock of exotic wild and domestic sheep. Journal of the American Veterinary Medical Association 198: 1052–1056.

COGGINS, V. L. 1988. The Lostine Rocky Mountain bighorn sheep die-offs and domestic sheep. Proceedings of the Biennial Symposium of Northern Sheep and Goat Council 6: 57–64.

CRAIG, F. F., J. G. COOTE, R. PARTON, J. H. FREER, AND N. J. GILMOUR. 1989. A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. Journal of General Microbiology 135: 2885–2890.

DASSANAYAKE, R. P., S. SHANTHALINGAM, C. N. HERNDON, P. K. LAWRENCE, E. F. CASSIRER, K. A. POTTER, W. J. FOREYT, K. D. CLINKENBEARD, AND S. SRIKUMARAN. 2009. *Mannheimia haemolytica* serotype A1 exhibits differential pathogenicity in two related species, *Ovis canadensis* and *Ovis aries*. Veterinary Microbiology 133: 366–371.

———, D. R. CALL, A. A. SAWANT, N. C. CASAVANT, G. C. WEISER, D. P. KNOWLES, AND S. SRIKUMARAN. 2010. *Bibersteinia trehalosi* inhibits growth of *Mannheimia haemolytica* by a proximity-dependent mechanism. Applied and Environmental Microbiology 76: 1008–1013.

———, SHANTHALINGAM, S., C. N. HERNDON, R. SUBRAMANIAM, P. K. LAWRENCE, J. BAVANANTHASIVAM, E. F. CASSIRER, G. J. HALDORSON, W. J. FOREYT, F. R. RURANGIRWA, D. P. KNOWLES, T. E. BESSER, AND S. SRIKUMARAN. 2010. *Mycoplasma ovipneumoniae* can predispose bighorn sheep to fatal *Mannheimia haemolytica* pneumonia. Veterinary Microbiology (Epub ahead of print). doi: 10.1016/J.vetmic.2010.04.011.

FEDOROVA, N. D., AND S. K. HIGHLANDER. 1997. Plasmids for heterologous expression in *Pasteurella haemolytica*. Infection and Immunity 186: 207–211.

FOREYT, W. J. 1989. Fatal *Pasteurella haemolytica* pneumonia in bighorn sheep after direct contact with clinically normal domestic sheep. American Journal of Veterinary Research 50: 341–344.

———. 1990. Pneumonia in bighorn sheep: Effects of *Pasteurella haemolytica* from domestic sheep and effects on survival and long term reproduction. Proceedings of the Biennial Symposium of the Northern Wild Sheep and Goat Council 7: 92–101.

———. 1994. Effects of controlled contact exposure between healthy bighorn sheep and llamas, domestic goats, mountain goats, cattle, domestic sheep, or mouflon sheep. Proceedings of the Biennial Symposium of the Northern Wild Sheep and Goat Council 9: 7–14.

———, AND D. A. JESSUP. 1982. Fatal pneumonia of bighorn sheep following association with domestic sheep. Journal of Wildlife Diseases 18: 163–168.

———, K. P. SNIPES, AND R. W. KASTEN. 1994. Fatal pneumonia following inoculation of healthy bighorn sheep with *Pasteurella haemolytica* from healthy domestic sheep. Journal of Wildlife Diseases 30: 137–145.

GENTRY, M. J., AND S. SRIKUMARAN. 1991. Neutralizing monoclonal antibodies to *Pasteurella haemolytica* leukotoxin affinity-purify the toxin from crude culture supernatants. Microbial Pathogenesis 10: 411–417.

GEORGE, J. L., D. J. MARTIN, P. M. LUKACS, AND M. W. MILLER. 2008. Epidemic Pasteurellosis in a bighorn sheep population coinciding with the appearance of a domestic sheep. Journal of Wildlife Diseases 44: 388–403.

JAWORSKI, M. D., D. L. HUNTER, AND A. C. S. WARD. 1998. Biovariants of isolates of *Pasteurella* from domestic and wild ruminants. Journal of Veterinary Diagnostic Investigations 10: 49–55.

JONES, G. E., W. A. KEIR, AND J. S. GILMOUR. 1985. The pathogenicity of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in ovine and caprine tracheal organ cultures. Journal of Comparative Pathology 95: 477–487.

KELLEY, S. T., E. F. CASSIRER, G. C. WEISER, AND S. SAFAEE. 2007. Phylogenetic diversity of Pasteurellaceae and horizontal gene transfer of leukotoxin in wild and domestic sheep. Infection, Genetics and Evolution 7: 13–23.

MARCIEL, A. M. 2001. Genetic tools for studying the transcription of *Mannheimia (Pasteurella) haemolytica*: Identification of cis-, trans-, and environmental factors modulating leukotoxin expression. PhD Thesis, Baylor College of Medicine, Houston, Texas, pp. 129–132.

MILLER, M. W. 2001. Pasteurellosis. *In* Infectious diseases of wild mammals, 3rd Edition, E. S. Williams and I. K. Barker (eds.). Iowa State University Press, Ames, Iowa, pp. 330–339.

NIANG, M., R. F. ROSENBUSCH, M. C. DEBEY, Y. NIYO,

J. J. Andrews, and M. L. Kaeberle. 1998. Field isolates of *Mycoplasma ovipneumoniae* exhibit distinct cytopathic effects in ovine tracheal organ cultures. Zentralbl Veterinary Medicine A 45: 29–40.

Odugbo, M. O., L. E. Odama, J. U. Umoh, and L. H. Lombin. 2004. The comparative pathogenicity of strains of eight serovars and untypable strains of *Mannheimia haemolytica* in experimental pneumonia of sheep. Veterinary Research 35: 661–669.

Onderka, D. K., and W. D. Wishart. 1988. Experimental contact transmission of *Pasteurella haemolytica* from clinically normal domestic sheep causing pneumonia in Rocky Mountain bighorn sheep. Journal of Wildlife Diseases 24: 663–667.

———, S. A. Rawluk, and W. D. Wishart. 1988. Susceptibility of Rocky Mountain bighorn sheep and domestic sheep to pneumonia induced by bighorn and domestic livestock strains of *Pasteurella haemolytica*. Canadian Journal of Veterinary Research 52: 439–444.

Petras, S. F., M. Chidambaram, E. F. Illyes, S. Froshauer, G. M. Weinstock, and C. P. Reese. 1995. Antigenic and virulence properties of *Pasteurella haemolytica* leukotoxin mutants. Infection and Immunity 63: 1033–1039.

Pitt, T. L. 1999. Molecular typing in practice. Journal of Hospital Infection 43: S85–S88.

Valdez, R., and P. R. Krausman. 1999. Mountain sheep of North America. University of Arizona Press, Tucson, Arizona, pp. 19–20.

Ward, A. C., M. R. Dunbar, D. L. Hunter, R. H. Hillman, M. S. Bulgin, W. J. Delong, and E. R. Silva. 1990. Pasteurellaceae from bighorn and domestic sheep. Proceedings of the Biennial Symposium of the Northern Wild Sheep and Goat Council 7: 109–117.

———, D. L. Hunter, M. D. Jaworski, P. J. Benolkin, M. P. Dobel, J. B. Jeffress, and G. A. Tanner. 1997. *Pasteurella* spp. in sympatric bighorn and domestic sheep. Journal of Wildlife Diseases 33: 544–547.

Yakubu, D. E., J. R. Fariborz, R. Abadi, and T. H. Pennington. 1999. Molecular typing methods for *Neisseria meningitidis*. Journal of Medical Microbiology 48: 1055–1064.

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