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PCR ASSAY DETECTS *MANNHEIMIA HAEMOLYTICA* IN CULTURE-NEGATIVE PNEUMONIC LUNG TISSUES OF BIGHORN SHEEP (*OVIS CANADENSIS*) FROM OUTBREAKS IN THE WESTERN USA, 2009–2010

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ABSTRACT: *Mannheimia haemolytica* consistently causes severe bronchopneumonia and rapid death of bighorn sheep (*Ovis canadensis*) under experimental conditions. However, *Bibersteinia trehalosi* and *Pasteurella multocida* have been isolated from pneumonic bighorn lung tissues more frequently than *M. haemolytica* by culture-based methods. We hypothesized that assays more sensitive than culture would detect *M. haemolytica* in pneumonic lung tissues more accurately. Therefore, our first objective was to develop a PCR assay specific for *M. haemolytica* and use it to determine if this organism was present in the pneumonic lungs of bighorns during the 2009–2010 outbreaks in Montana, Nevada, and Washington, USA. *Mannheimia haemolytica* was detected by the species-specific PCR assay in 77% of archived pneumonic lung tissues that were negative by culture. Leukotoxin-negative *M. haemolytica* does not cause fatal pneumonia in bighorns. Therefore, our second objective was to determine if the leukotoxin gene was also present in the lung tissues as a means of determining the leukotoxicity of *M. haemolytica* that were present in the lungs. The leukotoxin-specific PCR assay detected leukotoxin gene in 91% of lung tissues that were negative for *M. haemolytica* by culture. *Mycoplasma ovipneumoniae*, an organism associated with bighorn pneumonia, was detected in 65% of pneumonic bighorn lung tissues by PCR or culture. A PCR assessment of distribution of these pathogens in the nasopharynx of healthy bighorns from populations that did not experience an all-age die-off in the past 20 yr revealed that *M. ovipneumoniae* was present in 31% of the animals whereas leukotoxin-positive *M. haemolytica* was present in only 4%. Taken together, these results indicate that culture-based methods are not reliable for detection of *M. haemolytica* and that leukotoxin-positive *M. haemolytica* was a predominant etiologic agent of the pneumonia outbreaks of 2009–2010.

Key words: *Bibersteinia trehalosi*, Bighorn sheep, culture, die-off, leukotoxin, *Mannheimia haemolytica*, *Pasteurella multocida*, PCR.

INTRODUCTION

Pneumonia has played a significant role in the drastic decline of the bighorn sheep (*Ovis canadensis*) population in North America (Valdez and Krausman 1999; Miller 2001). Bacterial pathogens commonly detected in pneumonic bighorn lungs include *Mannheimia* (*Pasteurella*) *haemolytica*, *Bibersteinia* (*Pasteurella*) *trehalosi*, *Pasteurella multocida*, and *Mycoplasma ovipneumoniae* (Miller 2001; Besser et al. 2008; Wolfe et al. 2010). Of these bacteria, *M. haemolytica* has been most extensively studied as a cause of fatal pneumonia in bighorns (Foreyt et al. 1994; Dassanayake et al. 2009). Surprisingly, *B. trehalosi* and *P. multocida* have been detected by culture-based methods more frequently than *M. haemolytica* from pneumonic lung tissues of bighorns that expired during the die-offs of 2009–2010 and in the previous decades (Ward et al. 1990; Foreyt et al. 1994; Weiser et al. 2003). This study was designed to address this discrepancy. We reasoned that detection of

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M. haemolytica by culture-based methods was hindered by the inhibition of its growth by *B. trehalosi* and *P. multocida*. This notion was supported by our recent finding that *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica* when cocultured in vitro in broth cultures (Dassanayake et al. 2010a; Bavananthasivam et al. 2012). We hypothesized that an assay more sensitive than the culture-based methods would detect *M. haemolytica* in pneumonic lung tissues more accurately. Previously, we developed a PCR assay to detect *M. haemolytica* (Dassanayake et al. 2010a). However this assay detected *Mannheimia glucosida* (*P. haemolytica* serotype 11, according to the earlier nomenclature) as well. Therefore our first objective was to develop a PCR assay specific for *M. haemolytica* and use it to determine the presence of this organism in the pneumonic lungs of bighorns that died in the 2009–2010 outbreaks in Montana, Nevada, and Washington, USA.

Our previous experimental studies in bighorns with the wild-type and leukotoxin-negative mutants of *M. haemolytica* and *B. trehalosi* revealed that the leukotoxin deletion mutants do not cause fatal pneumonia in bighorns, indicating that leukotoxin is the most important virulence factor of these organisms (Dassanayake et al. 2009, 2013). Therefore, our second objective was to determine the presence of leukotoxin-A gene (*lktA*) in the pneumonic lungs of bighorns that expired in the 2009–2010 die-offs.

MATERIALS AND METHODS

In this study we developed a PCR assay specific for *M. haemolytica* and used it toward two goals. The first was to determine the presence of *M. haemolytica* in archived lung tissues from bighorns that died during the 2009–2010 epizootic and from bighorns that were killed to prevent disease spread when they showed clinical signs. These lung tissues were previously found to be negative for *M. haemolytica* by culture. The second goal was to determine the presence of selected members of *Pasteurellaceae* in the nasopharynx of bighorns from populations that did not suffer an all-age die-off during the past 20 yr.

Assay development and validation

To specifically detect *M. haemolytica*, O-sialoglycoprotein endopeptidase (*gcp*) sequences of *M. haemolytica* (GenBank accession numbers AY839677–839681 and U15958), *M. glucosida* (AY839680), and *B. trehalosi* (AY839681) in GenBank were aligned by the ClustalW program (Larkin et al. 2007). *Mannheimia haemolytica gcp*-specific primers were designed from regions with the lowest sequence identity. Sequences of leukotoxin of *M. haemolytica* (GenBank accession numbers AF414141, AF314503, AF314506, AF314508–314515, M20730, M24197, and U01215), *M. glucosida* (AF314517–314522), and *B. trehalosi* (AF314523–314526) were also aligned by the ClustalW program. *Mannheimia* (*M. haemolytica* and *M. glucosida*) leukotoxin-specific primers were designed from regions with the lowest sequence identity. The primers designed (gcp and lktA set-1) are shown in Table 1. The PCR was carried out in a final volume of 50 μL that contained Go*Taq*® PCR Green Master Mix (Promega Inc., Madison, Wisconsin, USA) with each primer at 0.2 μM concentration and 100 ng of genomic DNA. To enhance sensitivity, 5% dimethylsulfoxide (DMSO; ATCC, Manassas, Virginia, USA) was added to the PCR mixture. The PCR assay consisted of an initial denaturation step at 95 C for 5 min and a final extension at 72 C for 10 min for both PCR assays. Cycling conditions were 40 cycles of 95 C for 30 sec, 54 C for 30 sec, and 72 C for 30 sec for *M. haemolytica* PCR; and 95 C for 30 sec, 52 C for 30 sec, and 72 C for 40 sec for leukotoxin PCR. Genomic DNA extracted from the lung of an animal known to be infected with *M. haemolytica*, and a leukotoxin-positive *M. haemolytica* colony (Murphy et al. 1995), were used as positive controls for *M. haemolytica*-specific and leukotoxin-specific PCR assays, respectively. Lung tissue from a clinically healthy animal was used as negative control for both PCR assays. Specificity of PCR primers for *M. haemolytica* was evaluated using multiple reference strains and field isolates of *M. haemolytica* (Murphy et al. 1995), *M. glucosida* (CCUG38457), *B. trehalosi* (ATCC), and *P. multocida* (ATCC). Specificity of PCR primers for *lktA* was determined using multiple field isolates and reference strains of *M. haemolytica*, *M. glucosida*, and *B. trehalosi*. To confirm that the PCR specifically amplified *gcp* of *M. haemolytica*, PCR amplicons from five lung tissue samples, a positive control, and *M. haemolytica* serotype A1 were sequenced. To

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TABLE 1. The PCR primers used to detect the members of *Pasteurellaceae*, leukotoxin gene, and *Mycoplasma ovipneumoniae* in lung tissues of bighorn sheep (*Ovis canadensis*) involved in pneumonia outbreaks in Montana, Nevada, and Washington, USA, 2009–2010.

Species or gene	Target gene	Sequence (5′→3′)	Fragment size (bp)	Reference
Mannheimia haemolytica	O-sialoglycoprotein endopeptidase (*gcp*)	(F)[a] TGG GCA ATA CGA ACT ACT CGG G (R)[b] CTT TAA TCG TAT TCG CAG	227	This study
Bibersteinia trehalosi	manganese-dependent superoxide dismutase (*sodA*)	(F) GCC TGC GGA CAA ACG TGT TG (R) TTT CAA CAG AAC CAA AAT CAC GAA TG	144	Dassanayake et al. 2010a
Pasteurella multocida	*KMT1*[c]	(F) ATC CGC TAT TTA CCC AGT GG (R) GCT GTA AAC GAA CTC GC AC	456	Townsend et al. 1998
Mycoplasma ovipneumoniae	16S rRNA	(F) TGA ACG GAA TAT GTT AGC TT (R) GAC TTC ATC CTG CAC TCT GT	361	McAuliff et al. 2003
Leukotoxin (lkt) set-1	Leukotoxin-A (*lktA*)	(F) CTT ACA TTT TAG CCC AAC GTG (R) TAA ATT CGC AAG ATA ACG GG	497	This study
Leukotoxin (lkt) set-2	Leukotoxin-C, A, B	(F) TCA AGA AGA GCT GGC AAC (R) AGT GAG GGC AAC TAA ACC	3,053	Davies et al. 2001

[a] Forward primer.

[b] Reverse primer.

[c] *KMT1* = gene encoding a protein involved in the synthesis of capsular polysaccharide.

confirm that the PCR specifically amplified *lktA*, PCR amplicons of three lung tissue samples and a positive control were sequenced.

Assessment of samples from all-age epizootics

Bacterial isolation from pneumonic bighorn lungs and nasopharynx was performed by the Washington Animal Disease Diagnostic Laboratory (WADDL, Pullman, Washington, USA), according to standard protocols (Jaworski et al. 1993, 1998), prior to the initiation of this study. Lung tissues of 212 bighorns found dead or killed in Montana, Nevada, and Washington over 7 mo during the 2009–2010 die-offs were archived by WADDL. Of these, 70 samples negative for *M. haemolytica* by culture were selected. Examination of lungs of dead and killed bighorns for gross and histologic lesions consistent with pneumonia was performed by WADDL pathologists or veterinarians from the respective Departments of Fish and Wildlife. *Mannheimia haemolytica* and *B. trehalosi* colonies isolated from lungs and nasopharynx of dead and killed bighorns from the above three states during the 2009–2010 die-offs were obtained from WADDL for detection of *lktA* and secretion of leukotoxin protein.

For genomic DNA isolation, proteinase-K was added to ground lung tissue samples, along with the buffer provided by the manufacturer (Qiagen, Valencia, California, USA), and incubated at 56 C until tissues were completely lysed. Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). A PCR to detect *M. haemolytica* and *lktA* in the bighorn lungs was performed using genomic DNA as the template and the primers gcp and lktA set-1, respectively. The PCR conditions were as described above.

For detection of *lktA* in *M. haemolytica* and *B. trehalosi* isolated by culture from lung tissues and nasopharynx of dead and killed bighorns, PCR was performed using leukotoxin set 2 primers (lktA set-2; Davies et al. 2001) given in Table 1. These primers detect the *lktA* of *M. haemolytica* as well as of *B. trehalosi*. *Mannheimia haemolytica* or *B. trehalosi* cultures known to have intact *lktA* and an *lktA* deletion-mutant of *M. haemolytica* (Murphy et al. 1995) were used as positive and negative controls, respectively. The MTT dye-reduction cytotoxicity assay for detection of leukotoxin-induced cytolysis of target cells by culture supernatant fluid from bighorn sheep bacterial isolates was performed as previously described (Gentry and Srikumaran 1991). Prior to initiation of this study, detection of *M. ovipneumoniae* in the bighorn lung tissue samples was performed by WADDL, by PCR (n=48), and by culture (n=20) as described previously (McAuliffe et al. 2003; Besser et al. 2008).

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Assessment of samples from nonepizootic populations

Forty-five healthy bighorns from two populations (Sheep Ridge and Torry Rim) in Wyoming were selected for detection of bacterial species in the nasopharynx. These two populations are not known to mix on either their summer or winter ranges. These populations have not experienced pneumonia-associated all-age die-offs in the past 20 yr but have experienced poor lamb recruitment.

For genomic DNA isolation, nasopharyngeal swabs were incubated in 1 mL of 1X phosphate-buffered saline for 24 hr at room temperature, and bacteria were pelleted by centrifugation for 10 min at 5,000 $\times$ G. Genomic DNA was extracted as described above (see Assessment of samples from all-age epizootic). Nasopharyngeal swabs were streaked onto brain-heart infusion agar plates and incubated at 37 C for 16 hr. Thirty colonies per swab were selected for further analysis. Multiplex PCR assay was performed to identify *M. haemolytica*, *B. trehalosi*, and *P. multocida* using the species-specific primers (gcp, soda, and KMT1, respectively) given in Table 1, following cycling conditions described previously (Dassanayake et al. 2010a). All *M. haemolytica* and *B. trehalosi* isolates were tested for *lktA* using the primer lktA set-2 (Davies et al. 2001). In a separate experiment, PCR for *M. haemolytica* (gcp primers), *B. trehalosi* (sodA primers) and lktA (lktA set-1 primers) was performed on genomic DNA directly extracted from the swabs.

The PCR for detection of *M. ovipneumoniae* in the nasopharynx of healthy bighorns from Wyoming was performed by the Wyoming Game and Fish Wildlife Disease Laboratory, Laramie, Wyoming. An enhanced enrichment procedure was used to amplify cultures prior to DNA extraction. A 250-μL aliquot was removed and centrifuged at 13,000 $\times$ G for 1 min and the supernatant was discarded. DNA was extracted and purified using the E.Z.N.A. tissue DNA kit (Omega Biotek, Norcross, Georgia, USA) according to the manufacturer's instructions. *Mycoplasma ovipneumoniae*-specific 16S rRNA PCR was performed on purified DNA using the primers in Table 1 under previously described conditions (McAuliffe et al. 2003) with few modifications. The master mix for the PCR reaction included Accuprime GC-rich Buffer A and GC-rich DNA polymerase (Life Technologies, Carlsbad, California, USA) as well as PCR-grade water, dNTPs, $MgCl_2$, and 4.5 μL DNA template.

RESULTS

Bacteria isolated by WADDL from the lung tissues of bighorns that died of pneumonia or were killed in Montana, Nevada, and Washington over 7 mo during the die-offs of 2009–2010 are summarized in Figure 1. *Mannheimia haemolytica* was isolated from lung tissues of only six of 212 animals (3%). *Bibersteinia trehalosi* and *P. multocida* were isolated from the lung tissues of 23% and 31% of the animals, respectively. Therefore, we developed a PCR assay specific for *M. haemolytica* to detect this organism in the archived lung tissues that had been tested for this organism by culture prior to initiation of this study.

Assay development and validation

We examined 50 *M. haemolytica*, 33 *B. trehalosi*, 12 *P. multocida*, and 11 *M. glucosida* isolates to evaluate the specificity of *M. haemolytica gcp*-specific primers used in PCR assay. These primers recognized the *gcp* of *M. haemolytica* but not that of other species. Sequences of five amplicons of *gcp* of *M. haemolytica* obtained from lung tissues, a positive control, and *M. haemolytica* serotype A1 reference strain were 100% identical to that of *gcp* of *M. haemolytica* in GenBank (AY839677–839679 and U15958), confirming that the band observed at 227 base pairs (bp) was the *gcp* of *M. haemolytica*.

Leukotoxin gene-positive isolates of *M. haemolytica* (15 isolates), *B. trehalosi* (10 isolates), and *M. glucosida* (9 isolates) were used in the PCR assay to evaluate the specificity of the *lktA*-specific primers. Leukotoxin gene of *M. haemolytica* and *M. glucosida*, but not *B. trehalosi*, was amplified by these primers. Sequencing of three amplicons of *lktA* obtained from lung tissues and a positive control further confirmed that the band observed at 497 bp is the *lktA* of *M. haemolytica*.

Assessment of samples from all-age epizootics

Fifty-four of the 70 samples (77%) of lung tissues were positive for *M. haemolytica* by

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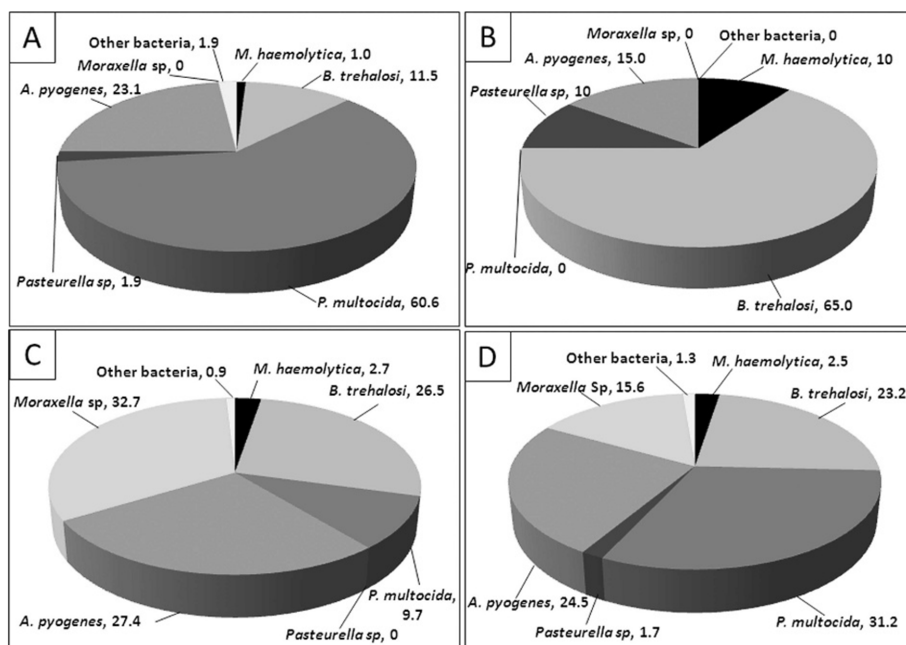

FIGURE 1. Members of *Pasteurellaceae* and other respiratory pathogens isolated from lung samples collected from bighorn sheep (*Ovis canadensis*) that died or were euthanized during die-offs associated with a pneumonia outbreak in the western USA, 2009–2010. Panels A, B, and C represent the samples from Montana, Nevada, and Washington states, respectively. Two hundred and twelve samples from Montana (n=116), Nevada (n=20), and Washington (n=76) were analyzed. Panel D combines findings of all three states. *Pasteurella* sp = *Mannheimia haemolytica* or *Bibersteinia trehalosi*. Other bacteria: *Staphylococcus aureus*, coliform or *Bacillus* spp. *A. pyogenes* = *Arcanobacterium pyogenes*; *P. multocida* = *Pasteurella multocida*. Numbers represent the percentage of the total number of samples.

our species-specific PCR assay. *Mannheimia haemolytica* was detected by PCR in 22 of 34 (65%), 5 of 5 (100%), and 27 of 31 (87%) samples from Montana, Nevada, and Washington, respectively (Table 2; Supplementary Table 1 in the online version of this manuscript).

Genomic DNA from 70 lung tissues selected for detection of *M. haemolytica gcp* was used to detect *lktA*. The *lktA*-specific PCR detected *lktA* in 64 of the 70 samples (91%). Leukotoxin gene was detected in 31 of 34 samples from Montana, all 5 lung samples from Nevada, and in 28 of 31 samples from Washington (Table 2).

We screened *M. haemolytica* and *B. trehalosi* isolates, obtained from lung or nasopharynx of killed bighorns showing signs of pneumonia, for *lktA* using PCR and secretion of leukotoxin using MTT dye-reduction cytotoxicity. All *M. haemolytica* isolates (three from lungs and five from nasopharynx) had *lktA*. All of these isolates secreted leukotoxin protein. Only two of 52 *B. trehalosi* isolates (4%) from lungs of dead bighorns were *lktA* positive. Both secreted leukotoxin. None of the 26 *B. trehalosi* isolates from nasopharynx of dead bighorns possessed *lktA*.

Mycoplasma ovipneumoniae-specific PCR and culture-based methods together detected this organism in 43 of 68 (65%) lung tissue samples. As stated earlier, these assays were performed by WADDL prior to the initiation of this study.

Assessment of samples from nonepizootic populations

Forty-five healthy bighorns from two populations that did not suffer an all-age die-off in the past 20 yr were screened for

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TABLE 2. Detection of members of *Pasteurellaceae* and *Mycoplasma ovipneumoniae* in lung tissues of dead bighorn sheep (*Ovis canadensis*) involved in pneumonia outbreaks and from the nasopharynx of healthy bighorns. The results of healthy bighorns represent the detection of the organism either in the nasal or pharyngeal swabs, or both, whereas the results of dead bighorns represent the detection of the organism in the pharyngeal swabs.

State and area	No. samples	*Bibersteinia trehalosi*[a]	*Pasteurella multocida*[a]	*Mannheimia haemolytica*[b]	Leukotoxin[c]	*Mycoplasma ovipneumoniae*[d]
Dead bighorn sheep						
Montana						
East Fork Bitterroot	30	3	16	19	28	10
Anaconda	2	0	1	2	2	0
Lower Rock Creek	2	0	1	1	1	1
Total (%)	34	3 (9)	18 (53)	22 (65)	31 (91)	11 (32)
Nevada						
East Humboldt/Ruby Mountains	5	4	0	5	5	3
Total (%)	5	4 (80)	0	5 (100)	5 (100)	3 (100)[e]
Washington						
Yakima Canyon	31[f]	9 (29)	4 (13)	27 (87)	28 (90)	30 (97)
All three states						
Total (%)	70	16 (23)	22 (31)	54 (77)	64 (91)	44 (65)
Healthy bighorn sheep						
Wyoming						
Sheep Ridge	9	9	5	0	0	2
Torry Rim	36	36	13	3	2	12
Total (%)	45	45 (100)	18 (40)	3 (7)[g]	2 (4)[g]	14 (31)

[a] Determined by culture.

[b] Determined by PCR using *M. haemolytica*-specific primers (gcp).

[c] Determined by PCR using lkt set-1 primers.

[d] Determined by culture or PCR.

[e] Two samples were not tested for *M. ovipneumoniae*.

[f] Five bighorns that were killed in this group were visually healthy at the time of euthanasia. These animals had signs of mild bronchopneumonia in the lungs. Three bighorns that were killed had coughing but had no apparent gross or histologic evidence of pneumonia.

[g] Genomic DNA extraction failed in three samples; hence, total number of samples tested was 42.

Pasteurellaceae and *M. ovipneumoniae* in their nasopharynx. *Bibersteinia treholosi* was detected in the nasopharynx of 100% of the bighorns whereas *M. haemolytica* was detected in only 7% (3/45) by culture. By the species-specific PCR, one sample that was negative for *M. haemolytica* by culture was positive for leukotoxin-positive *M. haemolytica*, bringing the total number of animals positive for *M. haemolytica* to 4 (9%). As expected, all the samples were positive for *B. trehalosi* by PCR.

Of the 45 bighorns, only two (4%) carried leukotoxin-positive *B. trehalosi* and *M. haemolytica*. Therefore, as expected, >90% of healthy bighorns carried leukotoxin-negative members of *Pasteurellaceae* in their nasopharynx. *Mycoplasma ovipneumoniae* was detected in 31% of the animals (Table 2).

DISCUSSION

PCR-based methods yield high specificity and sensitivity for the detection of bacterial DNA (Klein 2002; Safaee et al. 2006), but they do not indicate the presence of viable organisms in the tissue.

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The ability to accurately detect *M. haemolytica* isolates (n=50) but not *B. trehalosi* (n=33), *P. multocida* (n=12), or *M. glucosida* (n=11) indicated the high specificity of our PCR assay. The 100% identity between the sequence of PCR amplicons and the GenBank sequence for *gcp* of *M. haemolytica* further confirmed high specificity.

Culture-based methods detected *B. trehalosi* and *P. multocida* from 23% and 31%, respectively, but *M. haemolytica* from only 3% of the lung tissue samples from the 2009–2010 epizootics. These results are consistent with our previous in vitro demonstration that *B. trehalosi* and *P. multocida* inhibit the growth of *M. haemolytica* (Dassanayake et al. 2010a; Bavananthasivam et al. 2012). Our finding that 77% of the lung tissue samples that were culture-negative for *M. haemolytica* were positive by our PCR assay supports our hypothesis that culture-based methods do not accurately detect *M. haemolytica* in lung tissues from pneumonic bighorns.

Previous studies (Dassanayake et al. 2009, 2013) demonstrated that leukotoxin-positive *M. haemolytica* and *B. trehalosi* cause severe lung lesions and fatal pneumonia in bighorns whereas leukotoxin-negative strains cause mild lung lesions but no death. Our leukotoxin-specific PCR assay detected *lktA* in 91% of lung tissues (Table 2). As stated earlier, *M. haemolytica* was detected by PCR in 77% of lung tissues. The primers designed to amplify leukotoxin detected not only leukotoxin of *M. haemolytica* but also that of other *Mannheimia* species such as *M. glucosida* and *Mannheimia ruminalis*. Therefore, the samples (n=13) that were negative for *M. haemolytica* but positive for *lktA* were likely to be leukotoxin-positive *Mannheimia* species other than *M. haemolytica*.

We could not confirm, by cytotoxicity assay, the secretion of leukotoxin by *M. haemolytica* detected by the PCR assay due to nonavailability of cultures. Even though only eight isolates were tested, the fact that 100% of *M. haemolytica* from the lungs and nasopharynx of dead or killed bighorns isolated by culture secreted leukotoxin indicates that *M. haemolytica* was an important pathogen responsible for the fatal pneumonia in bighorns during 2009–2010.

Previously, Besser et al. (2012b) found leukotoxin-positive *Pasteurellaceae* (*M. haemolytica* and *B. trehalosi*) in only 23% (10/44) of bighorns affected during the 2009–2010 epizootic in contrast to 91% (64/70) of leukotoxin-positive *Mannheimia* species (*M. haemolytica* and *M. glucosida*) in our study. Animals examined in the previous study and in our study were different except for five animals. Of these five animals, results of *M. haemolytica*-specific PCR were the same for four animals in both studies. However, we found all five samples to be leukotoxin-positive while the previous study found them to be leukotoxin-negative. One possible reason for this discrepancy is that different primers were used to amplify *lktA*. Generally, the DNA sample is sheared to enhance the chances of accessibility of target region. This procedure is more likely to chop a larger target region (as in the previous study) than a shorter target region (as in this study). A second reason for the discrepancy could be the different PCR conditions (i.e., addition of DMSO and a larger number of cycles in this study). Besser et al. (2012b) found 100% of healthy bighorns negative for leukotoxin-positive *M. haemolytica*, which is consistent with our finding that the great majority of healthy bighorns do not carry leukotoxin-positive *M. haemolytica*.

It is logical to question how *M. haemolytica* could cause fatal pneumonia in bighorns if it is inhibited by *B. trehalosi* and *P. multocida*. Leukotoxin-induced lysis of alveolar macrophages and polymorphonuclear leukocytes (PMNs) abrogates or weakens the host defense mechanisms. Furthermore, lysis and degranulation of alveolar macrophages and PMNs, edema, and fibrin deposition enrich the nutrient

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supply in the microenvironment of the lung, which enables *B. trehalosi* and *P. multocida* to outgrow and inhibit *M. haemolytica*. This notion is supported by two observations: 1) The great majority of *B. trehalosi* isolates from bighorns are leukotoxin-negative and, hence, cannot successfully overcome the phagocytic effects of macrophages and PMNs; ruminant strains of *P. multocida* do not possess any virulence factor similar to the leukotoxin of *M. haemolytica*; and 2) *Bibersteinia trehalosi* grows much faster than *M. haemolytica* (doubling time for *B. trehalosi* is ~14 min compared to ~27 min for *M. haemolytica*; Dassanayake et al. 2010a). The inevitable loss of time between sample collection and laboratory analysis provides additional time for *B. trehalosi* and *P. multocida* to overgrow and inhibit *M. haemolytica* in the lung tissue. Our *M. haemolytica*-specific PCR assay circumvents the problem with detection of this organism by cultural methods.

Bibersteinia trehalosi isolated from these animals was unlikely to be the pathogen that caused the fatal pneumonia because only 3% (2/78 [lungs=52 isolates, nasopharynx=26 isolates]) of these isolates secreted leukotoxin. Moreover, two bighorn lung tissues that were positive for leukotoxin-producing *B. trehalosi* by culture were also positive for leukotoxin-producing *M. haemolytica* by PCR. Our finding that the majority of *B. trehalosi* isolates from bighorns do not produce leukotoxin is in agreement with that of previous studies (Tomassini et al. 2009).

Although *P. multocida* has been isolated from pneumonic bighorn lungs, experimental inoculation of bighorns has not been performed to determine whether it can cause fatal pneumonia. It is our view that *P. multocida* is not a significant pathogen in bighorn pneumonia because of the absence of virulence factors in this organism that can cause the type of lesions routinely observed in pneumonic bighorn lungs. Experimental inoculation studies are being planned to confirm this.

Detection of *M. ovipneumoniae* in the lung tissues of 65% of pneumonic bighorns by PCR or culture-based methods raises the question as to whether it was responsible for the fatal pneumonia in bighorns during the die-offs of 2009–2010 (Table 2). In a previous study, repeated inoculation of high doses (1×10^7 colony-forming units) of in vitro-propagated *M. ovipneumoniae* did not result in fatal pneumonia in two bighorn lambs (Besser et al. 2008). In another study, inoculation of oronasal washing from *M. ovipneumoniae*-positive domestic sheep into two *M. haemolytica*-negative adult bighorns did not result in fatal pneumonia. In the same study, inoculation of two adult bighorns with lung homogenate from *M. ovipneumoniae*-positive lambs resulted in fatal pneumonia in one animal but not in the other. The animal that developed fatal pneumonia did carry leukotoxin-positive *M. haemolytica* in its nasopharynx from the onset of the experiment (Dassanayake et al. 2010b). In another study, one of four bighorns commingled with four *M. ovipneumoniae*-negative domestic sheep developed fatal pneumonia (Besser et al. 2012a). On necropsy, *M. haemolytica* but not *M. ovipneumoniae* was isolated from the lungs. The other three animals did not develop pneumonia. However, it is important to note that these animals developed high titers of *M. haemolytica* leukotoxin-neutralizing antibodies (S. Srikumaran, unpubl. data). Furthermore, Weiser et al. (2012) found 50% of healthy bighorns tested were positive for *M. ovipneumoniae* in the nasopharynx. Their finding is supported by the detection of the presence and shedding of *M. ovipneumoniae* in healthy bighorns in a Colorado herd (L. Wolfe, Colorado Division of Wildlife, pers. comm.) and the detection of *M. ovipneumoniae* in the nasopharynx of 31% of healthy bighorns in this study (Table 2). Taken together, these results suggest that although *M. ovipneumoniae* can predispose bighorns to bacterial pneumonia, by itself it does not typically cause fatal

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pneumonia in bighorns, and that *M. haemolytica* causes fatal pneumonia in bighorns in the presence or absence of *M. ovipneumoniae*. Presence of *M. ovipneumoniae* may increase severity or spread of the disease by impairing the mucociliary defense mechanism which, in turn, impairs clearance of inhaled bacteria (Niang et al. 1998). Because most domestic sheep carry both leukotoxin-positive *M. haemolytica* and *M. ovipneumoniae*, it is prudent to segregate bighorn populations from domestic sheep until strategies to prevent transmission of these organisms from domestic sheep are developed. In summary, we have developed a PCR assay specific for *M. haemolytica*. The results of PCR assays of lung tissues from bighorns that died or were killed during the pneumonia outbreak in Montana, Nevada, and Washington suggest leukotoxin-positive *M. haemolytica* is an important pathogen responsible for the death of most bighorns during the die-offs of 2009–2010.

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