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Research Article

Evidence for Strain-Specific Immunity to Pneumonia in Bighorn Sheep

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ABSTRACT Transmission of pathogens commonly carried by domestic sheep and goats poses a serious threat to bighorn sheep (*Ovis canadensis*) populations. All-age pneumonia die-offs usually ensue, followed by asymptomatic carriage of *Mycoplasma ovipneumoniae* by some of the survivors. Lambs born into these chronically infected populations often succumb to pneumonia, but adults are usually healthy. Surprisingly, we found that introduction of a new genotype (strain) of *M. ovipneumoniae* into a chronically infected bighorn sheep population in the Hells Canyon region of Washington and Oregon was accompanied by adult morbidity (100%) and pneumonia-induced mortality (33%) similar to that reported in epizootics following exposure of naïve bighorn sheep. This suggests an immune mismatch occurred that led to ineffective cross-strain protection. To understand the broader context surrounding this event, we conducted a retrospective analysis of *M. ovipneumoniae* strains detected in 14 interconnected populations in Hells Canyon over nearly 3 decades. We used multi-locus sequence typing of DNA extracts from 123 upper respiratory tract and fresh, frozen, and formalin-fixed lung samples to identify 5 distinct strains of *M. ovipneumoniae* associated with all-age disease outbreaks between 1986 and 2014, a pattern consistent with repeated transmission events (spillover) from reservoir hosts. Phylogenetic analysis showed that the strain associated with the outbreak observed in this study was likely of domestic goat origin, whereas strains from other recent disease outbreaks probably originated in domestic sheep. Some strains persisted and spread across populations, whereas others faded out or were replaced. Lack of cross-strain immunity in the face of recurrent spillovers from reservoir hosts may account for a significant proportion of the disease outbreaks in bighorn sheep that continue to happen regularly despite a century of exposure to domestic sheep and goats. Strain-specific immunity could also complicate efforts to develop vaccines. The results of our study support existing management direction to prevent contacts that could lead to pathogen transmission from domestic small ruminants to wild sheep, even if the wild sheep have previously been exposed. Our data also show that under current management, spillover is continuing to occur, suggesting that enhanced efforts are indicated to avoid introducing new strains of *M. ovipneumoniae* into wild sheep populations. We recommend looking for new management approaches, such as clearing *M. ovipneumoniae* infection from domestic animal reservoirs in bighorn sheep range, and placing greater emphasis on existing strategies to elicit more active cooperation by the public and to increase vigilance on the part of resource managers.

KEY WORDS bighorn sheep, disease ecology, Hells Canyon, livestock-wildlife interface, molecular epidemiology, multi-locus sequence typing, *Mycoplasma ovipneumoniae*, *Ovis canadensis*.

Pneumonia in bighorn sheep (*Ovis canadensis*) is a population-limiting disease associated with transmission of pathogens from domestic sheep and goats (Foreyt and Jessup 1982, Besser et al. 2013, Cassirer et al. 2013). As with many other wildlife diseases, a lack of comprehensive, system-specific information hampers disease management (Besser et al. 2013, Joseph et al. 2013). Limiting contact with domestic sheep and goats is the primary strategy for preventing disease emergence in bighorn sheep (Western Association of Fish and Wildife Agencies Wild Sheep Working Group 2012, The Wildlife Society 2015). Although currently the best management option available, implementation is politically and logistically difficult because of the natural movements of wild animals, unregulated presence of domestic sheep and goats on private lands, and concerns by the livestock industry about losing access to public grazing allotments. Reliable knowledge about health

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threats and potential solutions will help wildlife and land managers make appropriate risk management decisions that will succeed in resolving the problem of pneumonia in bighorn sheep.

Based on the hypothesis that *Mannheimia haemolytica* expressing leukotoxin is the key causal pathogen, researchers have tested numerous vaccines to boost immunity to disease in bighorn sheep. However, so far no vaccine has protected wild sheep commingled with domestic sheep or goats in captive settings or shown potential for efficacy in free-ranging animals (Callan et al. 1991, Kraabel et al. 1998, Cassirer et al. 2001, Subramaniam et al. 2011, Sirochman et al. 2012). There may be several reasons for the elusiveness of an effective vaccine. First, there is a basic question as to the role of *M. haemolytica* in the disease (Besser et al. 2013) and second there are significant technical difficulties associated with vaccine development and application. Experimental challenge with leukotoxin-positive *M. haemolytica*, a well-described respiratory pathogen in domestic ruminants, is lethal to bighorn sheep in captivity (Foreyt et al. 1994, Dassanayake et al. 2009). However, *M. haemolytica* is only weakly associated with pneumonia epizootics in free-ranging bighorn sheep populations (Besser et al. 2012*b*). The pathology, microbiology, and the course of disease experimentally induced with *M. haemolytica* also do not match observations from the field (Besser et al. 2014). Recently, application of sensitive molecular diagnostic techniques on high quality samples led to identification of *Mycoplasma ovipneumoniae*, a previously overlooked bacterium, as the pathogen most strongly supported as a primary causal agent of pneumonia in bighorn sheep (Besser et al. 2008, 2012*a*, *b*).

M. ovipneumoniae is host-specific to Caprinae, and is frequently carried asymptomatically by domestic sheep and goats (Martin and Aitken 2000). When introduced into naïve bighorn sheep populations, outbreaks of polymicrobial pneumonia ensue, sometimes resulting in high mortality in all age classes (Besser et al. 2008, 2014). After all-age pneumonia outbreaks, surviving adults usually maintain good health and normal life spans, although some individuals chronically carry *M. ovipneumoniae* in their upper respiratory tract (Besser et al. 2013). Both carriers and non-carriers are resistant to disease although this protection fails to prevent epizootics in lambs (Plowright et al. 2013, Manlove et al. 2016).

M. ovipneumoniae is also associated with mild and transient respiratory disease, usually in juveniles, in its normal domestic sheep and goat hosts (DaMassa et al. 1992, Martin and Aitken 2000). However, several investigators have reported that *M. ovipneumoniae* infections in domestic sheep and goats can cause severe pneumonia, particularly when multiple strains are present (Parham et al. 2006, Rifatbegović et al. 2011). This could be linked to a strain-specific immune response that fails to provide universal protection. Many pathogens are able to evade host immune responses by expressing a diversity of surface-exposed targets for neutralizing antibodies. From influenza virus to *Mycoplasma* spp., antigenic variation within and across strains enables immune escape by pathogens and also complicates development of vaccines (Citti et al. 2010, Vink et al. 2012, Quiñones-Parra et al. 2014).

We documented the effects of invasion of a novel strain of *M. ovipneumoniae* into a group of free-ranging bighorn sheep that had harbored adult carriers for nearly 20 years following an all-age pneumonia outbreak (Cassirer et al. 1996, Plowright et al. 2013). Our expectation was that these adults were immune to the pathogen and that invasion of a new strain would not cause disease.

STUDY AREA

We conducted this study near Heller Bar at the mouth of the Grande Ronde River in Asotin County, Washington, USA (46.079° N, −116.986° W). The area was located in low elevation (250–1,250 m) canyon grasslands and cliffs along the breaks of the Grande Ronde and Snake Rivers on the northern edge of Hells Canyon. Summers were hot ($\bar{x}$ highs in Jul and Aug = 26–32° C) and winters were mild ($\bar{x}$ lows in Dec and Jan = −2 to 2° C). Average annual precipitation was 31 cm. July and August were the driest months and peak precipitation occurred in May. Plant associations were dominated by perennial bunchgrass (*Pseudoroegneria spicata* and *Festuca idahoensis*) communities, with deciduous riparian shrub stringers and upland shrub-fields. Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*) stands occurred on northerly aspects. In addition to bighorn sheep, common ungulates in the study area included mule deer (*Odocoileus hemionus*), white-tailed deer (*O. virginianus*), and elk (*Cervus elaphus*). Potential predators of bighorn sheep included cougars (*Felis concolor*), bobcats (*Lynx rufus*), coyotes (*Canis latrans*), wolves (*C. lupus*), and black bears (*Ursus americanus*). Over 50% of the area was publicly owned and managed by federal and state agencies chiefly for wildlife, recreation, and seasonal (spring) cattle grazing. A low density, unincorporated rural community was scattered on adjoining private rangelands at the mouth of the Grande Ronde River and along the adjacent Snake River.

Following extirpation in the early 1900s, the Washington Department of Fish and Wildlife reintroduced bighorn sheep to the Joseph Creek Wildlife Area near Heller Bar. Between 1977 and 1989, 39 sheep were translocated from Washington, Oregon, and Montana to establish the Black Butte population (Johnson 1995). This became one of 16 interconnected populations that comprise the Hells Canyon bighorn sheep metapopulation. The Black Butte population increased to approximately 215 animals before a pneumonia outbreak occurred in 1995; 70% of the sheep died or were transferred to captivity in an attempt to stop the epidemic (Cassirer et al. 1996). The source of the outbreak was thought to be domestic sheep or goats on private lands within the Black Butte bighorn sheep population range (Rudolph et al. 2003). The population never recovered because of chronically low recruitment due to pneumonia-induced mortality in lambs (Plowright et al. 2013). By 2013, only 36 bighorn sheep were observed in surveys, and the population was estimated at 45 (Cassirer et al. 2013, Washington Department of Fish and Wildlife, unpublished data). Three

spatially distinct female groups occur in the population: Heller Bar, Shumaker, and Joseph Canyon. The groups are connected by movements of males, but we observed no female interactions across groups during this study. The Heller Bar female group was most accessible from the road and was the subject of this investigation.

METHODS

Observations

We monitored the Heller Bar female group during 2013 and 2014 as part of a study of contact patterns and lamb survival. We could individually identify 4 (31%) and 11 (85%) of the 13 adult females in 2013 and 2014, respectively, by numbered ear tags and color-coded and numbered very high frequency (VHF) radio-collars. One unmarked female was missing a horn, so all 13 sheep were individually identifiable in 2014. We located marked animals from the ground by radio-telemetry and then observed them through binoculars and a spotting scope. We conducted frequent and intensive observations between 1 May and 16 July to document productivity and neonatal survival (2013 median observation interval = 4 days, median duration of each observation = 3 hr; 2014 median observation interval = 1.5 days, median duration = 2 hr). Frequency of observation from 17 July through 26 August was every 10 days in 2013 and every 5 days in 2014, and from 26 August through the first week in October we observed the sheep once a month in 2013 and every 10 days in 2014. Median duration of observations from 17 July through October was 1 hour in both years. At each observation we recorded female and lamb health and behavior. Animals observed with nasal discharge, droopy ears, head shaking, or lethargy received a clinical score of 1, and animals observed coughing received a clinical score of 2. Sheep with no evidence of disease received a clinical score of 0.

Radio-collars on adults were equipped with a switch that triggered a fast pulse mortality signal if no movement was detected for 4 hours. We conducted site investigations, and where possible, retrieved carcasses whole when mortalities were detected. Where this was not possible, we conducted field necropsies and collected the head, the respiratory tract, and grossly abnormal tissues when available. We detected lamb mortalities through observation and retrieved whole dead lambs when autolysis was not too advanced for diagnostic testing (Cassirer and Sinclair 2007). We assigned lamb mortality dates as the midpoint between the last live observation and either the date when the carcass was found, or the date when the dam was first observed without a lamb if no carcass was located. We assumed a female had lost her lamb when it was found dead or when the number of lambs declined and she was never again observed with a lamb that year. All cadavers and tissues were submitted to the Washington Animal Disease and Diagnostic Laboratory (WADDL; Pullman, WA, USA) for analysis.

Health Sampling

We captured and sampled females in February ($n = 11$ of 13) and July ($n = 2$ of 11) 2014. In October 2014, we resampled all 8 remaining sheep when we transferred them to captivity. We conducted captures via helicopter netgun and by darting from the ground with chemical immobilizing agents. All capture and handling followed animal care protocols approved by the Washington Department of Fish and Wildlife. Sampling entailed collecting throat swabs and placing them in buffered glycerol or Port-a-cul transport media (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA, #220144, #221606) for aerobic culture to detect Pasteurellaceae, swabbing nasal passages and placing swabs in mycoplasma broth (#102, Hardy Diagnostics, Santa Maria, CA, USA) for culture enrichment and polymerase chain reaction (PCR) detection of *M. ovipneumoniae* (Ziegler et al. 2014), and collection of serum for detection of antibodies to *M. ovipneumoniae* (competitive enzyme-linked immunosorbent assay [ELISA]), bovine respiratory syncytial virus (virus neutralization [VN]), bovine virus diarrhea (VN), infectious bovine rhinotracheitis (VN), and bovine parainfluenza-3 (PI-3, VN). Diagnostic testing on the above samples was conducted by WADDL.

We also collected upper respiratory washes by flushing nasal passages with 50 ml of phosphate-buffered saline and swabbed the oropharynx. We kept samples cool and processed them within 48 hours of collection, or stored them at -20° C. We extracted DNA (DNeasy, Qiagen, Redwood City, CA, USA) from swabs, from 10-ml aliquots of nasal wash, and from lung tissues of animals that died during the study as well as 2 pneumonic lambs that died in adjacent populations in 2013 to test for presence of pneumonia agents. We used a multiplex PCR to detect Pasteurellaceae including *Bibersteinia trehalosi*, *Pasteurella multocida*, and *Mannheimia* spp. (Besser et al. 2012*b*) and performed PCR for *lktA*, the gene encoding leukotoxin A, the major virulence factor of *Mannheimia* spp. and *B. trehalosi* (Walsh et al. 2016). If an agent was detected by either PCR or culture on any sample, we classified the animal as positive for that agent.

Strain Typing

Health of the Hells Canyon bighorn sheep metapopulation has been intensively monitored since the 1995 pneumonia outbreak in the Black Butte population, and intermittently prior to this. Therefore, we had access to fixed, frozen, and fresh lung tissue and swab samples collected from 1995–2015 in the Heller Bar female group and from 1986–2015 in adjacent female groups and populations. We extracted DNA from a subset of these sources to detect and strain type *M. ovipneumoniae* within the study population and the metapopulation through time. Detection was based on conventional (McAuliffe et al. 2003) and realtime (Ziegler et al. 2014) PCR.

We used multi-locus sequence typing (MLST) to characterize strains using partial DNA sequences of the 16S-23S intergenic spacer region (IGS), the small ribosomal subunit (16S), and the genes encoding RNA polymerase B (*rpoB*) and gyrase B (*gyrB*). We amplified these targets with PCR using a suite of existing and newly developed primers (Table 1). Because of the high degree of DNA sequence

Table 1. Oligonucleotide primers for polymerase chain reactions (PCR) used to amplify *Mycoplasma ovipneumoniae* multi-locus sequence typing (MLST) targets. Nesting refers to external and internal primer sets used for nested PCR reactions for amplification of the MLST loci, IGS, *rpoB*, and *gyrB,* when amplification from the default (internal) primers produced insufficient DNA template for sequencing.

Target[a]	Nesting	Oligonucleotide primer sequence	Reference
LM-F		TGAACGGAATATGTTAGCTT	McAuliffe et al. (2003)
LM-R		GACTTCATCCTGCACTCTGT	McAuliffe et al. (2003)
Ex-IGS-F	External	GTTAACCTCGGAGACCATTG	This paper
Ex-IGS-R	External	GTTTGCTAGGTTGGGTTTCC	This paper
IGS-F	Internal	GGAACACCTCCTTTCTACGG	Besser et al. (2012*b*)
IGS-R	Internal	CCAAGGCATCCACCAAATAC	Besser et al. (2012*b*)
Ex- *rpoB* −F	External	AGTTATCACAATTTATGGATCAAA	This paper
Ex- *rpoB* −R	External	GCTCAAAGTTCCATTTCNCCGAA	This paper
rpoB −F	Internal	TCGGCTTCAGCAATTCCTTTCTT	This paper
rpoB −R	Internal	TCGGCTGTTGGGTTGTCTTCTC	This paper
Ex- *gyrB* −F	External	AAAACGWCCAGGKATGTATATTGG	This paper
Ex- *gyrB* −R	External	GGATCCATTGTTGTTTCTCATAATTG	This paper
gyrB −F	Internal	GGGTCAAACAAAAGCAAAACTAAA	This paper
gyrB −R	Internal	ACGGAATAAAAATGTCAAAAGTAA	This paper

[a] LM = small ribosomal subunit (16S) gene, IGS = 16S-23S intergenic spacer, *rpoB* = β subunit of RNA polymerase gene, *gyrB* = gyrase B gene, F = Forward, R = Reverse.

variation in *M. ovipneumoniae*, the biggest challenge in developing this ensemble of loci was identification of targets with sufficiently conserved primer binding site sequences to enable consistent PCR amplification for subsequent sequencing. The loci selected for this study each exhibited extensive sequence polymorphism and together provide a highly discriminatory test. We typed the concatenated sequences using multi-locus sequence analysis because of the large numbers and diversity of the alleles detected at each target locus. We aligned sequences using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/, accessed 06 Sep 2016). We report sequence divergence as the percentage non-identity.

All PCR protocols included a preliminary dissociation phase (95° C, 15 min), amplification, and a final extension phase (72° C, 7 min). We amplified the 16S target using 35 cycles of 30 seconds each of dissociation (95° C), annealing (58° C), and extension (72° C). We amplified the IGS target using 35 cycles of dissociation (95° C, 1 min), annealing (52° C, 2 min), and extension (72° C, 2 min). For nested IGS amplification, the parameters for the external primers were identical except that the annealing temperature was 54° C. For *rpoB* and *gyrB*, we used inner and nested reactions with identical amplification conditions: dissociation (95° C, 1 min), annealing (50° C, 1 min), and extension (72° C, 2 min). We treated the extracts with alkaline phosphatase and exonuclease I (FastAP and ExoI, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. We submitted extracts to Amplicon Express (Pullman, WA, USA) to determine forward and reverse DNA sequences using the same primers used for PCR amplification. Representative sequences of all 4 target loci for the gentoypes herein are available in Genbank: strain 393 (KU986495, KU986500, KU986503, KU986506, representing IGS, 16S, *rpoB*, and *gyrB*), strain 404 (KU986496, KU986501, KU986504, KU986507), strain 415 (KU986494, KU986499, KU986502, KU986505), strain 402 (KU986493, KU986498, representing IGS and 16S), and strain 419 (KU986492, KU986497).

Statistical Analysis

We used a Kaplan–Meier staggered entry estimator and a Cox proportional hazard model to analyze survival of females and lambs. We fit trend lines to 7-day moving averages of clinical scores using a lowess smoothing factor derived from locally weighted moving mean scores spanning 25% of the full dataset (2–3 weeks). We used a Fisher's exact test to analyze prevalence of pneumonia agents before, during, and after the outbreak and to compare agents present in adult and lamb mortalities. We used a Kruskal–Wallis rank-sum test to determine whether neutralizing titers to PI-3 differed before, during, and after the outbreak. We used a 1-way analysis of variance (ANOVA) and Tukey contrasts to test for differences in serologic antibody titers to *M. ovipneumoniae* over the course of the outbreak and a 2-sided *t*-test to compare pre-outbreak antibody titers to *M. ovipneumoniae* in animals that died and those that survived. We used a Wilcoxon rank sum test with continuity correction to analyze duration of clinical signs. We conducted analyses with package survival (Therneau 2015), and stats and base packages in R (R Core Team 2015).

RESULTS

Survival and Clinical Signs of Disease

In 2013, we observed all marked females with lambs, and median parturition date was 18 May (range = 5–20 May). In 2014, we observed 10 of 13 females with live lambs and median parturition date was 8 May (range = 27 Apr–17 May). One marked female and her lamb died, presumably of dystocia, on 16 May 2014 (e5 and L5, Fig. 1a), 1 marked female was observed with a dead 2-day old lamb (e10, Fig. 1a), and 1 unmarked female appeared to be pregnant but was never observed with a lamb (e22, Fig. 1a).

We observed clinical signs of pneumonia in lambs starting 1 June in 2013 and 26 May in 2014. Symptoms continued until the day the last lamb died, which was 30 June in 2013, and 2 July in 2014 (Fig. 1). Median lamb mortality date was 28 June in 2013 and 24 June in 2014, at a median age

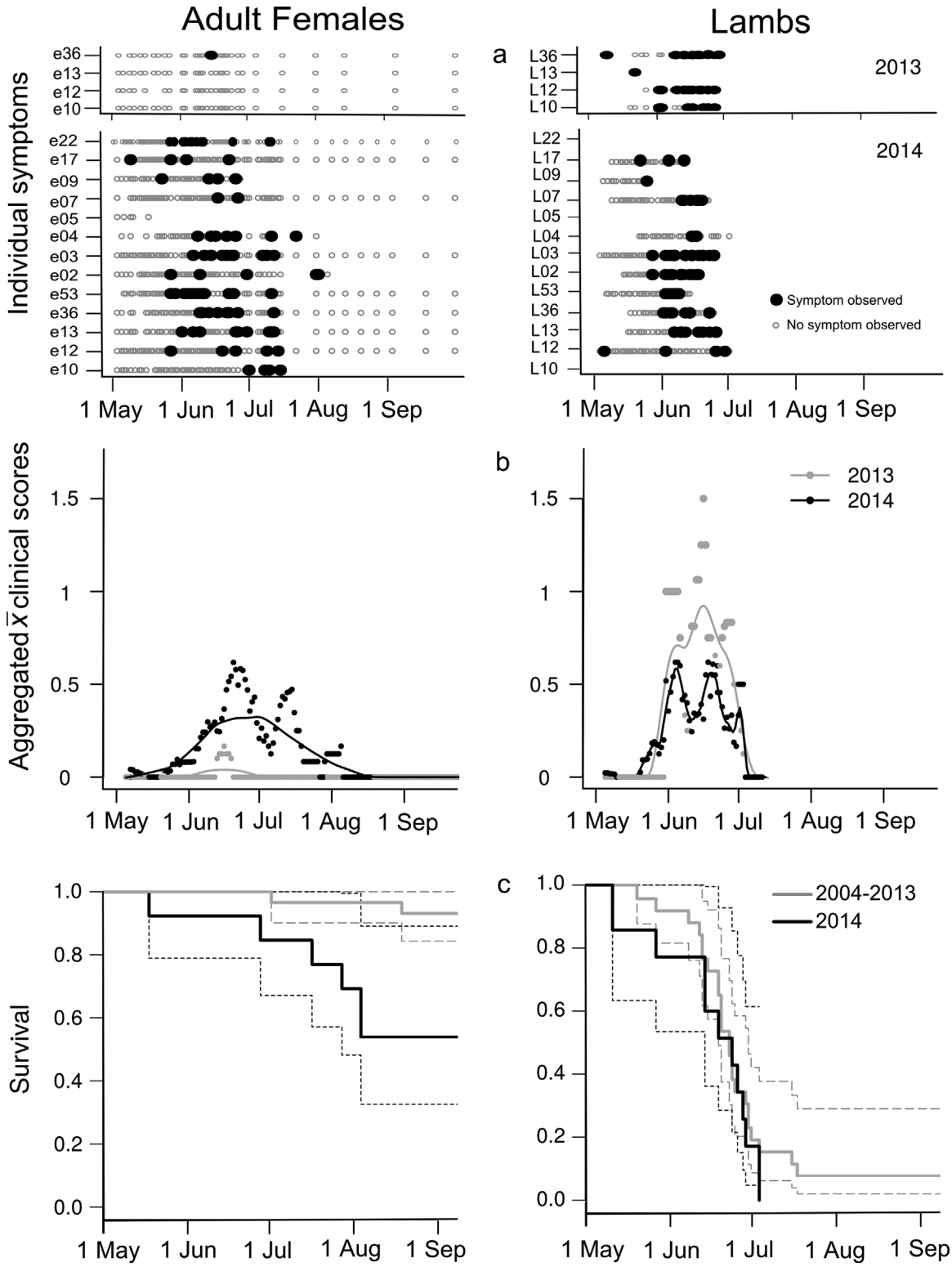

Figure 1. Clinical signs of pneumonia and survival of bighorn sheep in the Black Butte, Washington population during summers 2013 ($n = 4$ adult F and 4 lambs) and 2014 ($n = 13$ adult F and 10 lambs). (a) Time series of field observations of pneumonia symptoms in adult females (e) and their lambs (L). Observations ending prior to September indicate individual died. (b) Smoothed average daily clinical scores for adult females and lambs. (c) Adult female and lamb survival between May and October 2014 and survival between May and October during the 10 previous years, 2004–2013 (Kaplan–Meier curves and 90% CIs).

of 40 and 44 days, respectively. All lambs died both years and there were no differences in lamb survival curves between years (log $\text{rank}_1 = 0.01$, $P = 0.92$) or between 2014 and the previous 10 years (Fig. 1c). We submitted 11 lambs for necropsy (6 in 2013 and 5 in 2014). With the exception of 1 lamb that died at approximately 2 days of age in 2014 (L10, Fig. 1a), all lambs presented characteristic lesions of bighorn lamb respiratory disease, including moderate to

Table 2. Prevalence of *Mannheimia* spp. (Mh), *Bibersteinia trehalosi* (Bt), *Pasteurella multocida* (Pm), Pasteurellaceae leukotoxin encoding gene (*Lkt*A), and *Mycoplasma ovipneumoniae* (*Movi*) in asymptomatic bighorn sheep females (F) sampled before and after a pneumonia outbreak, symptomatic females during the outbreak, and in the lungs of pneumonic females that died in the Black Butte population and pneumonic lambs that died in Black Butte, (10) and adjacent populations (2), Washington and Oregon, USA.

Agent	F before ($n = 11$)	F during (symptomatic) ($n = 2$)	F pneumonia mortalities ($n = 3$)	Lamb pneumonia mortalities ($n = 12$)	F after ($n = 8$)
Mh	0.91	1.00	0.00	0.70	0.75
Bt	0.82	1.00	1.00	0.80	1.00
Pm	0.73	1.00	1.00	0.10	1.00
*Lkt*A	0.27	0.50	0.33	0.50	0.38
Movi	0.09	1.00	1.00	1.00	0.13

severe, subacute to chronic bronchopneumonia and otitis media.

Median age at first observation of clinical signs in lambs was 20 days (range: 7–35), median duration of clinical signs was 22 days (range: 1–59), and median age at death was 42 days (range: 22–66). Assuming that lambs were infected by 4 days of age and lung lesions were present by 10 days of age (Besser et al. 2008), we estimated a median latent period of 16 days (range: 3–31) between infection and first observation of disease symptoms in lambs and a median infection period of 38 days (range: 18–62) prior to death.

In adults, the only evidence of possible respiratory disease in 2013 was a single observation of coughing on 14 June, and all adults survived. In 2014, we observed clinical signs of pneumonia in adults starting in May. By June, all adults exhibited symptoms of pneumonia including severe prolonged coughing (Fig. 1a and b). Five adult females (38%) died between May and August 2014 (difference between summers, log $\text{rank}_1 = 5.98$, $P = 0.01$) and the hazard of an adult female dying in the summer of 2014 was 6.83 times higher (SE = 0.83, $P = 0.02$) than in any of the previous 10 summers (2004–2013, Fig. 1c). We did not observe pneumonia symptoms prior to the first adult death on 16 May 2014. Subsequent mortalities followed observation of clinical signs of pneumonia and occurred on 26 June, 18 July, 27 July, and 3 August (Fig. 1a). Median duration of clinical signs was 36 days (range = 18–81 days), which was longer than observed in lambs (median = 22 days, range = 1–59, W = 136.5, $P < 0.001$). Duration of clinical signs did not differ between adults that died and those that survived (Fig. 1a, Wilcoxon rank sum W = 20, $P = 1.0$). We submitted samples to WADDL from all (4 of 5) mortalities where sufficient tissues were available for diagnosis. No gross or histological evidence of respiratory disease was found in the female that died on 16 May, although evaluation of tissues at WADDL was limited because of autolysis. The 3 adult females submitted for necropsy between late June and early August were diagnosed with chronic, moderate to severe bronchopneumonia. The survivors appeared to make a full recovery with no evidence of ongoing disease.

Microbiology and Immune Responses

M. ovipneumoniae was the only pneumonia agent detected more frequently in the lungs of adults that died of pneumonia than in the upper respiratory tract of healthy adults before and after the outbreak ($\chi_1^2 = 26.66$, $P < 0.001$; Table 2). Prevalence of other suspected pneumonia agents in the upper respiratory tract of symptomatic adults sampled during the outbreak was similar to that detected in the lungs of adults that died of pneumonia except that *Mannheimia* spp. were detected in the upper respiratory tract of live adults but not in the lungs of adults that died. We found no differences in prevalence of pneumonia agents present in upper respiratory tract samples collected before and after the outbreak ($\chi_1^2 < 2.6$, $P > 0.2$) or in the lungs of lambs that died in 2013 and 2014 ($\chi_1^2 < 0.39$, $P > 0.5$). Detection of *Mannheimia* spp. was more common in lamb mortalities than in adult mortalities ($\chi_1^2 = 4.55$, $P = 0.07$), and *P. multocida* was more frequently detected in the lungs of dead adults than lambs ($\chi_1^2 = 8.78$, $P = 0.01$; Table 2).

We detected serologic evidence of exposure to PI-3 (VN titer ≥ 4) and *M. ovipneumoniae* (ELISA inhibition > 50%) in adults before, during, and after the 2014 pneumonia outbreak. We did not detect evidence of exposure to other respiratory viruses during the study. Average *M. ovipneumoniae* ELISA percent inhibition (%I) values prior to the outbreak (69%) increased significantly to 89%I and 82%I during and after the disease event, respectively ($F_{2,18} = 4.811$, $P = 0.02$; Fig. 2a). Individuals that died during the outbreak had higher *M. ovipneumoniae* ELISA % I values prior to the outbreak ($\bar{x} = 79\%$) than those that survived ($\bar{x} = 59\%$, $t_{7.5} = 3.02$, $P = 0.02$; Fig. 2b). Median PI-3 titers of 256 ($\log_2 7.7$) did not change significantly over the course of the outbreak ($\chi^2_2 = 4.55$, $P = 0.23$).

Strain Typing

We genotyped *M. ovipneumoniae* from all Heller Bar bighorn sheep where it was detected in 2013 ($n = 7$) and 2014 ($n = 11$). We found a single strain in 2013 based on identical DNA sequences of each of the 4 MLST loci, and that strain was also detected in the first lamb to die of pneumonia in 2014. All subsequent detections of *M. ovipneumoniae* differed from the 2013 strain at 3 MLST loci. The IGS sequences differed by 32 single nucleotide polymorphisms (SNP) and 3 base insertions or deletions (indels, 8.7% divergent), *rpoB* by 16 SNP (2.8% divergent) and *gyrB* by 16 SNP (4% divergent). The 16S sequences did not differ between strains.

We report strain differences by IGS sequences because we were unable to amplify *rpoB* and *gyrB* from DNA extracted

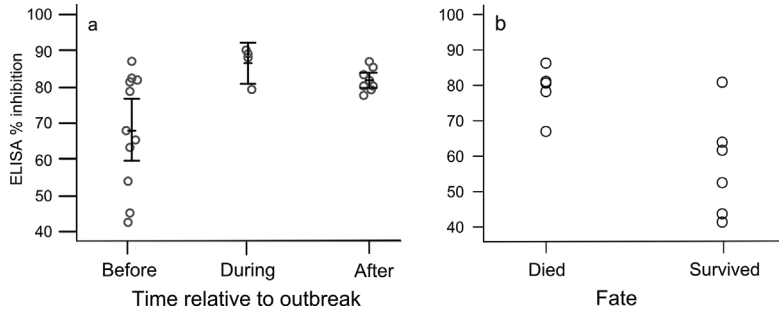

Figure 2. Serologic titers (competitive enzyme-linked immunosorbent assay [ELISA]) to *Mycoplasma ovipneumoniae* in bighorn sheep in Black Butte, Washington, February–October 2014. (a) Mean and 90% confidence intervals before, during, and after a pneumonia outbreak. (b) Antibody titer levels before the outbreak in adults that survived ($\bar{x} = 59\%$) and those that died ($\bar{x} = 79\%$).

from formalin-fixed, paraffin-embedded lung tissues, the only specimens available prior to 2006. Because each of these strains differed by indels in IGS, the strains are conveniently designated by their differing IGS lengths. The 2013 Black Butte strain, IGS 404, has been detected in this population since the 1995 pneumonia outbreak (Fig. 3b). The 2014 strain, IGS 393, had never previously been detected in this population or any other bighorn sheep population in Hells Canyon or elsewhere in the western United States (among >700 other isolates that have been IGS typed).

In October 2014, we removed survivors from the Heller Bar female group in an attempt to prevent further spread of this strain. Nonetheless, 1 month after the removal, we detected the IGS 393 strain type in an adult male removed from the town of Asotin, Washington and we detected it again 4 months later in a 9-month-old lamb in the Shumaker female group, located between the Joseph and Heller Bar

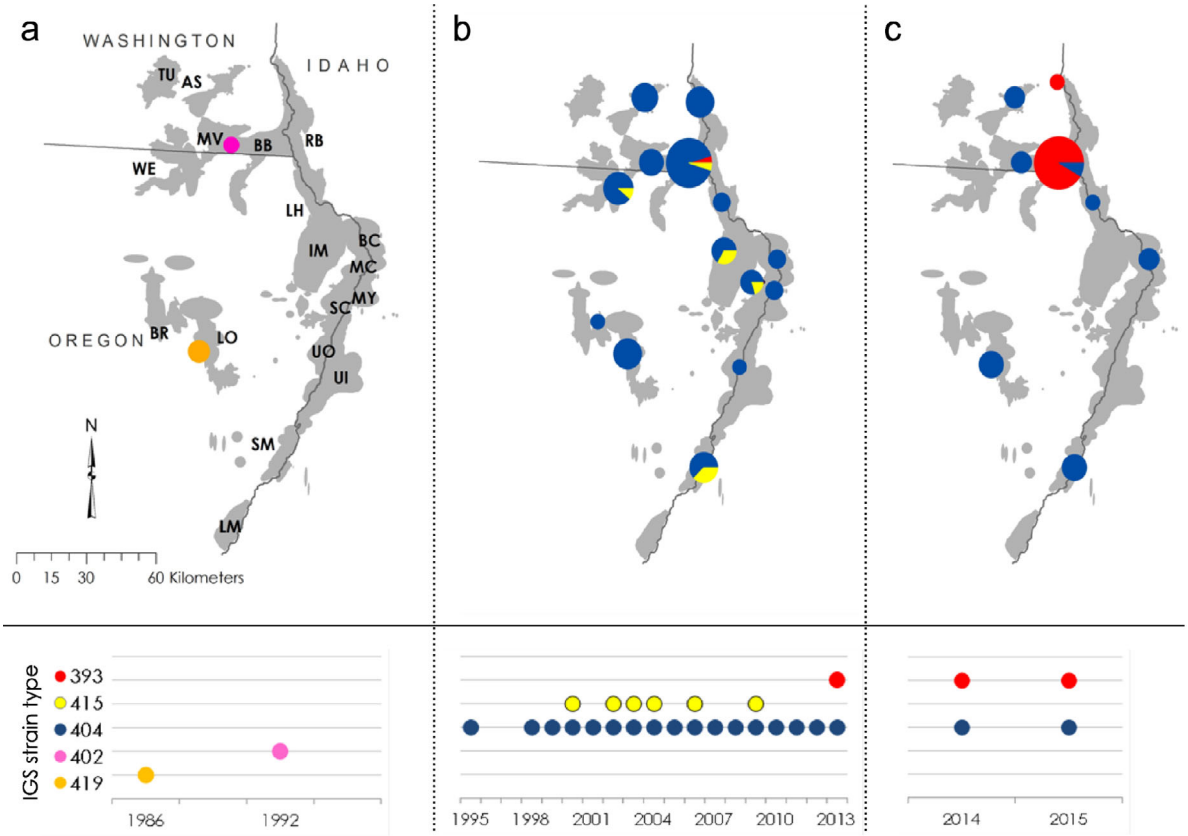

Figure 3. Spatiotemporal distribution of the 16S-23S intergenic spacer (IGS) genotypes of *Mycoplasma ovipneumoniae* in the Hells Canyon bighorn sheep metapopulation in Washington, Oregon, and Idaho, USA in (a) 1986 and 1992 (b) 1995–2013; and (c) 2014–2015. Each colored marker represents one strain type, pie charts display strain types within populations and are scaled by sample size. Pie charts containing >1 color indicate that 2 strain types were present in a population during that time interval but not necessarily detected in the same year. Gray shaded polygons denote bighorn sheep populations: AS = Asotin, BB = Black Butte, BC = Big Canyon, BR = Bear Creek, IM = Imnaha, LH = Lower Hells Canyon, LM = Lookout Mountain, MU = Muir, MV = Mountain View, MY = Myers Creek, RB = Redbird, SM = Sheep Mountain, TU = Tucannon, UO = Upper Hells Canyon, Oregon, UI = Upper Hells Canyon Idaho.

groups in the Black Butte population. Retrospective analysis revealed that this strain was present in the lungs of a male that died of pneumonia in the Joseph Creek group in December 2013, representing the index case of disease associated with the IGS 393 strain type. The IGS 393 strain has not been detected in any surrounding populations, which remain carriers of the original IGS 404 strain (Fig. 3c).

Two other strains were detected in specimens obtained during or after disease outbreaks in other Hells Canyon populations (Coggins 1988, Foreyt et al. 1990), and have not been detected since. These samples were from the Lostine population in 1986–1987 (IGS 419) and the Mountain View population in 1992 (IGS 402; Fig. 3a). A third strain (IGS 415) associated with a pneumonia outbreak in the Sheep Mountain population in 2000 was apparently replaced by the IGS 404 strain by 2006 based on samples typed from 2006 and 2015 in that population. The IGS 415 strain was subsequently infrequently detected in 4 other populations between 2003 and 2009 and has not been found since then (Fig. 3b).

Sequence divergence among the 3 recently detected strains where all 4 genes could be analyzed (i.e., IGS 393, 404, and 415) was between 9% and 10%. These strains were well dispersed across 20 genotypes of *M. ovipneumoniae* collected from 9 domestic sheep flocks in the western United States and Australia and 10 domestic goat flocks in the western United States and China. The IGS 404 and 415 types, first identified in the Black Butte and Sheep Mountain populations, respectively, were more closely related to the domestic sheep lineage, whereas the IGS 393 type detected in the Black Butte population in 2013 and 2014 clustered with the domestic goat clade (Fig. 4).

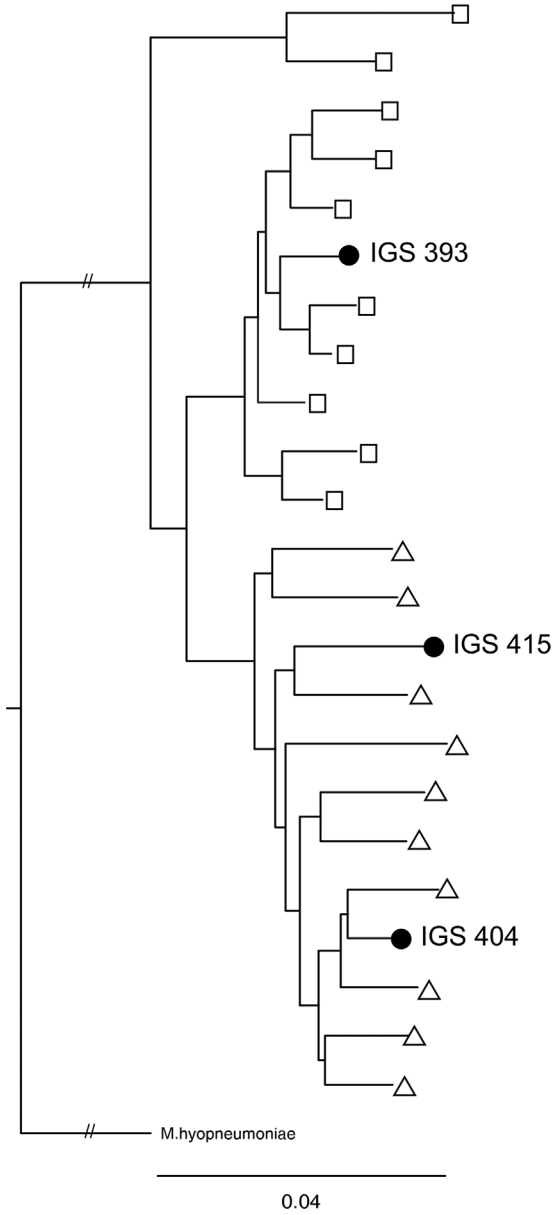

Figure 4. Phylogeny of 3 bighorn sheep strains of *Mycoplasma ovipneumoniae*, Hells Canyon, Washington, Oregon, and Idaho, USA, and randomly selected strains from domestic sheep (triangles) and domestic goats (squares), 1975–2015. Domestic sheep and goats appear to host divergent lineages of *M. ovipneumoniae* and the samples from bighorn sheep clustered with either the domestic sheep or domestic goat lineages. Neighbor-joining tree of concatenated partial 16S, 16S-23S intergenic spacer region (IGS), *rpoB*, and *gyrB* DNA sequences is rooted with homologous sequences from *M. hyopneumoniae*. Branch lengths are scaled by proportion non-identity (scale bar). Domestic sheep samples are from Washington, Oregon, Idaho, Nevada, Colorado, California, and an ATCC type strain from Australia. Domestic goat samples are from Washington, Idaho, California, and China (Yang et al. 2011, 2014).

DISCUSSION

This is the first study to report on an all-age pneumonia outbreak in an intensively sampled population of free-ranging bighorn sheep with health, survival, and observational data collected before, during, and after the disease event. The collection and analysis of this information, employing recently developed molecular methods for pathogen detection and genotyping, allowed us to attribute severe disease in a bighorn sheep population with long-standing *M. ovipneumoniae* carriage to introduction of a novel strain of *M. ovipneumoniae*. This conclusion is supported by the detection of the never before recorded IGS 393 strain in the pneumonic lungs of adults that died, and in the upper respiratory tract of adults with clinical signs during the outbreak. We also observed a significant increase in antibody titers to *M. ovipneumoniae* during the outbreak denoting an active immune challenge and we documented that previous exposure and ongoing carriage of the *M. ovipneumoniae* IGS 404 strain was not protective against disease. To the contrary, lower survival of adults with higher serologic titers prior to the disease outbreak could reflect a harmful autoimmune reaction associated with antibody response to *M. ovipneumoniae* in bighorn sheep as has been suggested for domestic sheep (Niang et al. 1998*a*). Strain-specific immunity, as measured by serologic antibody inhibition of *M. ovipneumoniae*, was similarly reported by Alley et al. (1999) for domestic sheep.

Adult mortality associated with this strain introduction was within the range previously observed during pneumonia outbreaks in naïve animals in this metapopulation (28–42%; Cassirer et al. 2013). Lamb mortality followed an identical time course regardless of strain, consistent with a lack of

protective immunity in neonates. The timing of the onset of clinical signs following infection in lambs (latent period) was similar to that reported in experimental challenge of adults (Besser et al. 2014). However, disease progression in lambs was more rapid and severe than observed in free-ranging adults in this study or in experimental exposure of naïve adults in captivity (Besser et al. 2014).

Although pneumonia in bighorn sheep is a polymicrobial disease, pathogens other than *M. ovipneumoniae*, including *lkt*A positive *Pasteurellaceae* and respiratory viruses, were either not detected or showed no association with disease. Whereas *M. ovipneumoniae* was present in all pneumonic adults and lambs, prevalence of *Pasteurellaceae* varied between age classes. This could be due to conditions associated with growth of an opportunistic pathogen or to other factors. Sample sizes were too small to draw broader inference. Anaerobic bacteria, not tested in this study, are a large component of the microbiome in pneumonic bighorn sheep lung tissue and may also play a larger role as secondary pathogens than previously suspected (Besser et al. 2008).

The novel strain of *M. ovipneumoniae* detected in this study differed from the resident strain by 52 independent genetic mutations on 4 loci. This unique strain was not a variant of a resident strain and had never before been detected in over 700 samples strain-typed from Hells Canyon and other bighorn sheep populations. Therefore, the most likely source of this Caprinae-specific pathogen was a domestic sheep or goat. Phlyogenetic analysis indicated that this strain was most likely of domestic goat origin.

This strain introduction likely occurred from a domestic goat on or from private lands within bighorn sheep range despite substantial efforts by wildlife managers and nongovernmental organizations to prevent contact. Management strategies included distributing educational material to flock owners and the general public, purchasing and removing a domestic sheep flock, and removing individual bighorn or domestic sheep and goats when they were at risk of contact. Our retrospective analysis showed that unique strains of *M. ovipneumoniae* were associated with 4 other epidemiologically unrelated all-age pneumonia outbreaks in Hells Canyon, as would be expected from similar spillover events. Two of these historical strains (IGS 402 and 419) apparently remained localized, one spread and subsequently disappeared (IGS 415), whereas the fourth (IGS 404) has persisted and proliferated over a span of $\geq$20 years. It is not clear why some strains of *M. ovipneumoniae* persist and others apparently do not. The IGS 404 strain may have driven fade-out of other extant strains when it spread, as introduction of the IGS 393 strain did in this study. Strain replacement might occur when the carrier host immune response is cross-reactive but protection is strain-specific. Under these conditions a new strain could have a competitive advantage and exclude the original strain.

Although evidence suggests that this epizootic was caused by introduction of a novel *M. ovipneumoniae* strain, it is also possible that pneumonia outbreaks could be precipitated in carrier bighorn sheep populations if appropriate mutations occur in strains to which resistance has previously been acquired. Mutations in key virulence genes or in genes coding for the *M. ovipneumoniae* capsule, which likely plays a role in adherence to host cells and in evading antibody recognition (Niang et al. 1998*b*, Razin et al. 1998), could cause disease and are unlikely to be detected by our strain-typing method. Another plausible mechanism of pneumonia resurgence would be reintroduction of the same strain of *M. ovipneumoniae* into a population following either pathogen fade-out and waning immunity (Sydenstricker et al. 2005) or recruitment of unexposed susceptible individuals. Finally, other factors may play a role in triggering outbreaks such as pathogen dose, host contact patterns, immunocompetence, and invasion of secondary pathogens. Identifying the conditions most frequently associated with pneumonia outbreaks in previously exposed populations and a wider investigation of the genetic diversity and host-specificity of *M. ovipneumoniae* strains would provide valuable insights into the adaptive immune response in bighorn sheep and the ecology of this disease.

MANAGEMENT IMPLICATIONS

Lack of cross-strain immunity to *M. ovipneumoniae* could be one explanation for the regular occurrence of pneumonia epizootics in bighorn sheep populations over a century after initial contact with domestic sheep. Single strain infection in bighorn sheep populations contrasts with *M. ovipneumoniae* carriage in domestic sheep where numerous strains typically coexist within a flock (Alley et al. 1999, Harvey et al. 2007). In the absence of cross-strain immunity, these flocks may serve as a constant source of novel strains capable of causing disease in bighorn sheep. Although vaccination could potentially reduce pathogen burden or prevalence within bighorn sheep populations, it is not clear that a vaccine would protect bighorn sheep from severe disease if exposed to new strains. Our results instead support preventing spillover as a primary strategy for managing disease in bighorn sheep. This could be accomplished by maintaining separation between bighorn sheep and domestic sheep and goats, by clearing *M. ovipneumoniae* infection from domestic hosts, and by exercising caution to avoid mixing *M. ovipneumoniae* strains among bighorn sheep populations during translocations. The management strategies implemented near the bighorn sheep in this study were apparently unsuccessful in preventing transmission, underscoring the difficulty of maintaining separation. New approaches, more active cooperation by the public, and greater vigilance on the part of resource managers may be key to preventing pneumonia outbreaks in bighorn sheep.

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