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CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ASSOCIATED WITH PNEUMONIA IN BIGHORN SHEEP

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ABSTRACT: *Pasteurella multocida* is a highly diverse group of bacteria recognized as important pathogens. Although *P. multocida* is not ordinarily associated with disease in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*), numerous isolates were cultured in high numbers from free-ranging bighorn sheep in the Hells Canyon area of Idaho, Washington, and Oregon (USA) during the winter of 1995–96. Animals captured in Hells Canyon and held in captivity, and their offspring, also harbored *P. multocida.* Biochemical utilization tests on 90 isolates identified three subspecies: *P. multocida multocida a* (n=54); *P. multocida multocida b* (n=13); and *P. multocida gallicida* (n=15); and a non-speciated biotype, U^6 (n=8). Genomic DNA digestion with restriction endonuclease *Hha* I separated the isolates into 62 unique restriction fragment length polymorphism profiles. Capsular type A was predominant (72% of isolates). Only one isolate type, which may have been transmitted from a feral goat, was capsular type D, possessed the structural gene, *toxA,* for dermonecrotoxin detected by polymerase chain reaction, and produced toxin as determined by monoclonal antibody immunoblot. In conclusion, bighorn sheep in this study carried diverse types of generally non-toxigenic *P. multocida* associated with epizootic pneumonia.
Key words: Capsular type, dermonecrotoxin, *Ovis canadensis canadensis, Pasteurella multocida,* pneumonia epizootic, polymerase chain reaction, restriction fragment length polymorphism.

INTRODUCTION

Pasteurella multocida, as defined by Rimler and Rhoades (1989), is a highly diverse group of bacteria recognized as important pathogens of animals. These organisms are widespread, colonizing both terrestrial and aquatic birds and mammals (Biberstein, 1979). Since the official recognition of the type strain as *P. multocida* by Rosenbusch and Merchant (1939), taxonomy of this group of organisms has undergone many changes (Mutters et al., 1989; Biberstein et al., 1991) resulting in assignment of isolates previously identified in the species to other species and non-speciated biotypes. Currently the species contains three subspecies designations: *P. multocida multocida, P. multocida septica,* and *P. multocida gallicida* (Mutters et al., 1989). *Pasteurella multocida multocida* is further divided into two variants, *a* and *b.* The broad complexity of the species involving antigenic variation, host predilection, and pathogenesis has been reviewed recently (Hunt et al., 2000) and indicates that effective classification should incorporate molecular tools including restriction enzyme analysis and polymerase chain reaction (PCR) procedures.

Each of the subspecies also consists of heterogeneous groups of organisms, some of which are primary pathogens (Christensen and Bisgaard, 2000). Many strains of *P. multocida* have been implicated as causes of severe outbreaks of respiratory disease in cattle, sheep, goats, pigs, and rabbits, atrophic rhinitis in pigs, and fowl cholera (Timoney et al., 1988). Other strains frequently are detected as normal commensals on the mucosa of the upper respiratory tracts of mammals. Many factors such as viral and mycoplasmal infections, poor nutrition, overcrowding, and shipping are associated with reduced physical and immunologic defenses which permit these commensal organisms to invade

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TABLE 1. Distribution of *Pasteurella multocida* subspecies and biotype U6 isolated from bighorn sheep in association with free-ranging or captive status and health of the animals.

P. multocida subspecies/biotype	Bighorn sheep characteristics and numbers				
	Healthy free-ranging	Healthy captive	Respiratory distress free-ranging	Dead[a] free-ranging	Dead captive
multocida a	2	1	4	20	8
multocida b	1	0	0	4	1
gallicida	0	0	0	7	1
U6	1	0	0	3	0
Toxigenic *multocida a*	0	0	0	1	0
multocida a + multocida b	0	1	0	6	0
multocida a + gallicida	0	0	1	4	0
multocida a + U6	1	0	0	2	0
multocida b + U6	0	0	0	1	0
multocida a + multocida b + gallicida	0	0	0	2	0

[a] All dead sheep showed evidence of lung consolidation at necropsy.

the lower respiratory tract and cause respiratory disease. Some strains appear to be normal commensals of the upper respiratory tracts of wild ruminants (Ward et al., 1997; Jaworski et al., 1998) but are rarely reported to be associated with respiratory disease in North American wild ruminants. Exceptions include hemorrhagic septicemia in North American bison (*Bison bison*) (Rimler and Wilson, 1994), septicemic pasteurellosis in elk (*Cervus elaphus nelsoni*; Wilson et al., 1995), and sporadic cases of respiratory disease affecting small numbers of deer (*Odocoileus hemionus*; Thorne, 1982) and bighorn sheep (Jaworski et al., 1998). Some strains producing hemorrhagic septicemia have characteristic restriction endonuclease profiles (Rimler, 2000). Multiple strains, some of which were shared with wildlife, were isolated from a single turkey flock exhibiting fowl cholera (Snipes et al., 1989). Dermonecrotoxins, proteins of approximately 112 to 160 kD which exhibit cytotoxic, dermonecrotic, and osteolytic activity, are produced by strains which possess *toxA* genes (Chanter and Rutter, 1989; Ghoshal and Niyo, 1993; Lichtensteiger et al., 1996).

A pneumonia epizootic affecting bighorn sheep in Idaho, Oregon, and Washington (USA) occurred in Hells Canyon and resulted in the death of 50–75% of the animals in four herds during the winter of 1995–96 (Cassirer et al., 1996). Samples collected by wildlife biologists from dead or dying bighorns were submitted to the Caine Veterinary Teaching Center (Caldwell, Idaho) for bacteriologic analysis. *Pasteurella haemolytica* and *P. trehalosi,* common commensals in bighorn sheep, were isolated from most animals. In addition, multiple strains of *P. multocida,* which are reportedly less common in bighorn sheep, were isolated from a high percentage of the animals. This study was conducted to determine if morbidity and mortality were associated with a single or multiple strains of *P. multocida,* their ability to produce dermonecrotoxin, or their particular capsular type.

MATERIALS AND METHODS

Pasteurella multocida isolates

Ninety *P. multocida* isolates from oropharyngeal or lung tissue samples of 72 bighorn sheep were evaluated. The condition of the animals (healthy, respiratory distress, or dead) at the time of sample collection was recorded (Table 1). Some of the isolates were from samples collected from live, free-ranging bighorn sheep when they were first noted to be exhibiting signs of respiratory distress and from animals

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which had died in their native habitat in Hells Canyon National Recreation Area (46°10″N, 117°00″W) (Cassirer et al., 1996). Other isolates were from live sheep that were captured in Hells Canyon and moved to pens at the Idaho Department of Fish and Game Wildlife Health Laboratory near Caldwell, Idaho during the later stages of the epizootic for monitoring and antibiotic treatment and some of those which subsequently died. Three isolates, 97-1205, 98-879, and 98-1230 were cultured from one of the surviving bighorn ewes and two lambs born in captivity at 22 and 31 mo after first detection of disease in the Hells Canyon population.

Samples from each animal were inoculated onto Columbia blood (5% ovine) agar (CBA) and Columbia blood (5% bovine) agar with antibiotics selective for Pasteurellaceae as previously described (Jaworski et al., 1993). Gram-negative isolates which were oxidase-, indole- and nitrate-positive and urease-negative were tentatively identified as *P. multocida.* Additional biochemical utilization tests were performed to confirm identification and differentiate isolates into subspecies and biotypes using the system of Biberstein et al. (1991). Isolates were subsequently maintained frozen at −70 C in a 60:40 (v/v) phosphate buffered saline (pH 7.2): glycerol cryoprotectant until they were propagated on fresh CBA for evaluation in this study.

Genomic DNA isolation

Bacteria grown on CBA were inoculated into 3 ml of brain-heart infusion (BHI) broth which was then incubated at 37 C with shaking. Genomic DNA was isolated from stationary phase (16 hr) BHI broth cultures containing approximately 5×10^8 colony forming units (cfu) ml^{-1} using the Puregene® DNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA) and quantified with Hoechst 33258 fluorometry (Labarca and Paigen, 1980).

Restriction fragment length polymorphism analysis

Purified DNA (1.5 μg) was digested with 5 U of *Hha* I restriction endonuclease (Promega Corp., Madison, Wisconsin, USA) for 3 hr at 37 C in a final reaction volume of 20 μl according to manufacturer's directions. Replicate digestates were electrophoresed in 1.0% equivalent agarose-Synergel (Diversified Biotech, Boston, Massachusetts, USA) gels for 16 hr at 30V and stained with ethidium bromide. Gels were photographed on a UV transilluminator and imaged with a Gel Doc™ 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, California, USA) for band analysis.

Data analysis

Imaged banding patterns were used to calculate relationship indices (Nei and Lei, 1979) between all isolates within each subspecies and biotype. Dendrograms were constructed using the unweighted pair-group method using arithmetic averages (UPGMA) cluster analysis (Sneath and Sokal, 1973).

Polymerase chain reaction (PCR) for dermonecrotoxin *toxA* gene

Isolates were tested for presence of the dermonecrotoxin structural gene, *toxA,* by PCR using the primers and a modified protocol originally designed by Lichtensteiger et al. (1996). Briefly, each 50 μl reaction contained 10 ng of DNA, in MasterAmp™ PCR Premix C (Epicentre Technologies, Madison, Wisconsin), 1.5 U RedTaq™ DNA polymerase (Sigma Chemical Co., St. Louis, Missouri, USA), and 0.75 mM each primer. Forty cycles, with each cycle consisting of denaturing at 94 C, annealing at 55 C and extension at 72 C for 30 sec each were performed in a thermal cycler (Perkin-Elmer Cetus Corp., Emeryville, California). The resulting PCR products were electrophoresed in 1.1% agarose gels for 45 min at 90V, stained with ethidium bromide (50 ppm) and imaged with a Gel Doc™ 2000 Gel Documentation System (Bio-Rad Laboratories) for band analysis.

Immunoblot analysis for dermonecrotoxin activity

Crude toxin preparations were made from selected isolates of *P. multocida* as described by Lichtensteiger et al. (1996). Each crude toxin preparation (200 μl) was vacuum filtered onto a nitrocellulose membrane using a Bio-dot apparatus (Bio-Rad Laboratories). Following filtration, the membrane was removed and treated with a blocking buffer (Tris buffered saline [TBS] with 3% gelatin) for 45 min at room temperature. The membrane was washed twice in TBS with 0.05% Tween-20 (TTBS) and incubated at room temperature overnight in a 1:50 dilution in TBS of anti-*P. multocida* dermonecrotoxin monoclonal antibody (Hybridoma α-DNT-1B2A3; American Type Culture Collection No. CRL-1965, Manassas, Virginia, USA). Following two additional 5 min washes in TTBS, the membrane was incubated 1 hr in a 1:2,000 dilution of peroxidase-conjugated goat anti–mouse immunoglobulins (Cappel, Organon Teknika, Durham, North Carolina). Subsequently, two washings were performed as above, followed by a third brief wash in TBS. The membrane was then incubated for 15 min in a chromogenic substrate for peroxidase

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(3mM 4-chloro-1-naphthol with 0.015% H_2O_2). Finally, the membrane was rinsed in distilled H_2O to stop the reaction and the membrane image recorded digitally (Gel Doc 2000; Bio-Rad Laboratories).

Polymerase chain reaction for capsular type genes

The capsular type of each isolate was determined with multiplex PCR using primers designed to amplify capsule determinant genes by Townsend et al. (2001). Each 50 μl reaction contained 10 ng of DNA, in MasterAmp™ PCR Premix B (Epicentre Technologies), 1.5 U RedTaq™ DNA polymerase (Sigma Chemical Co.) and 3.2 μM of each primer. Forty cycles, with each cycle consisting of denaturing at 94 C, annealing at 55 C and extension at 72 C for 30 sec each, followed by a final extension at 72 C for 5 min were performed in a thermal cycler (PerkinElmer Cetus Corp.). The resulting PCR products were electrophoresed in 1.1% agarose gels for 45 min at 90V, stained with ethidium bromide (50 ppm), and imaged with a computer coupled device (Gel Doc 2000, Bio-Rad Laboratories).

RESULTS

Pasteurella multocida multocida a was the predominant subspecies (54/90) isolated from 33 animals including three healthy, four with respiratory distress, and 26 dead bighorn sheep (Table 1). This subspecies was also found in 17 animals in combination with the other subspecies and biotype U[6]. Only three of the animals carrying these combinations were alive at sampling. The 54 isolates presented 29 unique restriction fragment length polymorphism (RFLP) patterns (Fig. 1). Eighty-two percent (44/54) of *P. multocida multocida a* isolates, one of which was dermonecrotoxigenic, were from dead animals. This dermonecrotoxigenic isolate (95-1519) was identical to an isolate cultured from a feral goat found near the bighorn sheep in Hells Canyon (K. Rudolph, pers. comm.) and clustered with a dermonecrotoxin-negative *P. multocida multocida a* isolate (95-1508) from an animal with respiratory distress with a similarity coefficient (SC) of 0.78. However, these two isolates were not closely related to the remaining 52 *P. multocida multocida a* isolates based on RFLP profiles. Another cluster of five identical organisms, 4/5 isolated from dead animals, was distantly related to the remaining 47 *P. multocida multocida a* isolates with a similarity coefficient of 0.05. With only two exceptions (96-1103 and 96-997), the isolates from captive animals had identical RFLP patterns or clustered together. Only five isolates were obtained from healthy animals and none of these clustered together.

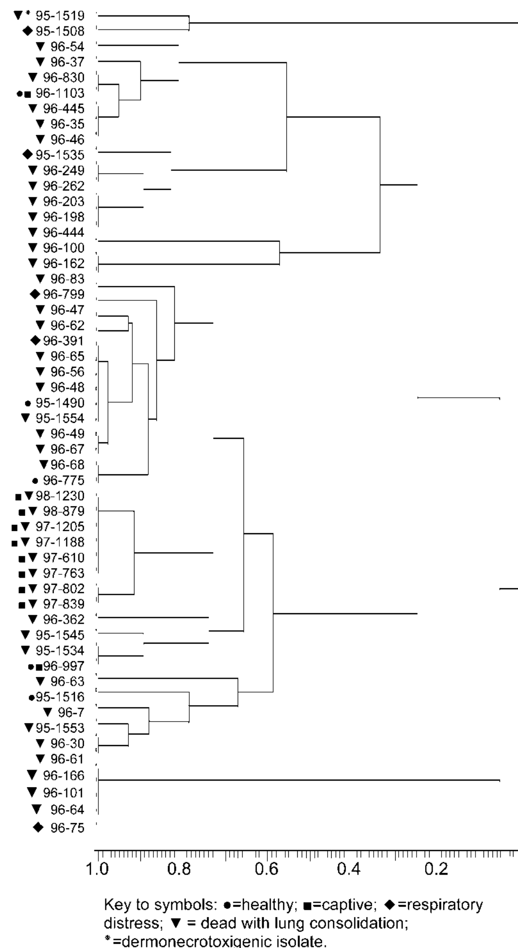

FIGURE 1. Genetic relationships of *Pasteurella multocida multocida a* associated with bighorn sheep in Hells Canyon, 1995–96.

In contrast, *P. multocida multocida b* was isolated alone from only five dead, free-ranging, or captive animals and only one live animal, and in combination with

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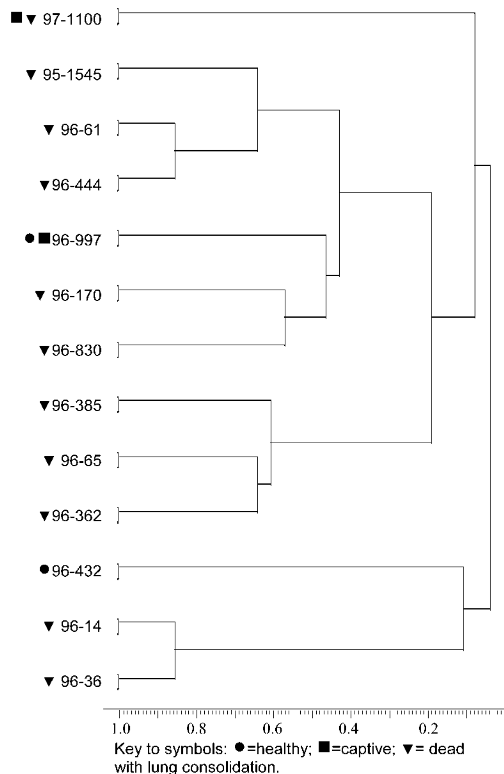

FIGURE 2. Genetic relationships of *Pasteurella multocida multocida b* associated with bighorn sheep in Hells Canyon, 1995–96.

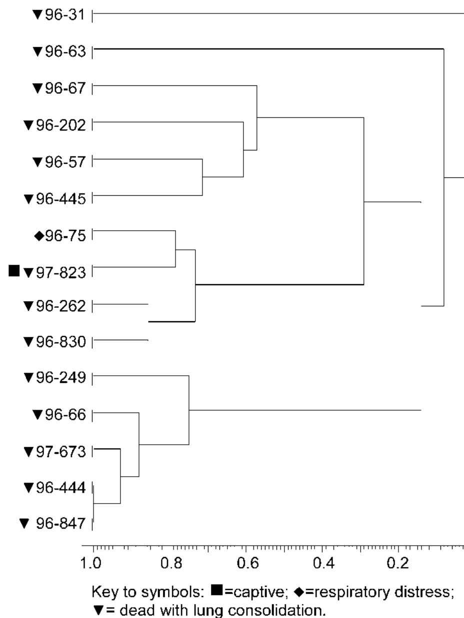

FIGURE 3. Genetic relationships of *Pasteurella multocida gallicida* associated with bighorn sheep in Hells Canyon, 1995–96.

the other subspecies or U6 biotype in one healthy and nine dead, free-ranging animals, from which *P. multocida multocida a* was also isolated (Table 1). Each of the 13 isolates gave a unique RFLP pattern with SCs ranging from 0.04–0.86 (Fig. 2). Only two *P. multocida multocida b* isolates were from healthy animals (96-432 and 96-997) and these clustered differently. The two isolates from captive animals (96-997 and 97-1100) were unrelated.

All but one (96-75) of the 15 *P. multocida gallicida* isolates was from dead animals and two (96-444 and 96-847) shared identical RFLP patterns (Fig. 3). Only one isolate (97-823) was carried by a captive animal and this had a SC of 0.78 with isolate 96-75 from a live animal showing signs of respiratory distress.

Eight *P. multocida* biotype U^6 isolates were found in this study and three (38%) shared identical RFLP patterns (Fig. 4). Two (25%) of these isolates were cultured from apparently healthy animals and these, especially 95-1490, were only distantly related to the rest of the U^6 isolates. As a group, the *P. multocida* biotype U^6 isolates were the most similar with seven of eight having SCs of 0.44 or greater.

Overall, 74% (67/90) of isolates carried the capsular type A gene and the remainder were acapsular, with one exception, 95-1519. This dermonecrotizing toxin producing isolate carried the capsular type D gene and was confirmed type D by Dr. Richard Rimler, USDA, Agricultural Research Service (Ames, Iowa, USA). Capsular types (A and acapsular) appeared to be distributed randomly throughout the taxonomic groups (data not shown).

DISCUSSION

The *P. multocida* isolates from lung tissue in this study were cultured in high numbers, suggesting that these organisms

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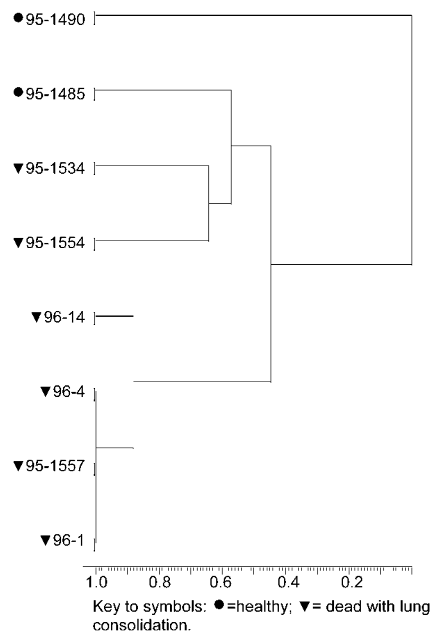

FIGURE 4. Genetic relationships of *Pasteurella multocida* biotype U^6 associated with bighorn sheep in Hells Canyon, 1995–96.

may be more pathogenic for bighorn sheep than previously recognized. Pasteurellaceae are commonly found among the normal flora of healthy-appearing wild ruminants, with *P. trehalosi* and *Mannheimia haemolytica* being isolated most frequently. In contrast, P. *multocida* is less frequently isolated from healthy wild ruminants (Ward et al., 1997; Jawarski et al., 1998). In the present study, Pasteurellaceae isolated from samples of individual animals were identified as *M. haemolytica, P. trehalosi,* and *P. multocida* (15%); *M. haemolytica* and *P. multocida* (1%); *P. trehalosi* and *P. multocida* (28%); or *P. multocida* only (55%), representing extremely high prevalences of the latter species. While respiratory disease in wild ruminant populations is most likely the culmination of several biotic and abiotic factors involved in pathogenesis (Rimler, 2000), *P. multocida* strains have been implicated previously in pneumonia in bighorn sheep and elk, (Thorne, 1982) and hemorrhagic septicemia in fallow deer (*Dama dama*; Eriksen et al., 1999) and bison.

Isolates in each of the *P. multocida* subspecies and biotype U^6 comprised multiple RFLP profile groups. Strains of *P. multocida multocida a* were isolated from more bighorn sheep than those in other subspecies and the U^6 biotype; however, a clearly-defined association with disease was not indicated for any subspecies or RFLP profile group. In addition, no synergistic association between organisms in any of the subspecies or biotype U^6 appeared to be associated with morbidity or mortality in these animals.

Strains of *P. multocida* that possess the *toxA* gene produce a dermonecrotoxin (Lichtensteiger et al., 1996). Infection with toxigenic strains results in progressive atrophic rhinitis in pigs (Chanter and Rutter, 1989; Goshal and Niyo, 1993; Gardner et al., 1994). Goats in Norway have been shown to carry dermonecrotoxigenic *P. multocida* and develop atrophic rhinitis (Baalsrud, 1987). The role of dermonecrotoxin in wild ruminants or its occurrence in *P. multocida* isolated from them has not been well-defined, although our results indicate such occurrences may be rare.

Presence of a capsule is an important virulence factor in *P. multocida* commonly found in domestic animals (Boyce and Adler, 2000). Capsular type A, predominant in the present study, was not particularly associated with any group of bighorn sheep, e.g., dead, live, or captive. Because type A strains are often found associated with avian hosts, these may serve as a reservoir of *P. multocida* in the Hells Canyon area.

Other studies on the genomic RFLP patterns of *P. multocida* (Zhao et al., 1992; Gardner et al., 1994; Donnio et al., 1999; Rimler, 2000) have shown sensitive levels of discrimination and remarkable genetic heterogeneity and are considered especially valuable in epidemiologic studies of pasteurellosis outbreaks (Hunt et al., 2000). Strains selected for analysis in those studies have been mostly derived from

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swine herds because of the economic impact of atrophic rhinitis. One study (Donnio et al., 1999) compared isolates from humans and swine, concluding that no apparent differences were discernible and that transmission may occur from pigs to humans working with them on production farms. Few studies on the genetic or phenotypic variability between strains of *P. multocida* isolated from wild ruminant populations have been published. Only one RFLP pattern was found with 70 isolates, all in the same serotype, implicated in pasteurellosis in an elk herd located at the National Elk Refuge (Jackson, Wyoming, USA; Wilson et al., 1995). In another study using six strains of the same serotype isolated from pronghorn (*Antilocapra americana*), moose (*Alces alces*), cattle, and swan each gave a unique RFLP pattern (Dunbar et al., 2000). Results from the present study indicate many unique RFLP patterns from bighorn sheep in the Hells Canyon area of Idaho, Washington, and Oregon.

The diversity of *P. multocida* strains isolated from bighorn sheep in this study is indicative of multiple origins rather than recent transmission from a single source. This is apparent even with *P. multocida multocida a* isolates which were cultured from the majority of the bighorn sheep. Although clusters of *P. multocida multocida a* isolates with identical SC values are suggestive of transmission between bighorn sheep, SC values below 0.8 appear to rule out recent transmission from a single source. The most notable suggestive evidence of transmission includes that of the dermonecrotoxigenic strain isolated from the feral goat and one of the bighorn sheep found in close proximity. Because this strain was not isolated from any of the other bighorn sheep in the study, it appears that the goat was the most probable source. In another group of animals, identical organisms were isolated from a ewe (97-1205) at the time of her death and the lungs of her two lambs which were euthanized while suffering from acute pneumonia 8 (98-879) and 10 (98-1230) mo later.

High prevalence of *P. multocida* in bighorn sheep populations during a period of high morbidity and mortality suggests that one or more of the strains may be pathogenic for these animals. Polymorphic DNA patterns and RFLP patterns of PCR amplicons from virulence genes of these isolates are being characterized further for possible use in predicting virulence potential of these organisms for bighorn sheep. Characterizations conducted on strains isolated from animals during health monitoring programs may be useful in averting *P. multocida* epizootics. Wildlife biologists should be aware of the presence of *P. multocida* strains in bighorns and the possible deadly consequences of unchecked transmission within and between bighorn populations.

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