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DETECTING NONHEMOLYTIC *PASTEURELLA HAEMOLYTICA* INFECTIONS IN HEALTHY ROCKY MOUNTAIN BIGHORN SHEEP (*OVIS CANADENSIS CANADENSIS*): INFLUENCES OF SAMPLE SITE AND HANDLING

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ABSTRACT: Effects of sampling procedures on ability to culture *Pasteurella* spp. from Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) were examined experimentally. Sample site influenced ($P < 0.0001$) recovery of *P. haemolytica* in adult bighorn sheep. We isolated nonhemolytic *P. haemolytica* from 18 of 19 tonsillar swabs and 18 of 19 tonsillar biopsies from adult sheep, yet only four of 19 nasal swabs yielded isolates. Sample handling also affected ($P < 0.0001$) recovery of *P. haemolytica*. Nonhemolytic *P. haemolytica* was cultured from 14 of 19 tonsillar swabs plated directly onto blood agar, but from only two of 19 swabs stored for 24 hr in modified Amies with charcoal; we failed to recover *P. haemolytica* from any of 19 swabs stored for 24 hr in modified Stuart's medium. We detected nonhemolytic *P. haemolytica* at least once in bronchial aspirates from four and in nasal swabs from three of six bighorn lambs. Based on direct cultures of tonsillar swabs and/or biopsies, all 26 bighorn sheep (seven lambs, 19 adults) sampled were infected with nonhemolytic *P. haemolytica*; only two lambs developed pneumonia during the study period. Thirty-four of 37 nonhemolytic *P. haemolytica* isolates tested were biotype T; three were biotype A. Serotypes 3; 4; 3, 4 and 3, 4, 10 were identified in a subsample of 17 isolates. Our data suggest tonsillar swabs or biopsies plated directly onto blood agar and incubated immediately offer the greatest probability of recovering nonhemolytic *P. haemolytica* from healthy bighorn sheep.

Key words: Rocky Mountain bighorn sheep, *Ovis canadensis canadensis*, *Pasteurella haemolytica*, pasteurellosis, pneumonia, diagnostics, epizootiology, carrier state, experimental study.

INTRODUCTION

Detecting *Pasteurella haemolytica* infections in bighorn sheep (*Ovis canadensis*) has emerged as an important wildlife management issue. *Pasteurella* spp. often contribute to a pneumonia complex that impairs bighorn population performance (Potts, 1937; Marsh, 1938; Buechner, 1960; Post, 1962; Forrester, 1971; Spraker and Hibler, 1982; Thorne, 1982; Onderka and Wishart, 1984, 1988; Foreyt, 1989). Recent reports of possible transmission of *P. haemolytica* between bighorn and domestic sheep (Foreyt and Jessup, 1982; Coggins, 1988; Onderka and Wishart, 1988; Onderka et al., 1988; Foreyt, 1989) have collectively influenced federal land management policies for some western rangelands. These policies have been challenged because so many aspects of pasteurellosis in wild bighorn sheep are poorly understood. Before effective and equitable policies for managing pasteurellosis in bighorn sheep can be developed, fundamental questions regarding the origins, epizootiology and ecology of *P. haemolytica* in wild bighorn populations need to be answered.

Properly collecting and handling clinical samples is a prerequisite to obtaining reliable data on pasteurellosis in wild bighorn sheep. Postmortem diagnosis of pasteurellosis can be accomplished reliably using gross and histological lesions (Spraker and Hibler, 1982; Onderka et al., 1988) supported by culturing *Pasteurella* spp. from tissue samples. However, antemortem diagnoses often rely on culturing nasal swabs in field and laboratory situations (Spraker et al., 1986; Coggins, 1988; Onderka and Wishart, 1988; Onderka et al., 1988; Foreyt, 1989). Those results may be less reliable. Nasopharyngeal sampling de-

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tects only a fraction of the *P. haemolytica* carried in tonsils of healthy domestic sheep (Gilmour et al., 1974; Al-Sultan and Aitken, 1985); a similar pattern has been described in hunter-killed bighorn sheep (Onderka and Wishart, 1988). These observations suggest that sampling techniques might affect recovery of *Pasteurella* spp. from live bighorn sheep under some conditions. Here, we conducted experiments to examine effects of sample site and handling on detection of *Pasteurella* spp. infections in captive bighorn sheep.

MATERIALS AND METHODS

Effects of sampling site on recovering *Pasteurella* spp.

We conducted two experiments with captive Rocky Mountain bighorn (*O. canadensis canadensis*) lambs and adults to compare reliability of different sampling sites for recovering *Pasteurella* spp. Tame bighorn ewe/lamb pairs ($n = 7$) were housed in approximately 50 m^2 isolation pens at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (Fort Collins, Colorado 80526, USA; 40°35'N, 105°10'W). Alfalfa hay, pelleted feed (Baker and Hobbs, 1985), mineralized salt blocks and water were provided *ad libitum* throughout the experiment. Individuals were identified by numbered eartags. We performed health assessments and recorded clinical condition of each ewe and lamb at least daily, and weighed them weekly.

Our first experiment compared nasal swabs, tonsillar swabs and transoral bronchial aspirates for detecting *Pasteurella* spp. in bighorn lambs. Samples were collected weekly from 16 July to 28 August 1989; lambs were about 8- to 10-wk-old when collections began. We sedated lambs with 3–5 mg xylazine hydrochloride (HCl) (AnaSed®, Lloyd Laboratories, Shenandoah, Iowa 51601, USA) given intravenously (IV), then blindfolded them for sample collections. Deep nasal swabs were obtained by passing a 15 cm cotton-tipped swab (American Scientific Products, McGaw Park, Illinois 60064, USA) 10 to 14 cm into one nare. We used a laryngoscope to visualize the palatine tonsils, then swabbed the sinus and/or mucosal surface of one tonsil using a standard (American Scientific Products) or miniature-tipped cotton swab (Spectrum Laboratories, Inc., Los Angeles, California 90060, USA). Nasal and tonsillar samples were immediately swabbed onto 5% sheep blood agar and MacConkey's plates (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Maryland 21030, USA). Incubation began within 3 hr of collection.

To obtain bronchial aspirates, we viewed the arytenoid cartilages using a laryngoscope, then topically anesthetized them with about 0.3 ml lidocaine HCl 2% (Vedco, Inc., Overland Park, Kansas 66204, USA). We passed a sterile endotracheal tube (6 mm O.D.) about 15 to 20 cm into the trachea. We then passed polyethylene tubing (2.5 mm O.D.) aseptically through the endotracheal tube about 30 to 40 cm into the trachea using anatomical landmarks (Getty, 1975) to estimate the length of tubing required to enter a mainstem bronchus. Once the tubing was in place, we administered 5 mg (IV) yohimbine HCl (Antagonil®, Wildlife Laboratories, Fort Collins, Colorado 80524, USA), and proceeded with sampling only after a strong cough could be elicited. When the lamb could cough, usually within 2 min, we instilled 15 ml USP lactated ringers solution (LRS) (Abbott Laboratories, North Chicago, Illinois 60064, USA) followed by 2 ml air through the polyethylene tubing using a 20 ml syringe and 16 ga needle. Immediately after delivering LRS, we manipulated the trachea externally to induce coughing while applying suction to retrieve a sample. One drop of bronchial aspirate was placed onto blood agar and MacConkey's plates, and these were handled as described earlier.

Culture plates were incubated at 37 C and examined for bacterial growth at 24 and 48 hr. We estimated abundance of *Pasteurella* spp. growth and followed established procedures to obtain isolated bacterial colonies and identify isolates (Carter, 1984); nonhemolytic *P. haemolytica* isolates were further compared to Onderka et al.'s (1988) description of a unique "bighorn strain" of *P. haemolytica*. Small sample sizes and repeated measures structure precluded statistical analysis of data from this experiment.

In order to detect concurrent viral infections, we collected paired sera for titers to parainfluenza type 3 (PI-3), infectious bovine rhinotracheitis (IBR) and bovine respiratory syncytial virus (BRSV) from all lambs in July or August. Antibody titers were determined using serum neutralization for PI-3, BRSV (Carbrey, 1971) and IBR (Carbrey, 1971; Bitsch, 1978) by Colorado State University Diagnostic Laboratory (CSUDL; Fort Collins, Colorado 80523, USA). We also collected feces and counted *Protostrongylus* sp. larvae using a modified Baermann technique (Beane and Hobbs, 1983). Virus (Schachter and Dawson, 1979) and chlamydial (Hawkes, 1979) isolations from bronchial aspirates of two pneumonic lambs were attempted by CSUDL.

Lambs showing clinical signs of pneumonia were removed from the experiment and treated

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with antibiotics: we used subcutaneous (SC) injections of 3 mg/kg gentamicin sulfate (Elkins-Sinn, Inc., Cherry Hill, New Jersey 08003, USA) TID and 11 mg/kg amoxicillin (Amoxi-inject®, Beecham Laboratories, Bristol, Tennessee 37620, USA) BID for 5 days, followed by alternate-day SC injections of about 22 mg/kg long-acting oxytetracycline (Liquamycin®, LA-200®, Pfizer Agricultural Division, New York, New York 10017, USA) for another 10–14 days.

A second experiment compared nasal and tonsillar swabs and tonsillar biopsies for isolating *Pasteurella* spp. from 19 adult bighorn sheep. In addition to the seven ewes with lambs, we used seven tame rams and five tame ewes without lambs for this experiment; these additional animals were housed in two paddocks (each about 3 ha), but otherwise husbandry was as previously described. All bighorn ewes and rams were immobilized with IV ketamine HCl (Bristol Laboratories, Syracuse, New York 13201, USA) and xylazine mixed 10:1 (estimated doses: yearlings, 65–75 mg ketamine + 6.5–7.5 mg xylazine; ewes, 90 mg ketamine + 9 mg xylazine; rams, 100 mg ketamine + 10 mg xylazine), then blindfolded for sample collections. Each adult was sampled once in July or August 1989.

Nasal and tonsillar swabs were collected and processed as described for lambs. We used a laryngoscope to view palatine tonsils and biopsied them using a long-handled cup biopsy instrument with a 4 mm diameter cutting surface; all biopsies were obtained from within the tonsillar sinus. We checked biopsy sites for adequate hemostasis and then applied about 0.5 ml lidocaine topically to reduce pain at the biopsy site. Biopsy tissue was streaked onto blood agar, and *Pasteurella* spp. were isolated and identified. We tested for differences in recovery of *P. haemolytica* among sampling sites using Cochran's Q test (Marascuilo and McSweeney, 1977).

A subsample of 37 *P. haemolytica* isolates (19 from lambs, 18 from adults) was submitted to Department of Epidemiology and Preventive Medicine, University of California–Davis (Davis, California 95616, USA) for biotyping (Biberstein, 1978). Seventeen isolates (seven from lambs, 10 from adults) were submitted to the Wyoming State Veterinary Laboratory (University of Wyoming, Laramie, Wyoming 82070, USA) for serotyping (Frank and Wessman, 1978).

Effects of sample handling on recovering *Pasteurella* spp.

In conjunction with the above experiments, we examined effects of sample handling on results of bacterial cultures. Specifically, we compared recovery of *Pasteurella* spp. from bighorn tonsillar swabs plated directly onto blood agar with recoveries after storing swabs for 24 hr in two widely used transport media, modified Amies with charcoal (BBL Microbiology Systems, Becton Dickinson and Co.) and modified Stuart's media (Culturettes®, Marion Laboratories, Inc., Kansas City, Missouri 64114, USA).

From each of 19 tame bighorn sheep (six lambs, six ewes and seven rams), we collected three tonsillar swabs during the same sampling session. One swab from each sheep was randomly assigned to each of three treatments (direct plating, Amies or Stuart's). Samples swabbed directly onto blood agar and MacConkey's plates were streaked and placed in a 37 C incubator within 3 hr of collection. To simulate transportation from field to lab, we held swabs in Amies and Stuart's media for 24 hr at room temperature before streaking onto blood agar and MacConkey's plates and incubating at 37 C. Culture techniques and evaluation of results were as previously described. We tested for effects of sample handling on recovery of *P. haemolytica* using Cochran's Q test.

We also attempted to recover *Pasteurella* spp. from three biopsy samples held in modified Amies for 24 hr, then handled as fresh biopsies. These results were compared with tonsillar swabs taken from the same three animals at the same time and held in modified Amies for 24 hr.

Viability of *Pasteurella* spp. isolates in commercial transport media was tested using fresh pure cultures of nonhemolytic *P. haemolytica* isolated from our bighorn sheep. We collected isolated colonies on swabs and placed them in either modified Amies ($n = 5$) or modified Stuart's ($n = 5$) media. After 24 hr storage at room temperature, these were handled in the same manner as other samples and examined for bacterial growth.

RESULTS

Effects of sampling site on recovering *Pasteurella* spp.

Five of the seven bighorn lambs remained clinically normal or showed only mild signs of rhinitis during the 7-wk study period. One lamb developed pneumonia just as the study began, and was sampled only twice. Another lamb appeared healthy for 3 wk, then developed pneumonia. Both lambs recovered uneventfully after antibiotic therapy. All adult bighorn sheep remained healthy throughout the study period.

Viruses, chlamydiae or lungworms did not appear to contribute to respiratory disease in these bighorn lambs. All titers to

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IBR, BRSV and PI-3 were negative (<1: 2). Viruses or chlamydiae were not isolated from bronchial aspirates. Six of seven lambs were negative for *Protostrongylus* sp. larvae in feces. Based on these data and previous experience, we attributed mild rhinitis in the absence of coughing or depression to nasal bot (*Oestrus ovis*) infections, and clinical pneumonia to pasteurellosis.

We identified all *Pasteurella* spp. isolates (n = 92) from our captive bighorn sheep as *P. haemolytica*. These isolates were predominantly nonhemolytic, and resembled nonhemolytic *P. haemolytica* strains from wild bighorn sheep described by Onderka et al. (1988). However, hemolysis did occur directly under colonies of some isolates, and we observed more extensive hemolysis around two nasal isolates from one lamb. Thirty-four of 37 isolates tested were biotype T; two lamb and one adult isolates were biotype A. Four of seven lamb isolates were identified as serotype 3, 4; three isolates, including one from each pneumonic lamb, were serotype 4. Of 10 nonhemolytic *P. haemolytica* isolates submitted from adult bighorn sheep, two were serotype 3, three were 4, two were 3, 4, 10 and three were untypable.

Nonhemolytic *P. haemolytica* was isolated from tonsillar swabs of each apparently-healthy bighorn lamb at least once during the 7-wk sampling period (Table 1). We detected nonhemolytic *P. haemolytica* in bronchial aspirates at least once in four, and in nasal swabs at least once in three of six healthy lambs (Table 1). After pneumonia developed in two lambs, we isolated nonhemolytic *P. haemolytica* consistently from all three sites (Table 1). Overall, tonsillar swabs yielded only slightly higher recoveries of *P. haemolytica* than other sites in bighorn lambs (Table 1).

A somewhat different pattern emerged in comparing nasal and tonsillar swabs and tonsillar biopsies in healthy adult bighorn sheep, where *P. haemolytica* recovery rates differed among sampling sites (Q = 24.5, $P < 0.0001$). We isolated nonhemolytic *P. haemolytica* in tonsillar swabs and/or biopsies from each of 19 adults. Recovery rates of *P. haemolytica* from tonsillar swabs (18 of 19) and biopsies (18 of 19) were identical; agreement between these two techniques was about 89%. Nasal swabs yielded far fewer *P. haemolytica* isolates (four of 19) than corresponding tonsillar samples from healthy bighorn adults.

TABLE 1. Occurrence of *Pasteurella haemolytica* in nasal swabs, tonsillar swabs and bronchial aspirates from apparently-healthy and pneumonic bighorn lambs.

Eartag number	Sex[a]	Total samples/ site	*Pasteurella haemolytica* isolates		
			Nasal	Tonsillar	Bronchial
Healthy lambs					
A289	M	7	0	1	0
C89	F	7	0	3	3
E89	F	7	0	2	0
L789[b]	F	3	2	2	1
Q689	M	7	6	6	5
T89	M	7	4[c]	3	4
Pneumonic lambs					
L789[b]	F	1	1	1	1
Q889[d]	F	2	2	2	3

[a] M, male; F, female.
[b] Developed pneumonia during week 4.
[c] Two isolates showed marked hemolysis.
[d] Pneumonic when study began.

For most lamb samples, regardless of site, few *P. haemolytica* isolates were obtained on primary blood agar plates. The number of nonhemolytic *P. haemolytica* colonies on primary isolations tended to increase from all sites after lambs developed pneumonia, but colony numbers also increased on other occasions that could not be correlated with detectable changes in clinical condition. In samples from adults, *P. haemolytica* colonies were moderately to highly abundant on primary blood agar plates from tonsillar swabs and biopsies, as compared to adult nasal swabs and all samples from healthy lambs.

Effects of sample handling on recovering *Pasteurella* spp.

Sample handling also affected recovery of *P. haemolytica* (Q = 24.6, $P < 0.0001$).

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Storing tonsillar swabs in commercial transport media markedly reduced nonhemolytic *P. haemolytica* detection. We isolated nonhemolytic *P. haemolytica* from 14 of 19 tonsillar swabs plated directly onto blood agar, but from only two of 19 corresponding swabs stored for 24 hr in modified Amies with charcoal; we failed to recover *P. haemolytica* from any of the 19 swabs stored for 24 hr in modified Stuart's medium. Primary plates from tonsillar swabs stored in either transport medium showed moderate to heavy growth of other gram-negative bacteria, making identification of *P. haemolytica* colonies more difficult than those from direct plating.

Presence of tonsillar tissue appeared to improve ability to recover *P. haemolytica* from stored samples. We isolated nonhemolytic *P. haemolytica* from all three biopsy samples held for 24 hr in modified Amies, but from none of the corresponding tonsillar swabs obtained from those same animals and handled identically.

Both transport media supported nonhemolytic *P. haemolytica*. All swabs of pure cultures held in either modified Amies with charcoal or modified Stuart's media for 24 hr yielded viable cultures.

DISCUSSION

Both sampling site and subsequent handling of clinical samples influenced our ability to detect nonhemolytic *P. haemolytica* in apparently healthy bighorn sheep. Nasal swabs detected four of 19 (21%) nonhemolytic *P. haemolytica* carriers shown positive by culturing tonsillar samples. Moreover, only two of 14 (14%) positive tonsillar swabs stored in transport media for 24 hr yielded nonhemolytic *P. haemolytica*.

Effects of sample site and sample handling were separated by design in our experiments. In practice, however, effects of such factors on diagnostic results and their interpretation would have been multiplied. We surmise that combined influences of suboptimal sampling procedures could markedly lower the probability of detecting nonhemolytic *P. haemolytica* in healthy bighorn sheep. For example, estimated prevalence of nonhemolytic *P. haemolytica* infections in our tame bighorn "population" might have been 3% rather than 100% had we relied on culturing nasal swabs held overnight in commercial transport media.

Nasal swabs are widely used to detect *Pasteurella* spp. infections in wild bighorn sheep. Our data support other investigators' observations that using nasal swabs may lower rates for recovering *P. haemolytica* from healthy bighorn sheep. Culturing tonsillar tissue, Onderka and Wishart (1988) isolated nonhemolytic *P. haemolytica* from 15 of 61 healthy bighorn sheep harvested in Alberta after a pneumonia epizootic; their earlier survey using nasal and pharyngeal swabs yielded only one isolate in 240 harvested sheep. A similar disparity between tonsillar and nasal isolates was encountered in attempts to recover *P. haemolytica* from wild bighorn sheep after a pneumonia outbreak in Idaho (Dunbar et al., 1990).

Differences in tonsillar and nasal carriage of *Pasteurella* spp. in bighorn sheep are not surprising. Tonsillar tissue yielded about 50% more *P. haemolytica* isolates than corresponding nasopharyngeal swabs from slaughtered domestic sheep (Gilmour et al., 1974); moreover, biotype T isolates were about 15 times more common in tonsils. Tonsillar swabs from 37 of 37 domestic lambs and 25 of 28 related ewes were positive for *P. haemolytica* at least once during a 3-mo sampling period (Al-Sultan and Aitken, 1985); T biotypes also predominated in that study. Most nonhemolytic *P. haemolytica* isolates from our experiments were biotype T, regardless of source. Rarity of A biotypes in our tame bighorn sheep may have accentuated observed differences in *P. haemolytica* recovery among sampling sites.

All bighorn lambs in our study were infected with nonhemolytic *P. haemolytica*, but most remained healthy throughout the study. Lambs were probably infected

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shortly after birth: using only nasal swabs, we recovered nonhemolytic *P. haemolytica* from three of these same lambs at 1 wk of age (Miller and Hobbs, 1989). *P. haemolytica* can be detected in tonsils of domestic lambs less than 1-day-old (Al-Sultan and Aitken, 1985), and in nasal swabs of 2-day-old lambs (Shreeve and Thompson, 1970). Infection rates for *Pasteurella* spp. in wild bighorn lambs have not been examined, but such data may offer further insights into epizootiology of the bighorn pneumonia complex.

Recovery rates for nonhemolytic *P. haemolytica* varied among and within individual bighorn lambs sampled repeatedly. Overall, these differences did not appear related to clinical condition or susceptibility to pneumonia: two of three healthy lambs that yielded nonhemolytic *P. haemolytica* from nasal, tonsillar and bronchial samples failed to develop pneumonia. Moreover, occurrence of hemolytic *P. haemolytica* isolates from one lamb's nasal swabs did not appear correlated with increased pathogenicity because that lamb remained healthy throughout our study.

Whether *Pasteurella* spp. infect free-ranging populations prior to pneumonia outbreaks remains a pivotal question in understanding and managing pasteurellosis in bighorn sheep. It is possible that some strains of *Pasteurella* spp. are ubiquitous in wild bighorn sheep. Based on direct cultures of tonsillar swabs, all 26 bighorn sheep (adults and lambs) used in our experiments were infected with nonhemolytic *P. haemolytica*. However, many of our captive bighorn sheep had previously recovered from pasteurellosis (Miller, 1988; Miller and Hobbs, 1989), so this high prevalence estimate may be artificially inflated. About 25% of healthy bighorn sheep harvested in Alberta after a widespread pneumonia epizootic (Onderka and Wishart, 1984) carried nonhemolytic *P. haemolytica* in their tonsils (Onderka and Wishart, 1988), although the sampling frame for that survey was somewhat unclear.

Pasteurella spp. have been isolated from healthy wild bighorn sheep (Post, 1962; Thorne et al., 1979; Thorne, 1982; Onderka and Wishart, 1988; Foreyt, 1989), but Foreyt (1989, p. 343) contended that "routine isolation of *P. haemolytica* from healthy bighorn sheep is uncommon." We believe failure to isolate *Pasteurella* spp. from healthy bighorn sheep may be in part an artifact of sampling procedures. In light of experimental and field data, prevalence estimates for *Pasteurella* spp. in bighorn sheep that are based on culturing nasal swabs should probably be regarded as minimum estimates. Such data should also be interpreted with the understanding that failure to isolate *Pasteurella* spp. using nasal swabs does not necessarily ensure absence of *Pasteurella* spp. infections in sampled bighorn populations.

Our data suggest that tonsillar swabs or biopsies plated directly onto blood agar and incubated immediately offer the greatest probability of recovering nonhemolytic *P. haemolytica* from healthy bighorn sheep; once pneumonia develops, nasal swabs may be equally representative. If samples must be stored or transported before incubation, tonsillar biopsies may provide better results than swab samples. These recommendations may seem impractical for field situations, but tonsillar samples are not difficult to collect and portable incubators are available for holding and transporting field samples. Although slightly more time may be required to collect and process individual tonsillar samples, few samples are actually needed from finite populations to estimate prevalence of infections when rates are relatively high or low (DiGiacomo and Koepsell, 1986). In our opinion, a few properly collected samples will offer more reliable information on *Pasteurella* spp. infections in a bighorn population than numerous samples of questionable quality.

Wildlife scientists and managers may soon be directed to design research and management experiments intended to provide definitive knowledge about the epi-

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zootiology of pasteurellosis in bighorn sheep, particularly as related to interactions with domestic livestock. Collection of representative diagnostic samples will be essential in such investigations. Plans for future research should consider potential biases that might be introduced by sampling techniques. Using nasal swabs and/or modified Amies or Stuart's transport media would lead investigators to markedly underestimate prevalence or fail to detect presence of nonhemolytic *P. haemolytica* infections in bighorn sheep populations. It follows that scientific inferences and management policies based on those data may be equally unreliable.

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