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A Reliable Transport Method for Isolating *Pasteurella haemolytica* from Bighorn Sheep

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ABSTRACT: We compared three transport methods for the recovery of *Pasteurella haemolytica* from pharyngeal swabs collected under field conditions from 42 bighorn sheep (*Ovis canadensis*) in one captive and three free-ranging populations. Transport methods included: Amies medium with charcoal, transported on ice, and cultured on blood agar within 24 hr; direct plating on blood agar, transported on heating pads, and incubated at 37 C within 8 hr of collection; and phosphate buffered glycerol (PBG), transported on dry ice, and stored at −70 C for 10 days before culture. Isolates of *P. haemolytica* were recovered from all 42 bighorn sheep with a mean (±SE) of 1.2 ± 0.1 (Amies), 1.3 ± 0.1 (blood agar), and 1.3 ± 0.1 (PBG) isolates per swab. No statistical differences ($P > 0.05$) were observed in the recovery of *P. haemolytica* isolates among the transport methods. However, based on our experience and results of this study, we recommend that if submission of samples to the laboratory is likely to be delayed, pharyngeal swabs be transported in PBG on dry ice and kept frozen until they are cultured. Viable samples can be maintained in PBG at −70 C for several years.

Key words: *Pasteurella haemolytica*, bighorn sheep, *Ovis canadensis*, bacterial transport methods, pharyngeal swabs, phosphate buffered glycerol.

Management of bighorn sheep (*Ovis canadensis*) populations often involves their capture for introduction or reintroduction into vacant suitable habitats, translocation to supplement existing populations, herd health evaluation, and research. In many capture operations, biological samples are collected from animals for identifying specific pathogens and determining overall health. Failure to isolate pathogenic organisms or low recovery rates of organisms in the laboratory from specimens collected from free-ranging animals frequently is the result of faulty collection, inefficient handling techniques, or excessive transport times before arrival at the laboratory (Wild and Miller, 1991). Techniques requiring a minimum of equipment and instruction while ensuring arrival of high quality specimens to the laboratory are essential for accurate diagnosis.

Pasteurella haemolytica often is associated with epizootics of pneumonia in captive and free-ranging bighorn sheep (Onderka and Wishart, 1984; Foreyt, 1989; Miller et al., 1991) and has received considerable attention by wildlife managers and researchers as a major mortality factor of bighorn sheep. Dunbar et al. (1990) have shown that swabs collected from tonsils or the pharyngeal area, or tonsillar tissue are more reliable for detecting *P. haemolytica* in healthy bighorn sheep than swabs collected from nasal sinuses. However, once samples are properly collected under field conditions, transit time to a diagnostic laboratory often is delayed because of remote collection sites or delayed transportation, resulting in decreased detection of *P. haemolytica*. Because of the importance of *P. haemolytica* in bighorn sheep, our objective was to compare recovery of *P. haemolytica* from pharyngeal swabs collected from bighorn sheep under field conditions by three transport methods.

Pharyngeal swabs were collected from 42 healthy bighorn sheep between December 1992 and March 1993 from three free-ranging populations and one captive herd; samples included 35 Rocky Mountain (*O. canadensis canadensis*) and seven California (*O. canadensis californiana*) bighorn sheep. Free-ranging Rocky Mountain bighorn sheep were sampled from the Hall Mountain population ($n = 14$) (seven adult ewes, two yearling ewes, two ewe lambs, one adult ram, two ram lambs) in Pend Orielle County in northeastern Washington (USA, 48°50′N, 117°15′W), and the

Lostine herd in the Wallowa Mountains (n = 11) (three adult ewes, three yearling ewes, one adult ram, two yearling rams, two ram lambs) in Wallowa County in northeastern Oregon (USA, 45°24'N, 117°24'W). We also sampled a captive herd maintained in a 2.5 ha enclosure at Washington State University (n = 10) (six adult ewes, two ewe lambs, one adult ram, one yearling ram) in Pullman, Washington (46°45'N, 117°10'W). Seven California bighorn sheep (four adult ewes, one ewe lamb, two adult rams) were free-ranging on Vulcan Mountain in Ferry county in north-central Washington (48°50'N, 118°40'W).

Pharyngeal swabs were obtained by holding the mouth open with a harp speculum; the tongue was secured gently between the speculum and lower jaw. A standard transport rayon-tipped swab on plastic shaft (Spectrum Laboratories, Inc., Houston, Texas, USA) was grasped in a 23-cm hemostat and guided to the posterior pharynx, using care not to touch the lateral walls of the buccal cavity or tongue to minimize contamination. The swab was rubbed firmly against the tonsillar and pharyngeal region and removed. Three pharyngeal swabs were collected from each of 23 sheep, and two swabs from each of 19 sheep. Of the 23 swabs collected in triplicate, one was cultured directly onto 5% sheep blood agar (Benton Dickinson Microbiology Systems, Cockeysville, Maryland, USA) and streaked for isolation using a 10 µl disposable inoculating loop. A second swab was placed in modified Amies medium with charcoal (Spectrum Laboratories, Inc.), and the third swab was placed into a 4-ml glass vial containing 2 ml of sterile phosphate buffered glycerol (PBG). Of the 19 swabs collected in duplicate, one was placed in PBG; the second was placed in modified Amies medium with charcoal (n = 11) or cultured directly onto a blood agar plate (n = 8). The PBG was prepared by mixing together glycerol and buffer at a ratio of 3:2 in a water bath at approximately 40 C. The buffer was prepared by dissolving 5.23 g of K_2HPO_4 in 300 ml distilled H_2O, and 4.1 g of KH_2PO_4 in 300 ml distilled H_2O in separate flasks, and then adding the KH_2PO_4 to the K_2HPO_4 until the pH was in the range of 7.0 to 7.05. Two ml of PBG were placed in 4-ml glass vials that were loosely capped and sterilized with steam and pressure for 15 min. Vials of PBG were stored at 4 C after caps were tightened.

Specimens were transported from the field to the Washington Animal Disease Diagnostic Laboratory (WADDL) (Washington State University). Blood agar plates were transported in a plastic cooler containing a portable heating pad (Prism Technologies, Inc., Chicago, Illinois, USA) and placed in a 37 C incubator within 8 hr of collection. Swabs in modified Amies medium with charcoal were placed on ice in a plastic cooler and transferred to a 4 C refrigerator within 8 hr of collection. Swabs were streaked onto a 5% sheep blood agar plate within 24 hr of collection. Specimens in PBG were placed on dry ice in a plastic cooler and transferred to a −70 C freezer within 8 hr of collection. The swabs in PBG were removed from the freezer 10 days later and streaked onto a blood agar plate.

All blood agar plates were incubated at 37 C in a CO_2 incubator for 24 to 48 hr and examined for bacterial growth. Suspected *Pasteurella* spp. colonies were identified based on morphological characteristics (Carter, 1990). Different suspected colonies of *P. haemolytica* were isolated based on differences in color, texture, size and degree of hemolysis. Bacterial isolates which were Gram-negative, urea negative, spot-indole negative, oxidase positive, nitrate positive, grew as small colonies on MacConkey's agar, and produced acid reactions when inoculated into triple-sugar-iron agar slants were confirmed to be *P. haemolytica*. Biotyping according to sugar fermentation reactions (Kilian and Frederiksen, 1981), and serotyping by rapid plate agglutination (Frank and Wessman, 1978) were performed on isolates of *P. haemolytica*. McNemar's

symmetry test (Bishop et al., 1975) was used to test for differences in recovery of *P. haemolytica* isolates among the transport methods.

Pasteurella haemolytica was isolated from all 42 bighorn sheep; 135 isolates of *P. haemolytica* were recovered from the three transport methods (107 swabs) including 41 isolates from 34 swabs ($\bar{x} = 1.2 \pm 0.1$ SE/swab) from modified Amies medium with charcoal, 40 isolates from 31 swabs ($\bar{x} = 1.3 \pm 0.1$ SE/swab) inoculated directly onto blood agar plates, and 54 isolates from 42 swabs ($\bar{x} = 1.3 \pm 0.1$ SE/swab) from PBG. One hundred thirty-two (98%) isolates were non-hemolytic *P. haemolytica*, characteristic of strains previously described from bighorn sheep (Wild and Miller, 1991), and three were beta-hemolytic colonies. All non-hemolytic isolates were biotype T, including two isolates (1.5%) identified as serotype 3; seven (5.2%) were serotype 4; 27 (20%) cross-reacted in antisera to serotypes 3 and 4; one (0.7%) cross-reacted in antisera to serotypes 3 and 10; 72 (53%) cross-reacted in antisera to serotypes 3, 4, and 10; and 26 (19%) were untypeable. All three beta-hemolytic isolates were biotype T, but untypeable for serotype. We failed to recover *P. haemolytica* from two swabs placed in modified Amies medium with charcoal and two swabs streaked directly onto blood agar plates, but recovered at least one *P. haemolytica* isolate from all swabs transported and stored in PBG. A maximum of three different *P. haemolytica* isolates were recovered from any one swab, with two or three isolates recovered from 24% of the swabs in modified Amies medium with charcoal, 32% from blood agar plates, and 26% from PBG. No significant differences ($P > 0.05$) were observed in the recovery of *P. haemolytica* isolates among the transport methods.

Pharyngeal swabs streaked directly onto 5% sheep blood agar and transported for ≤ 12 hr in a container with a portable heating pad proved to be a reliable method for the recovery of *P. haemolytica*. While this technique was reliable under ideal conditions, equipment, expertise, and a short transport time are not always amenable to a field situation. Comparing the effects of sampling procedures on the ability to recover *Pasteurella* spp. from bighorn sheep, Wild and Miller (1991) concluded that tonsillar swabs or biopsies plated directly onto blood agar and immediately incubated had a high probability of recovery of nonhemolytic *P. haemolytica*. They reported only two of 19 tonsillar swabs stored in modified Amies medium with charcoal and 0 of 19 tonsillar swabs stored in modified Stuart's medium for 24 hr at room temperature yielded nonhemolytic *P. haemolytica* isolates, while this organism was recovered from 14 of 19 tonsillar swabs plated directly onto blood agar. We experienced relatively good recovery of *P. haemolytica* from pharyngeal swabs placed in modified Amies medium with charcoal, stored at 4 C within 8 hr of collection and then cultured on blood agar within 24 hr of collection. Although simple and convenient to use, ability to recover bacteria from these self-contained transport swabs can decrease rapidly over time (Wild and Miller, 1991). The small volume of media (approximately 0.5 ml) in these transport systems may result in desiccation of the swabs if the cap is not replaced tightly or plating is delayed, and other organisms in contaminated specimens may exceed the buffering capability of the media, resulting in overgrowth and death of fastidious organisms. Therefore, prompt delivery of these transport systems to the laboratory is essential if results of culture are to be representative.

Phosphate buffered glycerol provided a reliable method for transporting and storing swab specimens until processed. Potential loss of microorganisms due to desiccation of media and swabs, insufficient equipment, improper storage during transport or delays in submission to a laboratory were minimized or eliminated with the use of PBG. Also, the need for immediate transportation to a laboratory or

for additional field equipment was eliminated. Phosphate buffered glycerol is a nonnutritional media used primarily for freezing preservation of microorganisms. In our laboratory, actively growing colonies of *P. haemolytica* placed in this medium and stored at −70 C have produced viable colonies >3 yr later (W. Foreyt, unpubl.). Specimens placed in PBG must be kept cool; therefore a cooler containing ice (preferably dry ice for longer preservation) is required. If it is not possible to freeze specimens, refrigeration at 4 C for several days also has resulted in a relatively high recovery rate of *P. haemolytica* (W. Foreyt, unpubl.). Although all three transport methods evaluated resulted in good isolation results, we recommend the use of PBG as transport and storage media for isolation of *P. haemolytica* from bighorn sheep, especially under field conditions when delivery of specimens to a laboratory for culturing will be delayed. This technique is an effective, simple, inexpensive and convenient method for wildlife projects.

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