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Detection of *Mycoplasma ovipneumoniae* and *M. arginini* in Bighorn Sheep Using Enrichment Culture Coupled with Genus- and Species-Specific Polymerase Chain Reaction

Glen C. Weiser,[1,4] Mark L. Drew,[2] E. Frances Cassirer,[3] and Alton C. S. Ward[1] [1] Caine Veterinary Teaching Center, University of Idaho, 1020 East Homedale Road, Caldwell, Idaho 83607, USA; [2] Idaho Department of Fish and Game, 16569 South 10th Avenue, Caldwell, Idaho 83607, USA; [3] Idaho Department of Fish and Game, 3316 16th Street, Lewiston, Idaho 83501, USA; [4] Corresponding author (email: gweiser@uidaho.edu)

ABSTRACT: *Mycoplasma* species are of interest as possible primary pathogens in the pneumonia complex of bighorn sheep (*Ovis canadensis*). Previous investigations have not commonly detected low frequencies of *Mycoplasma* spp. from free-ranging bighorn sheep, possibly due to the fastidious and slow growth of these organisms. We developed a culture protocol that employed an average initial 3-day enrichment culture in liquid Hayflick broth in a CO_2-enhanced atmosphere. The broth was plated to solid Hayflick medium and the cultures observed for growth for up to 30 days. Polymerase chain reaction (PCR) was performed on DNA isolated from the enrichment broth and on isolates obtained from culture using *Mycoplasma* genus-specific PCR assays and species-specific PCR assays for *M. arginini* and *M. ovipneumoniae*. Some cultures that grew on Hayflick plates were picked as single colonies but were mixed because two organisms may grow together and appear as a single colony. Culture and PCR tests produced similar results for *M. arginini*, but for *M. ovipneumoniae*, culture alone was less accurate than PCR. Use of genus-specific primers also may allow detection of other species in samples negative for *M. arginini* and *M. ovipneumoniae*. Two methods of transport from field to laboratory (Port-a-Cul™ tubes, cryoprotectant in liquid N_2 and Fisher Transport System) gave similar results under our study conditions.

Key words: Bighorn sheep, culture technique, *Mycoplasma arginini*, *Mycoplasma ovipneumoniae*, *Ovis canadensis*, polymerase chain reaction.

Mycoplasma species are associated with multiple pathologic conditions, including respiratory disease, in most mammalian and avian species (Whithear and Browning, 2004). They may act alone or in concert with other agents associated with pneumonia in bighorn sheep (Miller, 2001; Rudolph et al., 2003, 2007). *Mycoplasma ovipneumoniae* infections have been associated with chronic respiratory infections, poor growth, and exercise intolerance of lambs in domestic sheep herds (Sullivan et al., 1973; Timoney et al., 1988) and have been suggested as a possible cause of population-limiting pneumonia in bighorn sheep adults and lambs (Besser et al., 2008).

Mycoplasmas are rarely detected on media routinely used in diagnostic bacteriology laboratories and they vary in their growth on selective culture media (Whitford, 1994). While culture is the gold standard for diagnosis, mycoplasmas, particularly *M. ovipneumoniae*, are generally difficult and time consuming to grow. Polymerase chain reaction (PCR) is a more rapid and sensitive detection technique (McAuliffe et al., 2003) and is especially useful in identifying mixed cultures. While also fastidious, *M. arginini* is fast growing and ubiquitous and may overgrow other mycoplasmas (McAuliffe et al., 2003).

Our objective was to identify test procedures that will be successful for culture and sensitive and specific for detection of *Mycoplasma* species in samples from bighorn sheep. Samples were inoculated into Hayflick broth medium (Atlas, 1993) to enrich and selectively propagate mycoplasmas. Broths were incubated at 37 C for 3 days, subcultured onto Hayflick agar, and incubated for 30 days for propagation of organisms and colony detection and characterization.

Some nonmycoplasma organisms, usually identified as "L-forms" (Domingue and Woody, 1997), react as true mycoplasmas in preliminary test procedures

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and may erroneously be identified as mycoplasmas if more definitive tests are not used. Use of a *Mycoplasma* genus-specific primer set employed in this study would help eliminate the reporting of L-forms.

We tried several transport media to determine agreement between PCR and culture on three submissions of oropharyngeal swabs ($n=13$, $n=5$, and $n=4$) collected from 14 bighorn sheep (Table 1). The bighorn sheep were darted using 3 mg of carfentanil and 45 mg of xylazine in 1-ml darts (Pneudart, Williamsport, Pennsylvania, USA) 14–19 November 2008 or captured by helicopter netgun on 3 December 2008 along the Salmon River, east of Riggins, Idaho (45°42′N, 116°32′W). Once restrained, the animals were given complete health examinations.

For case 08-1553 (Table 1) oropharyngeal swabs were placed into a Port-a-Cul™ tube (Becton, Dickinson and Co., Sparks, Maryland, USA) and a duplicate placed into a cryotube (Cryule vial, Wheaton, Millville, New Jersey, USA) containing a cryoprotectant (phosphate-buffered glycerol 2:3 v/v, pH 7.2) and the cryotubes placed in liquid nitrogen within 12 hr after collection. For case 08-1587, all swabs were transported in Port-a-Cul tubes. For case 08-1592, one animal (2015) had duplicate swabs placed into a Port-a-Cul tube and a Fisher system tube (Fisher Transport System, Fisher Healthcare, Houston, Texas, USA) containing Amies medium without charcoal. Swabs were transported to the Caine Veterinary Teaching Center at 4 C and inoculated into Hayflick's broth 24–72 hr after collection.

For PCR detection of *Mycoplasma*, 1 ml of Hayflick broth from each sample after 3 days of incubation was processed with Chelex® resin (Bio-Rad, Hercules California, USA; Ramirez et al., 2006). Briefly, each 1 ml of Hayflick sample was centrifuged at 9,500 × G for 2 min and DNA extracted from the pellet in 200 µl of extraction medium. A portion of the remaining Hayflick enrichment broth was then inoculated onto semisolid Hayflick medium for comparison to PCR results, and to obtain viable organisms for archiving and future study. The semisolid medium was observed for growth for 30 days. Selected colonies characteristic of mycoplasmas were subjected to PCR. All cultures were incubated in a CO_2-enhanced atmosphere (approximately 10%) at 37 C.

The PCR was optimized using authentic (American Type Culture Collection [ATCC] Manassas, Virginia, USA) *M. arginini* ATCC 23243 and *M. ovipneumoniae* ATCC 29419. A proprietary PCR premix (Product 206143, Qiagen Inc., Valencia, California, USA) was used according to the manufacturer's protocol with 2 µl of template in 25-µl reactions. The species-specific primer sets described below showed no cross-reactivity between *M. arginini* and *M. ovipneumoniae* ATTC accessions, nor did they amplify product from *Bibersteinia trehalosi*, *Pasteurella multocida*, or *Mannheimia haemolytica* DNA.

The PCR primers genus-specific for *Mycoplasma* used were GPO-1, 5′-ACT CCT ACG GGA GGC AGC AGT A-3′ and MGSO, 5′-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3′ (Dussurget and Roulland-Dussoix, 1994). Positive samples were subsequently tested for *M. ovipneumoniae* and *M. arginini* using primers specific for these two species,(i.e., MOVPF 5′-GTT GGT GGC AAA AGT CAC TAG-3′ and MOVPR 5′-CTT GAC ATC ACT GTT TCG CTG-3′ and MAGF 5′-GCA TGG AAT CGC ATG ATT CCT-3′ and GP4R 5′-GGT GTT CTT CCT TAT ATC TAC GC-3′, respectively; Lauerman, 1998).

The first submission (case 08-1553) included samples from seven animals (Table 1). Duplicate swabs (with one exception) were collected as described above. Where results from the duplicates differed, an advantage of one transfer protocol over the other was not evident.

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TABLE 1. Results of *Mycoplasma* culture and polymerase chain reaction (PCR) using primers specific for the genus *Mycoplasma* and the species *M. ovipneumoniae* and *M. arginini* on 22 oropharyngeal samples from 14 bighorn sheep, *Ovis canadensis*, sampled along the Salmon River, Idaho, USA, in 2008.

Case number and sheep ID	Transport medium[a]	Culture	PCR results with primers[b]					
			Mycoplasma		*M. ovipneumoniae*		*M. arginini*	
			Broth[c]	Isolate	Broth	Isolate	Broth	Isolate
08-1553								
8416	Port-a-Cul	Negative	−	na	−	na	−	na
8416	LN_2	Negative	−	na	−	na	−	na
8400	Port-a-Cul	Positive	+	+	−	−	+	+
8400	LN_2	Positive	+	+[d]	+	+	−[e]	+
8405	Port-a-Cul	Positive	+	+	−	−	+	+
8405	LN_2	Positive	+	−	−	−	+	−
8415	Port-a-Cul	Negative	−	na	−	na	−	na
8415	LN_2	Negative	−	na	−	na	−	na
7389	Port-a-Cul	Negative	+	na	−	na	−	na
7389	LN_2	Negative	−	na	−	na	−	na
7392	Port-a-Cul[f]	Negative	+	na	−	na	−	na
8410	Port-a-Cul	Positive	+	+	−	−	+	+
8410	LN_2	Positive	+	+	−	−	+	+
08-1587								
6769	Port-a-Cul[g]	Negative	+	na	+	na	−	na
6770		Negative	−	na	−	na	−	na
2011		Positive	+	+	+	−	+	+
2430		Positive	+	+	+	−	+	+
2017		Positive	+	+	+	+	+	+
08-1592								
2015	Port-a-Cul	Negative	−	na	−	na	−	na
2015	Fisher system[h]	Positive	+	+	+	−	+	+
6764		Positive	+	+	−	−	+	+
6764 duplicate		Positive	+	+	+	−	+	+

[a] LN_2 = samples inserted into liquid nitrogen for transport, Port-a-Cul = samples inserted into this transport medium.

[b] na = not applicable; + = positive; − = negative.

[c] Broth refers to DNA amplified from the enrichment Hayflick broth formulation (Atlas, 1993), while isolate refers to DNA amplified from colonies growing from broth plated on solid Hayflick plates.

[d] Positive isolate results for both species-specific primer sets suggests a mixed culture was isolated.

[e] This preparation gave repeated negative results even though culture was positive, perhaps indicating sample degradation.

[f] A sample transported in LN_2 was not taken from this animal.

[g] Samples from this group were all transported in Port-a-Cul.

[h] Fisher Transport System.

The second set of samples (case 08-1587) was from five bighorn sheep. Three of five animals (60%) were culture positive for *Mycoplasma*. Using *M. ovipneumoniae* primers, PCR from broth DNA indicated all three with positive cultures were also positive for *M. ovipneumoniae*, but only one of the cultures appeared to be mixed.

The third set of samples (case 08-1592) included two OP swabs from each of two animals. Duplicate swabs from animal 6764 were transported in Fisherfinest System tubes. One of the duplicates was positive in broth DNA for *M. ovipneumoniae*. In contrast, duplicate swabs from animal 2015 gave different results.

The data in Table 1, taken together, indicate that the enhanced culture protocol described in this paper, followed by genus- and species-specific PCR-based

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identification to species is a reliable way to screen bighorn sheep for *Mycoplasma*. This protocol should perhaps most reasonably be conducted on a herd basis rather than for individual animals because not every animal in a herd is infected. Overall, 11 of 14 animals (78%) were PCR positive for *Mycoplasma*. Culture alone was a reasonably reliable way to detect *M. arginini*. *Mycoplasma* cultures were grown from samples of eight of 14 animals (57%). All cultures, with one exception, were PCR positive for *M. arginini*, and no animal was culture negative and PCR positive for *M. arginini*. Although *M. arginini* and *M. ovipneumoniae* are most frequently detected in bighorn sheep (Rudolph et al., 2007; Besser et al., 2008), the genus primers used in this study allow for the detection of additional species. Cultures of any *Mycoplasma* species also would be useful in strain typing and virulence factor studies. For these reasons, we have continued to culture, even though it is difficult and sometimes unproductive. Cultures may be useful in determining the pathogenicity of mycoplasmas in wild sheep. For example, virulence factors have been studied in some species isolated from domestic animals (Whithear and Browning, 2004) but have not been studied in isolates from bighorn sheep.

Based on our limited data, the choice of transport media and the use of liquid nitrogen did not noticeably impact ability to detect *Mycoplasma* when samples were kept cool and inoculated into broth within 24–72 hr. Port-a-Cul tubes for oropharyngeal swabs, which can also be used to culture for Pasteurellaceae, are used commonly in free-ranging bighorn sheep. The use of liquid nitrogen did not hinder the isolation of *Mycoplasma*, but liquid nitrogen is difficult to use under field conditions.

Our enhanced culture protocol, coupled with PCR tests on enrichment broths, is a reliable way to screen bighorn sheep for *Mycoplasma*, particularly *M. ovipneumoniae*. When compared with PCR, culture gave a false-negative result for *M. ovipneumoniae*. *Mycoplasma ovipneumonae* was isolated only in mixed culture with *M. arginini*. Clearly, improved culture techniques for isolating viable cultures of *M. ovipneumoniae* from wild sheep would be useful. Further research including a comparison of media formulations is indicated.

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