

## An Improved Method for Culturing *Mycoplasma ovipneumoniae* from Field Samples

**JESSICA E. JENNINGS-GAINES**, Wyoming Game and Fish Department, Wildlife Health Laboratory, 1174 Snowy Range Road, Laramie, Wyoming 82070, USA;  
*jessica.jennings@wyo.gov*

**WILLIAM H. EDWARDS**, Wyoming Game and Fish Department, Wildlife Health Laboratory, 1174 Snowy Range Road, Laramie, Wyoming 82070, USA

**MARY E. WOOD**, Wyoming Game and Fish Department, Thorne-Williams Research Center, 2362 Hwy 34, Wheatland, Wyoming, 82201, USA

**KAREN A. FOX**, Colorado Division of Parks and Wildlife, Wildlife Health Program, 4330 Laporte Avenue, Fort Collins, Colorado 80521-2153, USA

**LISA L. WOLFE**, Colorado Division of Parks and Wildlife, Wildlife Health Program, 4330 Laporte Avenue, Fort Collins, Colorado 80521-2153, USA

**MICHAEL W. MILLER**, Colorado Division of Parks and Wildlife, Wildlife Health Program, 4330 Laporte Avenue, Fort Collins, Colorado 80521-2153, USA

**HALCYON J. KILLION**, Wyoming Game and Fish Department, Wildlife Health Laboratory, 1174 Snowy Range Road, Laramie, Wyoming 82070, USA

**ABSTRACT** To better understand the distribution, occurrence, and role of *Mycoplasma ovipneumoniae* in the epidemiology of respiratory disease in bighorn sheep, techniques that accurately and consistently detect this organism in wild sheep populations are needed. We reviewed published techniques and compared commercially available growth media to optimize the growth of *M. ovipneumoniae* in our laboratory. Penicillin, amphotericin B, thalium acetate, and phenol red were added to tryptone soya broth. Incubating field samples at 37 C in 10 % CO<sub>2</sub>, for 48 hr, followed by direct plating onto solid media, improved detection, culture success, and overall agreement between culture and detection by polymerase chain reaction.

*Biennial Symposium of the Northern Wild Sheep and Goat Council 20:83-88.*

**KEY WORDS** bighorn sheep, culture, modified TSB-1, mountain goat, *Mycoplasma ovipneumoniae*, *Ovis canadensis*, *Oreamnos americanus*, polymerase chain reaction (PCR) assay, pneumonia

*Mycoplasma ovipneumoniae* causes persistent infections that are difficult to detect, diagnose, or cure in susceptible hosts (Razin 1996). Some investigators consider *M. ovipneumoniae* to be a primary cause of bronchopneumonia in North American bighorn sheep (*Ovis canadensis*; Besser et al. 2008, 2012, 2013), whereas others consider this agent a contributing pathogen that, when present can predispose to or exacerbate respiratory disease caused by pathogenic *Pasteurellaceae* (Dassanayake et al. 2010;

Wolfe et al. 2010). Recent efforts to better understand the occurrence, distribution, role, and perhaps control of *M. ovipneumoniae* in bighorn respiratory disease have been hampered by inconsistency in detection methods and between investigating laboratories. It follows that such efforts would collectively benefit from more reliable approaches for detecting this agent in field samples. Here we describe work focused on optimizing media, techniques and environmental conditions for growing and detecting *M. ovipneumoniae* in diagnostic

samples collected from bighorn sheep under field conditions.

## Sample area

We collected samples from seven free-ranging bighorn sheep and three free-ranging mountain goat herds from Wyoming, and fourteen free-ranging bighorn sheep herds from Colorado. Captive bighorn sheep from both the Wyoming Game & Fish Department and the Colorado Parks & Wildlife research facilities; three mule deer, two domestic sheep, and one domestic goat from Wyoming were also sampled.

## Methods

In total, we analyzed 1,186 samples in this study, including 1,005 nasal swabs and 181 various tissues and swabs. Samples were collected from October of 2010 through July of 2015 and included animals of both sexes and all age classes. Samples were received and processed within 48 hr of collection.

### SP4-G

Using a general *Mycoplasma* spp. culture protocol provided by Dr. Tom Besser (Washington State University, personal communication) and procedures outlined by Nicholas et al. (2008), we used nasal swabs (sterile polyester tipped applicators; Puritan#25-806 1PD, Guilford, Maine, USA) and “SP4 with glucose” broth (SP4-G; Hardy Diagnostics #R86, Santa Maria, California, USA) for *M. ovipneumoniae* detection. Samples were transported on cold packs, in Port-A-Cul™ tubes (modified Carey-Blair; Becton Dickinson #221606, Franklin Lakes, New Jersey, USA;  $n = 703$ ), or early during the study, in 3ml Amies media without charcoal in a 15 x 103mm Triforest culture tube (Triforest Enterprises, Irvine, California, USA;  $n = 153$ ). Swabs were removed from the transport media and placed into individual tubes of SP4-G. Tubes were incubated with caps loosened at 37 C with 10% CO<sub>2</sub> for 4 days. After incubation, one plate of Columbia

Blood Agar (CBA) with 5% sheep blood (Hardy Diagnostics #A16, Santa Maria, California, USA) was inoculated with 100 µl of broth. Inoculum was spread across half of the plate with a dry sterile polyester swab, and then streaked for isolation across the remaining half of plate. We incubated plates at 37 C with 10 % CO<sub>2</sub> for 7 days, and checked for growth daily. A 250 µl aliquot of broth was removed for DNA extraction on day four. DNA was extracted according to extraction kit instructions (E.Z.N.A. Tissue DNA Kit, Omega Bio-Tek, Inc, Norcross, Georgia, USA). DNA was analyzed using primers and PCR protocol published by McAuliffe et al. (2003), and optimized in our lab using the following protocol: initial denaturation for five minutes at 94 C, 32 denaturation cycles for 30 sec each at 94 C, annealing at 57.5 C for 30 sec, and extension at 72 C for 30 sec. The final extension was 72 C for 5 min. Samples were kept at 4 C until analyzed by agar gel electrophoresis.

### TSB-1

As an alternative, we experimented with switching from SP4-G to tryptone soya broth (TSB-1; Patel et al. 2008), and incorporated amphotericin B, penicillin, and thallium acetate (Razin 1996) to inhibit contamination. Samples were cultured in 2 ml of this modified TSB-1 (mod TSB-1) in 5 ml round-bottom tubes (BD Falcon 352054, Franklin Lakes, New Jersey USA). Culture and PCR protocol remained the same.

During this study, we made minor changes to improve the culture protocol. Spreading inoculum on the CBA plate was facilitated by a polyester swab soaked in corresponding broth sample instead of a dry polyester swab. Culture plates were read for 5 days, instead of 7 days. Also, 1 ml (i.e., 1,000 µl) of broth was aliquoted for PCR instead of 250 µl, because this matched the protocol used by the Washington Disease Diagnostic Laboratory (WADDL) in Pullman, WA, USA. A negative control of modified TSB-1 was

also used to ensure no contamination of the stock modified TSB-1. This control consisted of 1 ml of modified TSB-1 that was incubated and analyzed via PCR under the same conditions and protocols as samples.

## Results

### SP4-G

Several initial *Mycoplasma* culture isolates were identified by gross characteristics (i.e., small, round, center-less areas of hemolysis) and confirmed as *M. ovipneumoniae* via sequencing of the polymerase chain reaction (PCR) product and comparison with published *M. ovipneumoniae* sequences (National Center for Biotechnology Information [NCBI] Basic Local Alignment Search Tool [BLAST]; Wyoming State Public Health Laboratory, Cheyenne, Wyoming). We identified all subsequent culture isolates based on gross characteristics only. Using the initial *M. ovipneumoniae* enrichment protocol, with SP4-G (Table 2) as the standard culture broth and a 4- day incubation period, 33% (42/129) of nasal swabs were positive for *M. ovipneumoniae* by PCR. This culture method provided 26% (11/42) culture success (recovery rate) when compared to PCR results. In addition to a low recovery rate, this method was also associated with substantial bacterial contamination with 42% (53/125) of CBA plates examined exhibiting gross evidence of contamination.

### Modified TSB-1

Switching from SP4-G to tryptone soya broth (TSB-1; Patel et al. 2008), and incorporating amphotericin B, penicillin, and thallium acetate (Razin 1996) nominally increased culture success with a 2-day incubation period (Fisher's exact  $P=0.582$ ; Table 1); there was a significant difference in amount of contamination (Fisher's exact  $P=0.0108$ ; Table 1). A final formulation for modified TSB-1 is found in Table 3. To assess our optimized modified TSB-1 media and incubation time, a total of 856 nasal swabs, collected from routine surveillance of

free-ranging and captive animals, were enriched with modified TSB-1 and incubated for 48 hr. Of these, *M. ovipneumoniae* was detected by PCR in 32.6% (279/856) samples. In addition, 65.6% (183/279) of the PCR-positive samples also yielded observable *M. ovipneumoniae* via CBA culture. Various tissues and swabs obtained from necropsy were also enriched in modified TSB-1 for 48 hr, cultured and analyzed by PCR. Culture success rates are summarized in Table 4.

## Discussion

We modified established media and protocols in order to optimize growth of *M. ovipneumoniae* in our laboratory. Samples incubated for 48 hr in our modified TSB-1 at 37 C and 10 % CO<sub>2</sub> appeared to optimize growth and detection of *M. ovipneumoniae*. We note, however, that we were unable to identify the individual components of the different protocols that may have contributed to the improvement we observed. Because information from the field was often limiting, we were also unable to characterize our samples by the level (if any) of physical manifestation of disease. We compared 1,037 samples over five years using this improved protocol and culture broth to enhance the culture and PCR detection of *M. ovipneumoniae* to better understand its prevalence and distribution in bighorn sheep and mountain goat herds.

## Acknowledgements

Funding for this work was provided by the Wyoming Game and Fish Department, Colorado Division of Parks and Wildlife, and the Wyoming Governor's Big Game License Coalition. We thank T. Besser for consultation and sharing of laboratory protocols, and R. A. J. Nicholas for suggesting improvements to the manuscript. Also, many thanks to numerous agency personnel throughout Wyoming and Colorado for assistance with sample collections.

Table 1. Culture recovery and contamination in duplicate swabs enriched in SP4-G or modified TSB-1 (bighorn sheep from Wyoming and Colorado). Culture recovery rate is the number of culture positive divided by the number PCR positive. Days incubated is designated by 'd'.

Incubation Time and Enrichment broth	Total Samples	PCR Positive	Culture Positive	Culture Recovery Rate	Contamination Positive	Percent Contamination
2 d – SP4-G	13	9	7	77.8%	5	38.5%
2 d - mod TSB-1	13	10	9	90.0%	0	0%
4 d – SP4-G	23	13	2	15.4%	12	52.2%
4 d - mod TSB-1	23	13	5	38.5%	2	8.7%

Table 2. SP4 with glucose broth (SP4-G; Hardy Diagnostics, Santa Maria, California, USA).

Amount	Component
10g	Pancreatic Digest of Casein
5.0g	Pancreatic Digest of Gelatin
3.5g	PPLO Broth without CV
50mg	Polymixin B
5mg	Amphotericin B
170ml	Fetal Bovine Serum
1,000,000units	Penicillin
50mL	CMRL 1066 Medium (10X)
35.0ml	Yeast Extract
20ml	Yeastolate 10%
5g/L	Glucose
690mL	Deionized H <sub>2</sub> O

Table 3. Modified TSB-1. Shelf life is 3 months at 4°C.

Amount	Component
30g	Tryptone Soya Broth (Oxoid Ltd, Basingstoke, Hampshire, England) (pancreatic digest of casein (17.0g/L), enzymatic digest of soya bean (3.0g/L), sodium chloride(5.0g/L), di-potassium hydrogen phosphate(2.5g/L), glucose(2.5g/L))
10g	D-Lactose Monohydrate (Sigma, St. Louis, Missouri, USA)
200mL	Porcine Serum – heat inactivated (Rocky Mountain Biologicals, Missoula, Montana, USA)
7.25mg	Amphotericin B (Sigma, St. Louis, Missouri, USA)
1,323,661units	Penicillin G Potassium Salt (Sigma, St. Louis, Missouri, USA)
23.27mL	Thallium Acetate solution (10mg/mL(de-ionized H <sub>2</sub> O) (Sigma, St. Louis, Missouri, USA)
18mg	Phenol Red (Sigma, St. Louis, Missouri, USA)
1,000mL	Deionized H <sub>2</sub> O

Table 4. Overall culture recovery in samples enriched in modified TSB-1 with thallium acetate for 48 h (Wyoming: bighorn sheep (721), mountain goats (40), mule deer (3), domestic sheep (1) and domestic goat (3); Colorado: bighorn sheep (267), domestic sheep (2)).

Sample Type	Culture Positive	PCR Positive	Culture Recovery Rate	Total Samples Tested
Nasal Swab	183	279	65.6%	856
Lung	32	78	41.0%	123
Bulla	5	19	26.3%	33
Sinus	1	3	33.3%	11
Liver	0	1	0%	12
Sinus Tumor	0	1	0%	1
Pericardium	0	1	0%	1

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