WAFWA Wildlife Health Committee 2014 BIGHORN SHEEP HERD HEALTH MONITORING RECOMMENDATIONS

January 3, 2015

Photo: Peregrine Wolff, Nevada Department of Wildlife

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Background

In September of 2013, a bighorn sheep disease sampling/health assessment workshop was conducted at the request of the Western Association of Fish and Wildlife Agencies, Wildlife Health Committee (WAFWA WHC) to prioritize and standardize testing protocols for respiratory pathogens of bighorn sheep. Specific concerns included that numerous tests for a variety of pathogens are available but interpretation of results is challenging, laboratories do not have standard methodology and the 2009 WAFWA WHC Sheep Sampling Guidelines required updating.

The workshop included wildlife health professionals from nine Western states and two Canadian provinces. The workshop was hosted by the Colorado Division of Wildlife and held in Fort Collins. Funding was secured from the Wild Sheep Foundation to support attendees with travel restrictions.
WAFWA Wild Sheep Working Group members were surveyed concerning their primary concerns surrounding testing for bighorn sheep pneumonia complex prior to the workshop. Their feedback was addressed in the agenda and discussion points.

The group produced the following documents that have been incorporated into the updated WAFWA WHC Sheep Sampling Guidelines:

A. Standardized definitions
    A. Sampling recommendations based on herd management plan and examples of commercially available tests for each pathogen
B. Recommendations for sample collection, processing and diagnostic techniques for *Pasteurellaceae spp*
C. Recommendations for sample collection processing and diagnostic techniques for *Mycoplasma spp.*
D. Necropsy protocol
E. Links to additional documents

Also identified were several tests/protocols requiring future research as well as topics/techniques for agency staff training to support consistent approaches to sample collection and handling. These products will support recommendations across agencies for different management practices and provide a valuable resource and reference for all wildlife health and management professionals.

Bighorn Sheep Respiratory Pathogen Sampling and Health Assessment Workshop Participants

Mark Drew, *Idaho Department of Fish and Game, Wildlife Health Laboratory, Caldwell, ID*

Hank Edwards, *Wyoming Game and Fish Department, Laramie, WY*

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Anne Justice-Allen, *Arizona Game and Fish Department, Phoenix, AZ*

Hally Killion, *Wyoming Game and Fish Department, Laramie, WY*

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Leslie McFarlane, *Utah Division of Wildlife Resources, Salt Lake City, UT*

Michael W. Miller, *Colorado Division of Wildlife, Wildlife Research Center, Fort Collins, CO*

Margo J. Pybus, *Alberta Fish and Wildlife Division, Edmonton,AB*

Jennifer Ramsey, *Montana Fish, Wildlife and Parks, Bozeman, MT*

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RECOMMENDATIONS

WILD SHEEP HERD HEALTH MONITORING

Western Association of Fish & Wildlife Agencies
Wildlife Health Committee
2014

Introduction:

This document is a condensed and updated version of the 2009 WAFWA, Wildlife Health Committee's, *Wild Sheep Herd Health Monitoring Recommendations* and provides current recommendations for monitoring herd health based on the best available science. To access the 2009 Guidelines go to:
(http://www.wafwa.org/documents/wswg/WAFWA_WS_Herd_Monitoring_9_09.pdf)
Assessing and monitoring herd health is an essential element of wild sheep management in North America. This document has been prepared for wildlife management agencies to provide standardized definitions and methods, protocols and recommendations for key elements of a wild sheep herd health plan. These elements are attached as appendices to this document to allow them to remain flexible and to account for scientific advances.

Collaboration among wildlife managers, diagnostic laboratories and wildlife health professionals is critical. It is vital for wildlife agencies to develop and maintain relationships with diagnostic laboratories and to use the appropriate protocols for collection and handling of samples. Wildlife managers should work directly with wildlife health staff and consult the WAFWA WHC for guidance in the appropriate health testing and response to disease outbreaks, management and response guidelines.

Wildlife management agencies should develop protocols and response plans that can be implemented prior to or in the event of observed clinical disease and mortality events. Standardized sampling protocols, training, and testing standards should be followed as closely as possible to ensure quality, and consistency in interpretation of results.
Evaluation of wild sheep population demographics or selected population characteristics such as adult or lamb survival is essential for assessments of herd production or performance and health and should be performed as often as possible.

- A review of past and present herd performance is critical as the first step in assessing the health status of a wild sheep population or its subsidiary herd units.

- Herd size and composition (adult survival and lamb recruitment) should ideally be assessed annually.

- Cause(s) of change(s) in herd size, composition and adult survival and lamb recruitment should be investigated in high priority herds.
- The degree of investment an agency may choose to make in monitoring individual wild sheep herds likely will depend upon the management objectives and relative importance of each herd.

Herd management plans should include a classification of health based on herd status and performance and supported by disease sampling results derived from opportunistic sampling, disease investigation or monitoring. This classification would assist in prioritizing herd health monitoring and other management activities.
For example, when considering translocation as a management action, herd performance evaluations should be done as a part of a complete health assessment both for potential source herds and for potential recipient (or adjacent) herds prior to the translocation. To underscore the importance of this recommendation, the requirement for such evaluations should be embedded in agency policy. In the absence of these data, herd health assessments may need to rely more heavily on other less practical approaches (e.g., extensive diagnostic sampling, quarantine).

The following appendices are included in this document to assist wildlife managers and health professionals with development and implementation of herd health plans and disease investigation, surveillance and monitoring. It is recognized that technology is constantly advancing and these appendices are meant to be fluid documents that will be updated to incorporate the latest science:

B. Standardized definitions
C. Sampling recommendations based on herd management plan and examples of commercially available tests for each pathogen
D. Recommendations for sample collection, processing and diagnostic techniques for *Pasteurellaceae spp*
E. Recommendations for sample collection processing and diagnostic techniques for *Mycoplasma spp.*
F. Necropsy protocol
G. Links to additional documents and examples of forms

APPENDICES:

APPENDIX A - DEFINITIONS

When discussing herd health status it is crucial that all wildlife professionals use consistent terminology. The following are recommended definitions.

HEALTHY HERD:

1. All age classes appear healthy (good body condition and no clinical signs of disease) and are present in expected proportions,
    a. And have lamb:ewe ratios > than 20 lambs:100 ewes,
    b. And have population numbers are stable or increasing.

NON-HEALTHY HERD:

1. One or more age classes are not present in expected proportions or the population is declining or below levels expected for the observed quality of habitat,
2. And the herd shows one or more of the following characteristics,
    a. Poor lamb recruitment, defined as < 20 lambs: 100 ewes over consecutive surveys, or
    b. Current or recent history of clinical disease.

DISEASE EVENT

Observation of clinical signs consistent with a disease or disease related mortality within a population.

DIE-OFF OR MORTALITY EVENT

Loss of an unusual portion or proportion of the population attributable to disease

All age die-off

Loss of an unusual proportion of the population across all age classes attributable to an infectious Disease

Lamb mortality event

Loss of significant proportion of young of the year and attributable to an infectious disease.

CLINICAL DISEASE

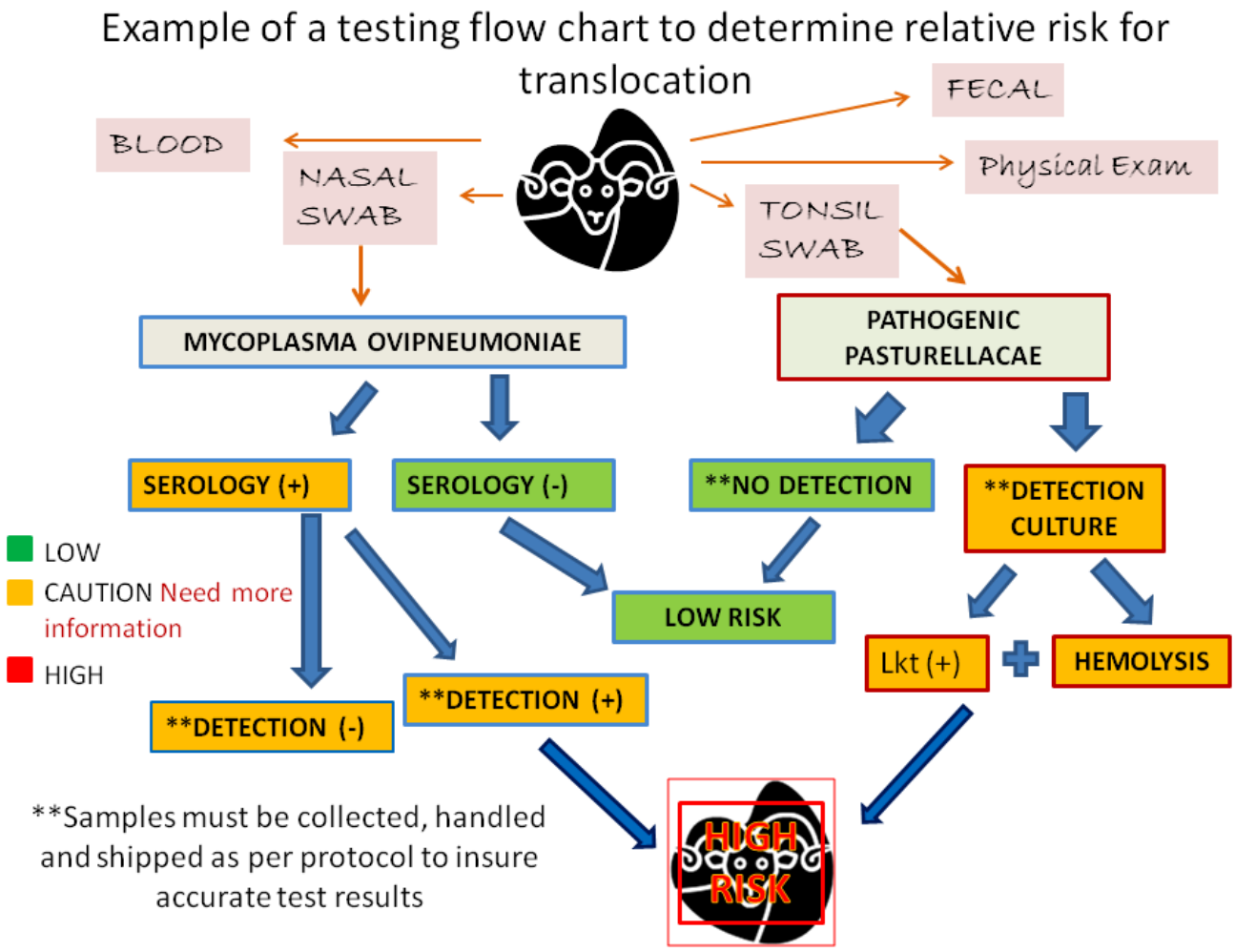
Clinical signs (symptoms) of disease in sheep may include:

- Coughing in a sustained manner (more than clearing of the mouth/throat)
- Nasal discharge – clear or cloudy
- Head shaking
- Droopy ears
- Lethargy or reluctance to move
- Exercise intolerance
- Poor body condition
- Diarrhea
- Skin abnormalities
- Eye abnormalities
- Sudden death

APPENDIX B: SAMPLING RECOMMENDATIONS BASED ON HERD MANAGEMENT PLANS WITH EXAMPLES

- The spreadsheets in **Appendix B:** contains both the recommended diagnostic sampling regimens for wild sheep herds under several categories as well as detailed bacteriology testing recommendations. The regimens may be used for routine herd health surveillance of high priority herds, pre-translocation screening, disease investigation or general opportunistic monitoring. Results of sampling efforts, whether opportunistic, for specific surveillance or disease investigation, may be used in a risk assessment to assign a relative risk score to a herd (*see figure 1. for an example flow chart to determine relative risk of translocation*).
- It is recommended that collection of blood and other biological samples (e.g., feces, hair, and tissue) from live or dead wild sheep be done at every opportunity. Frozen sera, tissues, swabs and air-dried skin, blood and hair may be stored indefinitely for retrospective use in disease, genetic and forensic studies. Nasal and tonsil / pharyngeal swabs collected by the standard techniques described in the appendices can be placed in brain heart infusion (BHI) broth with 10% glycerol and stored at –70 C for later culture if needed.
- Interpretation of diagnostic results must take into account the specific herd health history as well as contemporary observations. Some tests indicate active infection with a specific pathogen, while others only indicate prior exposure to a pathogen. Not all disease agents are significant to wild sheep population dynamics. Some agents are capable of causing illness and mortality when present alone but other agents appear to be more pathogenic when multiple agents are present. A variety of factors can impact how laboratory results should be interpreted (e.g. the test may not be validated for bighorn sheep, a test may present as a false positive or negative and serological titers may not be significant, etc.) therefore, agencies should consult with the testing laboratory and wildlife health professionals for their assistance in interpretation of this data.

Figure 1: Flowchart to determine relative risk for translocation



APPENDIX B: SAMPLING RECOMMENDATIONS BASED ON HERD MANAGEMENT PLAN							
DIAGNOSTIC METHOD / ASSAY	HERD MANAGEMENT PLAN						Test Commercially available at lab
	Healthy Herd Assessment Baseline (>20 lambs:100 ewes)	**Unhealthy Herd (<20 lambs:100 ewes)**	**Recipient Herd**	**Source Herd- Highest health**	**Disease Investigation**	**Disease Follow Up or Targeted Surveillance**	
	Minimum samples size for surveillance should be 10% of herd or sub-herd/ Combine with herd performance data						
Necropsy & histopathology	Opportunistic- even if it looks healthy	Recommended if feasible (consider collecting sick adults or lambs)	Opportunistic- even if it looks healthy	Opportunistic- even if it looks healthy	Recommended (collect carcasses or sick adults & lambs)	Recommended if feasible (consider collecting sick adults or lambs)	**See necropsy protocol. Be sure tissues and blocks are accessible for retrospective work**
Tissue Banking	Yes	Yes	Yes	Yes	Yes	Yes	
Nutrition & trace mineral assessment	Yes	Yes	Yes	Yes	Yes	Yes	Liver preferred, bank opportunistically
Serum banking	recommended	recommended	recommended	recommended	recommended	recommended	In ultralow in cryovials
Parasitology (internal & external parasites)	Recommended	Recommended	Recommended	Recommended	Recommended	Recommended	minimum 10% of herd

***Pasteurellaceae* detection**	Recommended	Live & dead	Recommended	Recommended	Recommended	Recommended	***See Appendix B Supplement below for details***
See Appendix B Supplement below for details	Refer to Appendix C: Recommendations For Sample Collection, Processing And Diagnostic Techniques For *Pasteurellaceae*						
***Mycoplasma ovipneumoniae* detection**	recommended	recommended	recommended	recommended	recommended	recommended	***See Appendix B Supplement below for details***
See Appendix B Supplement below for details	Refer to APPENDIX D: Recommendations for Sample Collection Processing And Diagnostic Techniques For *Mycoplasma* spp.						
Virus exposure (serology recommended)	recommended	recommended	recommended	recommended	recommended	recommended	
Parainfluenza 3 (PI3)	as indicated	as indicated	as indicated	as indicated	as indicated	as indicated	consult your state diagnostic lab for testing availability
Respiratory syncytial virus (RSV)	as indicated	as indicated	as indicated	as indicated	as indicated	as indicated	
Others (as indicated)							
Virus detection (by isolation, PCR, or IHC)	as indicated	as indicated	as indicated	as indicated	as indicated	as indicated	Consult your state diagnostic lab for protocol

APPENDIX B SUPPLEMENT: DETAILED BACTERIOLOGY TESTING RECOMMENDATIONS							
DIAGNOSTIC METHOD / ASSAY	APPLICATION TO HERD HEALTH MONITORUING & MANAGEMENT						Test Commercially available at lab
	Healthy Herd Assessment Baseline (>20 lambs:100 ewes)	**Unhealthy Herd (<20 lambs:100 ewes)**	**Recipient Herd**	**Source Herd-Highest health**	**Disease Investigation**	**Disease Follow Up or Targeted Surveillance**	
	Minimum samples size for surveillance should be 10% of herd / Combine with herd performance data						
***Pasteurellaceae* detection**	recommended						
Selective media-based bacteriology	Demonstrates presence of potentially pathogenic *Pasteurellaceae.*						Consider banking isolates / DNA
PCR - applied to tissue or swab (specify primers) for M. hemolytica	Demonstrates presence of potentially pathogenic *Pasteurellaceae.*						ISU -VDL
PCR for leukotoxin gene (*lktA*) - applied to tissue or swab (specify primers)?	Demonstrates presence of potentially pathogenic *Pasteurellaceae.*						Caine veterinary lab
PCR for leukotoxin gene (*lktA*) - applied to swab or plate growth or isolate (specify primers)?	Demonstrates presence of potentially pathogenic *Pasteurellaceae.*						WADDL
PCR for species-specific leukotoxin gene	Demonstrates presence of potentially pathogenic *Pasteurellaceae.*						For M. hemolytica Lkt 1 and 6, Newport

fragments?		Labs, MN
Speciation/strain typing - "biogrouping"	May be helpful in interpreting initial sceening results	Caine veterinary lab
PCR for Speciation for M. hemolytica	May be helpful in interpreting initial sceening results	Caine veterinary lab
Speciation/strain typing - 16S rRNA	Before using refer to specific references. May be helpful in interpreting initial sceening results	Newport Labs, MN
Speciation/strain typing - MALDITOF	Replaces selective media based bacteriology Before using refer to specific references	ISU-VDL, WADDL
Speciation/strain typing - Pulse field gel electrophoresis (PFGE)	Before using refer to specific references. May be helpful in interpreting initial sceening results	contact your VDL
	Refer to Appendix C: Recommendations For Sample Collection, Processing And Diagnostic Techniques For *Pasteurellaceae*	
***Mycoplasma ovipneumoniae* detection**	recommended	
Serology	Demonstrates prior exposure to potentially pathogenic *M. ovipneumoniae.*	WADDL
PCR - applied to tissue	Demonstrates presence of potentially pathogenic *M. ovipneumoniae.*	WADDL Caine veterinary Lab
PCR - applied to nasal swab	Demonstrates presence of potentially pathogenic *M. ovipneumoniae.*	WADDL Caine veterinary Lab

PCR Mycoplasma species identification (nasal swab or tissue)	Demonstrates presence of *Mycoplasma* species (*M. arginini* or *M. ovipneumoniae*)						Caine Veterinary Lab
	Refer to APPENDIX D: Recommendations for Sample Collection Processing And Diagnostic Techniques For *Mycoplasma* spp.						
***Mycoplasma ovipneumoniae* characterization (strain typing)**	May be beneficial to determine origin or relationships of *M. ovipneumoniae* infections in different herds or over time.						
Speciation - 16S rRNA sequencing							Not commercially available. Consult with Tom Besser (WSU/WADDL) for availability
Strain typing - 16S-23S intergenic spacer							
Necropsy & histopathology	Recommended to aid in interpreting lab findings.						**See necropsy protocol. Be sure tissues and blocks are accessible for retrospective work**
Tissue Banking	Yes	Yes	Yes	Yes	Yes	Yes	

ISU - VDL	Iowa State Veterinary Diagnostic Lab, Ames IA, 515-294-1950, http://vetmed.iastate.edu/diagnostic-lab
WADDL	Washinton Animal Disease Diagnostic Lab, Pullman, WA, 509-335-9696, http://waddl.vetmed.wsu.edu/
Caine Laboratory	Caine Veterinary Teaching Center, Caldwell, ID 208-454-8657, http://www.cainecenter.uidaho.edu/wildlife.htm
Newport Labs	Newport Labs, Worthington, MN, 800-220-2522, http://www.newportlabs.com/
Dr. Tom Besser	Dr. Tom Besser, Washington Animal Disease Diagnostic Lab, Pullman, WA, 509-335-9696, tbesser@vetmed.wsu.edu

APPENDIX C: RECOMMENDATIONS FOR SAMPLE COLLECTION, PROCESSING AND DIAGNOSTIC TECHNIQUES FOR PASTEURELLACEAE

WESTERN ASSOCIATION OF FISH AND WILDLIFE AGENCIES
WILDLIFE HEALTH COMMITTEE
2014

BACKGROUND

Pathogenic *Pasteurellaceae* are components of the respiratory disease complex of bighorn sheep in many jurisdictions. Information gathered to date from numerous animals, herds, and locations has shown the importance of some species and strains of these bacteria in bighorn respiratory disease outbreaks.

PASTEURELLACEAE DIAGNOSTIC TECHNIQUES: APPLICATIONS, ADVANTAGES, & LIMITATIONS

Because the *Pasteurellaceae* family includes both disease-causing and relatively benign species of bacteria, herd screening data may be relatively uninformative (and possibly misleading) unless some effort is made to focus on detecting evidence of potentially pathogenic species and strains.

Culture-based approaches

These approaches all require the presence of viable bacteria in the sample of interest.

Basic bacteriology

Principle: Bacteria grown from samples & identified (usually to species).

Uses: Applicable for tissue and live-animal sampling.

Advantages:

- Techniques for growing *Pasteurellaceae* in culture are well-established & widely available.
- Live bacteria grown from field samples can be further identified and compared using a variety of techniques.

Limitations:

- Sample condition & handling can influence results & interpretation (see details below).
- Laboratories handling mostly livestock submissions may be less successful in recovering and correctly identifying some bighorn *Pasteurellaceae*.
- Proper live-animal sampling requires special equipment & techniques, and can be logistically difficult (see details below).
- Difficult to deliver samples to laboratory in 24-36 hours when sampling remote herds. Delays in shipping and freezing/high temperatures can render samples worthless.

- Use of TSB/glycerol (frozen) may fail to recover some organisms (e.g., *Pasteurella multocida*

Specialized bacteriology: Biogrouping

Principle: Bacteria grown from samples & identified to species subgroups using specialized media.

Uses: Applicable for bacteria isolated from tissue and live-animal sampling.

Advantages:

- Techniques are well-established & have been used for decades.
- Live bacteria can be further identified & compared.

Limitations:

- Only one laboratory currently offers this type of screening.
- Interpretation of data somewhat subjective & correctly identifying some bighorn *Pasteurellaceae* may be problematic.
- Sample condition & handling can influence results & interpretation (see details below).
- Proper live-animal sampling requires special equipment & techniques, & can be logistically difficult (see details below).

Lab: University of Idaho, Caine Veterinary Teaching & Research Center, 1020 E Homedale Rd, Caldwell, ID 83607

Cost: Contact Dr. Glen Weiser for current fees (208) 454-8657; gweiser@uidaho.edu

Specialized bacteriology: Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry[1]

Principle: Bacteria grown from samples & identified to species subgroups using mass spectrometry https://ahdc.vet.cornell.edu/news/malditof.cfm
Uses: Applicable for bacteria isolated from tissues and live-animal sampling.

Advantages:

- Relatively rapid: takes "minutes" to run sample (once bacteria have been isolated).
- Relatively inexpensive.
- In theory, could provide more complete or informative characterization than other culture-based methods.

Limitations:

- Testing requires live, isolated bacteria so limitations of culture (see above) also apply.

- Data from bighorn samples unavailable, so utility in field investigations unclear.
- Species characterizations appear largely (entirely?) based on domestic isolates, so classifications may not distinguish pathogenic from benign species or strains.
- Limited availability, as not many labs presently using MALDI-TOF.

Labs: Cornell University - Animal Health Diagnostic Center, UC Davis - CAHFS, Iowa State University – Veterinary Diagnostic Lab, Kansas State Veterinary Diagnostic Lab, Texas A &M – Veterinary Medical Diagnostic Lab, Washington – Animal Disease Diagnostic Lab (soon)

Cost: Varies by laboratory: Cornell $35.00 including culture and MALDI-TOF, Iowa State: $25.00 for the first sample and $5.00 for each additional sample for culture and MALDI-TOF

Culture-free approaches

These approaches do not depend on the presence of viable bacteria in the sample of interest.

Histopathology

Principle: Pathogenic *Pasteurellaceae* cause microscopic tissue damage that can be recognized in preserved lung tissue.

Uses: Applicable for postmortem tissue sampling.

Advantages:

- Does not depend on live bacteria.
- Techniques are well-established, widely available, & fairly robust to field conditions.
- Provides *in situ* perspective on damage caused by multiple pathogens.
- Preserved tissues can be examined retrospectively & subjected to additional testing to detect other agents.

Limitations:

- Applications limited to postmortem samples.
- Does not provide detailed information on species or strain of bacteria present.
- Sample condition & handling can influence results & interpretation.
- Pathologists with limited experience in respiratory disease pathology may be less familiar with lesions caused by *Pasteurellaceae* in bighorns.
- Proper sampling and tissue preservation are required to assure complete & reliable results.

Polymerase chain reaction (PCR) assays

Principle: Bacterial DNA can be extracted from samples and specific genes detected (to identify species or virulence factor[2,3,4,5,6]).

Uses: Applicable for tissue, live cultures, and (potentially) direct live-animal sampling.

Advantages:

- PCR techniques well-established & numerous published primer sequences available for *Pasteurellaceae*; new primers can be designed to answer specific questions.
- Relatively robust to sample handling & condition.
- Does not depend on live bacteria, although amplification via culture may help in applications where relatively few bacteria of interest are present.

Limitations:

- Sample condition & handling can influence results & interpretation (see details below).
- Assays using different primers & conditions may yield different results, so interpret data in context.
- Depending on primers used, may not provide detailed information on species or strain of bacteria present.
- Samples from live animals may yield less reliable data; work to improve live animal applications ongoing (see details below).
- Limited availability & sometimes expensive, few labs offering PCR, high cost of some PCRs, require primary culture

Labs: Newport labs currently offers Leukotoxin 1 and 6 gene PCR for *M.* hemolytica. WADDL also offers a leukotoxin A gene PCR in the near future

LIVE-ANIMAL SAMPLING FOR *PASTEURELLACEAE* SCREENING

The WAFWA, WHC previously developed testing protocols for health monitoring or interstate and international movement of bighorn sheep. http://www.wafwa.org/documents/wswg/WAFWA_WS_Herd_Monitoring_9_09.pdf Within these protocols, a tonsil swab is recommended for culture to detect potentially pathogenic *Pasteurellaceae* spp., especially leukotoxigenic *Bibersteinia trehalosi* and *Mannheimia haemolytica*. Below is the recommended protocol for the collection, preservation, and shipping of tonsil swabs to the diagnostic laboratory for *Pasteurellaceae* screening.

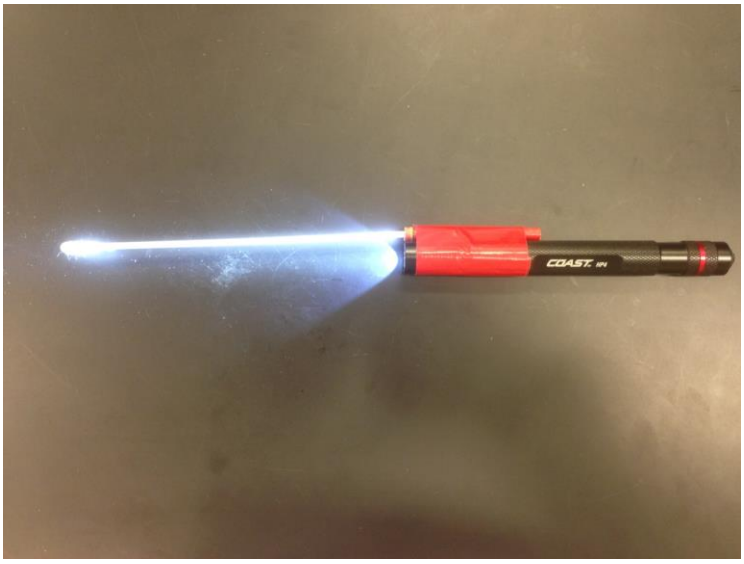
Standard sample collection (Use when only collecting tonsil swabs for shipment to laboratory)

- Use a clean oral speculum or mouth gag and an illuminated swab extender (see below) to allow the swab to reach the tonsillar region. Ideally, both tonsillar crypts should be

sampled with each swab. Be careful not to contaminate the sample by touching other parts of the oral cavity with the swab.

- Polyester-tipped swabs are recommended; do not use swabs with a cotton tip or wooden shaft.
- The speculum or mouth gag should be cleaned with soapy water and sterilized with 70% ethanol between animals.
- Immediately after collection, carefully withdraw the swab and place the swab into an 11 ml Port-A-Cul® tube (see below). Insert swab only about 25 mm (~1 in) into media or until polyester tip is immersed, break off the swab shaft and replace cap. If Port-A-Culs are not available, Amies without charcoal, Cary and Blair, or tryptic soy broth with 15% glycerol (TSB with glycerol, must be held on dry ice), can also be used, however, results may not be as reliable[7].

Swab extender with LED light (see supplies below)



<u>Enhanced sample collection</u>.

Enhanced sample collection is recommended as one option that limits variability of extended shipping times and pathogen survival. Enhanced sampling follows the standard collection, but a culture plate is inoculated immediately after specimen collection and placed into a mobile incubator. Culture plates are shipped directly to the laboratory if <24 hours after collection or individual colonies are isolated, purified, and preserved for later shipment.

- Inoculate one-third or a Columbia Blood Agar (CBA) or Columbia Selective Agar (CSA) plate immediately after sample collection and place in a mobile incubator held at 37°C. Streak for isolation in < 8hrs.

- If unable to send to the laboratory in <24 hours, remove suspect isolates in 24 hours and wash plate at 48 hours with Phosphate Buffered Saline (PBS) and freeze (for PCR).

Advantages**:**

- Enhanced recovery of organisms, consistent sample handling, able to save isolates for banking and research, media plates are inexpensive, mobile incubator inexpensive.

Limitations:

- Requires additional field equipment (bulky) and supplies, requires specialized training, significant time commitment to process cultures, difficult to keep plates from freezing in very cold weather.

Sample preservation and shipping

- Label the media tube and place in a small, insulated container containing ice packs. Insulate the media tube(s) from direct contact with ice packs during shipping. DO NOT FREEZE Port-A-Cul tubes.
- If shipment to the laboratory is expected to be delayed (will arrive at lab greater than 72 hours after collection), immediate freezing of swab in TSB\glycerol is recommended.
- Ship media tubes in an insulated box, ideally at 41 F (5 C)[8] as soon as possible after collection. Best results are obtained when samples reach the laboratory within 24-36 hours but not more than 72 hours after collection[8].
- Notify the receiving laboratory prior to shipping.
- Confirm overnight delivery with the package delivery service.
- Do not send packages that will arrive on weekends or holidays without making prior arrangements with the receiving laboratory.

Selected References for Recommended Sample Processing and Diagnostic Techniques

1. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D. 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clinical Infectious Diseases 49: 552–553.

2. Jaworski MD, Hunter DL, Ward AC, Wesley IV. 1993. Use of DNA analysis of Pasteurella haemolytica biotype T isolates to monitor transmission in bighorn sheep (Ovis canadensis canadensis). Journal of Clinical Microbiology 31:831-835.

3. Jaworski MD, Hunter DL, Ward AC. 1998. Biovariants of Isolates of *Pasteurella* from Domestic and Wild Ruminants. Journal of Veterinary Diagnostic Investigations 10:49-55.

4. Angen O, Thomsen J, Larsen LK, Kokotovic B, Heegaard PMH, Enemark JMD. 2009. Respiratory disease in calves: Microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response. Veterinary Microbiology 137: 165–171.

5. Miller MW, Hause BM, Killion HJ, Fox KA, Edwards, WH, and Wolfe LL. 2013. Phylogenetic and Epidemiologic Relationships among *Pasteurellaceae* from Colorado Bighorn Sheep Herds. Journal of Wildlife Diseases 49: 653-660.

6. Shanthalingam S, Goldy A, Bavananthasivam J, Subramaniam R, Batra SA, Kugadas A, Raghaven B, Dassanayake RP, Jennings-Gaines JE, Killion HJ, Edwards, WH, Ramsey JM, Anderson NJ, Wolff PL, Mansfield K, Bruning D, Srikumaran S. 2014. PCR assay detect Mannheimia haemolytica in culture-negative pneumonic lung tissues of bighorn sheep (*Ovis canadensis*) from outbreaks in the western USA, 2009-2010. Journal of Wildlife Diseases 50: 1-10.

7. Wild MA, Miller MW. 1994. Effect of modified Cary and Blair medium on recovery of nonhemolytic *Pasteurella haemolytica* from Rocky Mountain Bighorn sheep (*Ovis Canadensis Canadensis*) pharyngeal swabs. Journal of Wildlife Diseases 30: 16-19.

8. Wild MA, Miller MW. 1991. Detecting nonhemolytic *Pasteurella haemolytica* infections in healthy rocky mountain bighorn sheep (*Ovis canadensis canadensis*): influence of sample site and handling. Journal of Wildlife Diseases 27: 53-60.

9. Dassanayake RP, Shanthalingam S, Herndon CN, Subramaniam R, Lawrence PK, Bavananthasivam J, Cassirer EF, Haldorson GJ, Foreyt WJ, Rurangirwa FR, Knowles DP, Besser TE, Srikumaran S. 2010. *Mycoplasma ovipneumoniae* can predispose bighorn sheep to fatal *Mannheimia haemolytica* pneumonia, Veterinary Microbiology 145: 354–359.

Supplies for Enhanced Sample Collection:

Item	Catalog number	Price	Package	Supplier	Comments
Columbia Blood Agar	A16	12.57	pack 10	Hardy Diagnostics hardydiagnostics.com/ 800-266-2222	CBA
Columbia Selective Agar	A24	27.26	pack 10	Hardy Diagnostics	CSA
Culture loop	178CSR40	101.61	Pack 1000	Hardy Diagnostics	Disposable
Port-A-Cul	221606	23.86	pack 10	VWR https://us.vwr.com/store / 800-932-5000	https://www.bd.com/ds/productCenter/CT-PortACul.asp
CO2 packs	ref #260679			VWR	BD GasPak EZ CO2 Container System
Incubator heater	http://incubatorwarehouse.com/48-watt-incukit-dc.html			120 V	
Incubator	Any moderately sized cooler will work, we prefer the Coleman174-Classic-54-qt-Steel-Cooler for its durability				
Culture jar	http://www.sigmaaldrich.com/catalog/product/fluka/28029?lang=en&region=US				
Power inverter					12V to 120V
SUPPLIES FOR SWAB EXTENDER FOR TONSILLAR SAMPLING					
Oral speculum	Purchase at vet supply or construct				
LED light	Coast HP4 High Performance	21.21	Each	Amazon	95 Lumen LED Penlight
Dowel rods					¼" hardwood
Paint sticks					Tongue depressors

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APPENDIX D: RECOMMENDATIONS FOR SAMPLE COLLECTION, PROCESSING AND DIAGNOSTIC TECHNIQUES FOR MYCOPLASMA

WESTERN ASSOCIATION OF FISH AND WILDLIFE AGENCIES
WILDLIFE HEALTH COMMITTEE
2014

Background:

The finding of *Mycoplasma* spp. in bighorn sheep, especially lambs, as a cause of disease and mortality is of concern to wildlife managers. However, the culture and identification of *Mycoplasma* spp. is difficult as this bacteria is not robust and difficult to grow in the laboratory. Nevertheless, some laboratories attempt culture of the organisms using various methods. Several laboratories utilize PCR techniques to determine the presence of genetic material from various *Mycoplasma* spp. (e. g. *M. ovipneumoniae* and *M. argininni*). One laboratory is performing a competitive ELISA (cELISA) to measure the presence of antibodies to *M. ovipneumoniae*.

Recommendations:

For basic surveillance in herds where mycoplasma exposure is unknown, it is recommended that serology be used to screen a herd for the presence of antibodies to *M. ovipneumoniae.* Serology is also useful for determining historical exposure and prevalence in a particular herd. Nasal swabs can also be obtained for culture and/or PCR to determine the number of animals that may be actively infected/shedding *M. ovipneumoniae* (see below on sample collection recommendations) if serology indicates exposure to the bacteria. Surveillance has also been successful using hunter-killed animals, where sinus swabs are obtained from the skull when horns are removed for taxidermy. Nasal swabs taken from heads when horns are plugged for regulatory purposes may also be utilized if the head is presented fresh (P. Wolff, personal communication).

This document is meant to provide a standard method for the detection of Mycoplasma spp. in bighorn sheep. Revisions to this document are anticipated as methods evolve or are adapted.

1) **Live animals**
    a. Serology
        i. cELISA: Offered by Washington Animal Disease Diagnostic Lab. Measures antibody present in the sample as a % Inhibition (% I).

            Ziegler J.C., K. K. Lahmers, G. M. Barrington, S. M. Parish, K. Kilzer, K. Baker, and T. E. Besser. 2014. Safety and Immunogenicity of a *Mycoplasma ovipneumoniae* Bacterin for Domestic Sheep (*Ovis aries*) PLoS ONE 9(4): e95968. doi: 10.1371/journal.pone0095698.

ii. IHA: (Besser et. al. 2008). This method is not currently being offered commercially.

b. PCR

Sample collection, handling and shipment

i. Samples are collected by using sterile Dacron swabs with a plastic shaft (wood and cotton my leach some bacteria and *Mycoplasma spp*. growth inhibitors and may interfere with identification techniques like PCR). Samples are collected by gently inserting the swab deep into each nostril while slowly turning the swab. Care should be exercised not to touch the outside of the nares during sampling.

ii. The swab should then be placed into transport media (e.g. Port-A-Cul (modified Amies), or a Mycoplasma enrichment media (e.g. Hardy Diagnostics R102, hardydiagnostics.com/ 800-266-2222) or TSB/glycerol (Hardy Diagnostics D02). Insert swab about 1" into media and break off stem, replace cap on tube. **Check with the laboratory where you plan to submit the swabs to determine transport media and sample handling preference**

iii. Keep samples cool (4^{o}C) during collection and shipment to the laboratory. Samples should be received by the laboratory within 48 hrs after collection.

Samples collected in TSB/glycerol should be frozen and then can be held frozen indefinitely. Samples need to remain frozen during shipment which usually requires shipping on dry ice.

2) <u>Laboratory Diagnostics</u>

a. <u>PCR</u>:

At this point in time no single culture and/or PCR technique is being used consistently by the regional diagnostic laboratories. Below is a listing of the most common PCRs used as of 2014.

i. Mcauliffe, L., F. M. Hatchell, R. D. Ayling, A. I. M. King, R. A. J. Nicholas. 2003. Detection of *Mycoplasma ovipneumoniae* in Pasteurella-vaccinated sheep flocks with respiratory disease in England. Veterinary Record 153, 687-688

ii. Weiser, G.C., M.L. Drew, E.F. Cassirer, and A.C.S. Ward. 2012. Detection of *Mycoplasma ovipneumoniae* and *M. arginini* in Bighorn Sheep Using

Enrichment Culture Coupled with Genus- and Species-Specific Polymerase Chain Reaction. Journal of Wildlife Diseases 48(2) 449-453

iii. Mcauliffe, L., R.J. Ellis, J.R. Lawes, R.D. Ayling and R.A.J. Nicholas. 2005. 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species**.** Journal of Medical Microbiology 54(8) 731-739

b. Strain typing:
Research is just beginning in this area to determine the significance of strain types. If interested, we recommend that you contact Dr. Tom Besser at Washington State University (tbesser@vetmed.wsu.edu)

3) Dead animals

a. Tissue choice – Lung or swab from the tympanic bulla or sinus
b. Nasal swabs

Samples should be handled similarly to swabs from live animals and tissue can be submitted fresh or frozen for PCR. Tissues should also be formalin fixed for routine histology (see appendix E - Standardized Necropsy Protocols).

4) Archived tissue blocks

a. PCR on archived tissue blocks to confirm presence of *M. ovipneumoniae*. If requested strain typing can also be conducted (we recommend that you contact Dr. Tom Besser at Washington State University (tbesser@vetmed.wsu.edu)

Proficiency Testing:

In 2014 proficiency testing was completed for *M. ovipneumoniae* in the form of a ring test. The results of the test can be reviewed at (**need to provide link)**

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APPENDIX E: NECROPSY PROTOCOL FOR STANDARDIZED SAMPLE COLLECTION

Necropsy/Histopathology Proposed Protocol

1. Field Necropsy (when entire carcass cannot be retrieved from field)
    a. Training
        i. Video: Several videos exist for general necropsy procedures. One good general necropsy video is posted on youtube through Cornell University (may require permission to reproduce): http://video.vet.cornell.edu/virtualvet/bovine/chapters1-4.html
        ii. Handbook/field manual of images to be included in a "BHS field necropsy kit". One example of a bighorn sheep field necropsy manual is provided here (BHS FIELD NECROPSY MANUAL_CPW). No permission needed to reproduce.
    b. Field Necropsy Standardized Protocol
        i. Standardization of field observations
            1. Animal identification
            2. Body condition score (poor, fair, good, excellent) – based primarily on observations of omental fat
            3. Photos of lungs
            4. Percentage of lung fields affected by pneumonia
            5. Presence/absence of pleuritis and/or polyserositis
            6. Presence/absence of otitis interna, or pack head out for processing at lab
            7. Presence/absence of sinusitis or sinus tumor, or pack head out for processing at lab
        ii. Standardization of tissue collection for histopathology and archival – tissues saved in formalin until processed, blocks archived indefinitely
            1. Up to 4 sections of lung
                a. Pneumonic lung (or right cranioventral lobe)

b. Interface between pneumonic and non-pneumonic lung (or right middle lobe)

c. Grossly normal lung (or region of right mainstem bronchus)

d. Lungworm (*Protostrongylus*) nodule or dorsocaudal lobe if no nodule palpable.

2. 1 section each of liver, spleen, thymus, kidney, heart

3. Any additional grossly affected tissues

iii. Standardization o f tissue collection for ancillary diagnostics and archival – if archived, at least -20°C

1. 4 sections of lung, matched with formalin sections above

2. 1 section of liver (for possible trace mineral analysis or microbiology/molecular diagnostics for systemic diseases)

3. 1 section of spleen (for possible diagnostics of lymphotropic or erythrocyte-associated viruses, prions, etc.)

4. Any additional tissues as seen fit, or as recommended by agency, for example whole lungs, upper respiratory tract tissues, tonsil

iv. Standardization of swab collection

1. Swab of tympanic bullae in portacul

2. Optional ear swab for *Psoroptes* mites

3. If carcass is fresh enough for culture (48 hrs between death and delivery of samples to lab) fresh tissues listed above and refrigerated are preferable to swabs

4. Any additional swabs as seen fit, or as recommended by agency, for example tracheal swabs, bronchial swabs

2. Diagnostic Laboratory Necropsy (Performed by pathologist when entire carcass can be retrieved)

a. Necropsy performed as seen fit by pathologist

b. Diagnostic Objectives

i. Standardization of remarks

1. Include interpretation for possible role for leukotoxigenic bacteria (leukocytolysis/oat cells)

2. Include interpretation for possible role for *Mycoplasma* species (lymphocytic cuffing, bronchiolar epithelial hyperplasia, alveolar histiocytosis)

3. Include interpretation for possible role for non-leukotoxigenic bacteria (suppuration without leukocytolysis)

ii. Determination of geographic variation in pathology versus variation in lesion interpretation – slide swap between pathologists of various regions is recommended.

3. Research Objectives

a. IHC assays

i. *Mycoplasma ovipneumoniae* IHC

1. No currently offered as a diagnostic assay for bighorn sheep. Polyclonal antibody from rabbit has been validated and appears to be specific to *M. ovipneumoniae*, with no cross reaction with multiple other species of *Mycoplasma* (Ettorre et al. 2007).

ii. *Pasteurellaceae* IHC

1. *M. hemolytica* IHC commercially available in Canada

a. Methods published (Haines et al. 2004)

b. Monoclonal antibody against P12/D6/D5 has been tested for specificity only against *Mycoplasma bovis* and *Histophilus somni*

c. Antibody is not commercially available

d. No other *Pasteurellaceae* IHC assays are currently available.

b. PCR assays for tissues

i. *Mycoplasma ovipneumoniae* PCR direct from tissue (no culture) is commercially available; primers are published (McAuliffe et al. 2003). Real-time assay(s) are in development.

ii. Leukotoxin PCR direct from tissue (no culture) is not commercially available, although methods and validation are published (Fox et al. 2015), real-time assay(s) are in development, and primers designed for use in identifying cultured organisms have been used for detection in tissue (Besser et al. 2014).

References

1. Besser TE, Cassirer EF, Potter KA, Lahmers K, Oaks JL, Shanthalingam S, Srikumaran S, and Foreyt WJ. 2014. Epizootic pneumonia of bighorn sheep following experimental exposure to *Mycoplasma ovipneumoniae*. PLoS ONE 9(10):e110039. doi: 10.1371/journal.pone.0110039

2. Ettorre C.,Sacchini F, Scacchia M, and Della Salda L. 2007. Pneumonia of lambs in the Abruzzo region of Italy: Anatomopathological and histopathological studies and localisation of *Mycoplasma ovipneumoniae.* Vet Ital 43(1): 149-55.

3. Fox K.A., Rouse NM, Huyvaert KP, Griffin KA, Killion HJ, Jennings-Gaines J, Edwards,W.H.Quakenbush, S. L., and Miller M. W. 2015. Bighorn sheep (*Ovis **c**anadensis*) sinus tumors are associated with coinfections by potentially pathogenic bacteria in the upper respiratory tract. J Wildl Dis (in press).

4. Haines, D.M., Moline KM, Sargent RA, Campbell JR, Myers DJ, and Doig PA. 2004. Immunohistochemical study of *Hemophilus somnus, Mycoplasma bovis, Mannheimia hemolytica*, and bovine viral diarrhea virus in death losses due to myocarditis in feedlot cattle. Can Vet J 45(3):231-4.

5. McAuliffe L, Hatchell FM, Ayling RD, King AI, and Nicholas RA. 2003. Detection of *Mycoplasma ovipneumoniae* in Pasteurella-vaccinated sheep flocks with respiratory disease in England. Vet Rec 153(22):687-8.

APPENDIX F: List of available documents including field necropsy protocols and capture data sheets can be found on the following websites WAFWA sites:

Wild sheep working group
https://www.google.com/#q=wafwa+wswg

Wildlife Health Committee
http://www.wafwa.org/html/wildlifehealth.shtml