Appendix E: Working Group C—DISEASE OUTBREAK INVESTIGATION

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Facilitator:	*Michael Ziccardi*
Recorder:	*Heather Harris*
Participants:	*Tom Besser*
	Robert Briggs
	Sharon Hietala
	Hon Ip
	Hailu Kinde
	Howard Lehmkuhl
	Kathy Potter
	Mike Sharp
	Glen Weiser

Goals:

1. Disease outbreak diagnostic protocol
2. Pros/cons of existing diagnosis for outbreak
3. Research needs for diagnostic tools

Ben Gonzales Intro:

We need to collect the right data and determine the correct tools for diagnostics. Use the list of questions to stimulate thought.

Question 1—Value of monitoring healthy sheep herds to have baseline data

- Glen: Good for translocations, data subject to interpretations, varies by season, different results for different labs. Need to standardize the methodology for

bacterial cultures, microarrays for bacterial identification, best work right now to do a lot of presampling, may be able to reduce the quantity of samples…use epidemiology sampling to determine the statistically significant sample size rather than continuing to bulk sample.

- Ben: Want to see more standardization, western wildlife coop of state wildlife vets attempts to standardize protocols. Is serology testing comparable from different labs. CA diagnostic lab is financially the best for samples from California.
- Leslie: We should decide one place to send samples for specific tests so results will be consistent, different labs are different
- Mike: Important to determine how many sheep we are talking about
- Numbers of sheep vary by state and location. Small pops in southern CA identify lambs to ewes, endangered populations so can't handle lambs. Mojave small islands of habitat 60-120 sheep, mark and identify samples from sheep.
- Kathy: Hell's Canyon lambs not identified to ewe, running with pack of ewes.
- Tom: Sampling depends on goal…sampling for all upper respiratory flora in a population is impossible. Serosurveillance important for retrospective analysis, so important to bank as much as possible. How much resources to put into testing
- Hon: Microflora will always change, more longitudinal data can only help, microflora may change by proportion hourly and daily in same animal, good to look at long terms trends of isolates.
- Glen: Montana sampling tested all bighorn herds and some percent of domestics based on statistics. Need to agree what portion of samples will be tested per area, methods of sampling.
- Ben: Power calculation changes for each disease based on prevalence of that specific disease, so not the same for all sampling

Question 2—Post-mortem evaluation of live sick animals of greater diagnostic value than of mortalities recovered from the field, ethical considerations:

- Important to do, essential to determine disease, necessary to justify to permit agencies, all in agreement scientifically but may have issues ethically with endangered species.
- Ben: impossible to detect early stages of pneumonia clinically in wild population, but major political issue in CA with animal rights groups. This is herd health; individual may sometimes have to be sacrificed for the good of the herd. San Jacinto Mountains have such small population, hard to sample from that with few number of lambs.
- Ethical considerations- focus efforts on healthy pops with the most animals, not from endangered population unless necessary, easier to sacrifice lambs that will likely die anyway
- Leslie: Asking Ben if he ever treats sick lambs in the field, Ben responds that only in specific situations with small isolated pops. Desert herds often disappear after being reported sick, likely d/t scavenging.

Question 3—What additional diagnostic tools currently available in domestic livestock and human medicine that would be useful for?

- Mike: Microarrays, real challenge is getting samples in good condition, timing is critical with heat of the desert, should be thinking of ways to preserve samples in the field.
- Glen: Standard protocol, transport media, process immediately, plate, and archive samples in broths for future diagnostics. In Nevada, sympatric bighorn and domestic sheep were tested and a few organisms were found shared, but no outbreak occurred. In another instance, a few shared organisms were found in a domestic goat and three bighorn sheep found within 30 yards of the goat. An outbreak occurred in bighorn, but the shared organisms were not found in any of the other bighorn sheep, indicating that the shared organisms were probably not involved in the outbreak itself.
- Ben: Base camp operations, process animals and fed-ex samples the next morning vs. field crews processing on site with ice packs for samples, often samples are ruined
- Liquid nitrogen tank in field for immediate freezing. Swabs in glycerol tubes in the freezer able to get Pasteurella from them. Glen says that you do lose a lot of P. haemolytica with that method
- Hon: Expression arrays, good necropsy samples, immune status, genetics, nutritional status based on body condition, universal pathogen detection arrays snapshot everything to see what is present in sample, may identify things that are not culturable that could be missed. Wants to hear about what WA is doing. If the question is microbial flora, typical samples are swabs, lavage, get isolates. Viral- can use the same sample.
- Mike: With sick animals can control the situation, euthanize so in fresh condition, histopathology is the best tool and blocks are there for later DNA extraction, full necropsy to determine gross changes. Was concerned that young lambs did not have a thymus, important to look at the entire animal, not just the lung. Dead animals may not be in good postmortem condition for sampling.
- Tom: Virus isolation, get cell lines for potential for that. Also mycoplasma…need to get type strains to the appropriate lab, standardized to one lab is ideal.

Question 4—What potential diagnostic tools not currently available that would be valuable to determine the root causes of respiratory disease and what research is necessary to develop these tools?

- Glen: Hon and Glen have previous proposal for diagnostics but may be prohibitively expensive.
- Mike: Viruses have small window of opportunity to isolate virus, may still have nucleic acid and protein available, but this is unknown at this time.

Question 5—What diagnostic protocol would provide conclusive evidence of disease transmission from newly introduced bighorn sheep to domestic sheep

- Tom: Identical strain types would be strong evidence of the source to make link, but doesn't tell which direction of transmission.
- Hon: If evidence that domestics (reservoir flock) have diverse array of pathogens types and adjacent bighorn have one of those, may indicate a link.
- Glen: Many instances of some shared pathogens between domestic sheep and adjacent bighorn during outbreaks, but no proof of direction of transmission. Data is not available but anecdotal evidence, story is compelling.
- Robert: Marked strains used in cattle to prove transmission, put them in domestic flocks and prove that it transferred to bighorn. Can do with culture dependent or culture independent methods. Skeptical that hemolytica or trehalosi convert to pathogenic strains, sheep getting clinically sick from stress and other factors. Disease identification is usually late in the disease process so wants to know what is going on before that.
- Ben: Different environments will cause expression of different genes in pathogens. Concerned about culture methods not picking up the bugs, so probably need other methods.
- Howard: Pasteurella is commensal in nasopharyx but pathogen in the lung, overwhelmed innate immune system.
- Tom: Agrees that transmission studies are good and useful, much is known about the lab setting of putting domestics together with bighorns in a pen, but not the same situation in the wild. Molecular epidemiology to track a pathogen direction for transmission.

Question 6—Should specific tests be performed at one lab for consistency?

- Happens in the UK and US, standardized tests for quality control that are sent to all labs. Time to lab may be more important than which lab it goes to. Would be good to identify which labs are single sourced.

Question 7—How to make protocol as simple as possible to allow compliance at all labs that perform necropsies on BHS, simple list of tissues to be sent to clearing house for division and shipment with funding for shipping to appropriate labs?

- Increases time to the lab is concern
- Models with zoo communities like elephants
- Difference with technician help in labs, complicated protocols are a problem with the labs

Spreadsheet for Ben and Leslie for diagnostic protocols:

Want participants to add, looking not only at bugs but also nutritional status, etc. things that could affect disease. Assume the animals were live sick and euthanized in fresh postmortem condition. Would also want to do this sampling for healthy animals to serve as controls.

- Need to be able to take live animals for fresh samples, carcass collection has very limited value depending on condition of the carcass.
- Important to search for the etiology of respiratory disease, can still implement management changes while we continue research efforts to identify the cause.
- Workshop goal is to increase the level of scientific inquiry on this subject, circumstantial evidence is strong but need scientific evidence to prove the need for management changes.

Necropsy:

- Tissue block is great resource for future studies, can extract DNA at any time. Full histopathology is important
- Add middle ear, tympanic bullae
- Sheep: ileocecal valve in intestine sample, ileocecal LN, mesenteric LN, bronchial LN
- Tissues are stored in paraffin blocks, archived in each lab, may be worth considering storing the blocks all together somewhere but in US no national diagnostic system, labs reluctant to part with samples
- Website for virtual archive of where the blocks are stored, people can ask for material of interest with independent group to administer
- Freezer space is big issue, also with formalin fixed tissues are disposed of after 3-4 years.
- Madison NWHC seals blocks for long term preservation, issues with rodents and high temperatures affecting
- Would be good to have cryo-preserved lung tissue for long term studies, but would be difficult for field situations to have liquid nitrogen available
- Thymus: theory for lambs not having a thymus would be high fetal/perinatal stress causing atrophy or virus causing aplasia. Thymus should be easily identified at necropsy by pathologists and biologists. Should be looked at histologically.

Bacteriology:

- Nasal swab for aerobic culture for Pasteurella has been shown to be less ideal than a nasopharyngeal swab. Tracheal swabs similar to oropharyngeal swabs unless active pneumonia and then they are more similar to lung. Oropharyngeal is the best. If have lung, don't need the tracheal swab. Oropharyngeal swabs collected with a speculum in mouth so can directly sample the pharynx and

tonsils, better than a nasopharyngeal swab that touches all the nasal flora before it reaches the pharynx.

- Should be stored at -70, not -20.
- Mycoplasma culture is as important as molecular techniques, difficult to culture and issues with the best media and techniques, every lab has been missing some. Glen, Tom, and Leslie to work together on that. For sick animals, most important to find it in the lung and maybe one upper respiratory site. Would be good to archive swabs until an experiment is ready for PCR, take 2 swabs and freeze one for later PCR as a backup option. Again freezer space is a big issue for everyone. Tracheal samples and bronchoalveolar lavage not as useful as lung, but keep in.
- **Centralized sample bank** with request review, needs someone to determine who gets samples, what is being done with them, acknowledgement for agencies and people who collect the data to be included in all published studies. Zoo association for SSPs has advisory board for sample sharing for black rhinos with sparse samples to be shared throughout the US.
- Take one upper respiratory swab (oropharynx) and tonsilar biopsy to be frozen and one lower respiratory swab (lung). Remove nasal swab.
- Chlamydophila should be looked at but lower on priority list, has been isolated from one bighorn lung. Not sure which lab does Chlamydophila PCR
- Ureaplasma PCR available, may be more appropriate as a survey but not as part of primary protocol for outbreak investigation.
- Culture independent methods…archive samples. Scott Kelley or Tom could be doing it in their labs as research but don't have the resources to do as diagnostic lab. Would need to collect an additional sample to be archived…bronchoalveolar lavage has limited host nucleic acids so would be rich in microbiota and could be a good option, same techniques not applicable to lung tissue, cryo-preservation of BAL sample.
- Anaerobic culture- transition of bacteria in lung before death from commensal anaerobes happens late in disease, difficult to culture in labs. Does the growth of anaerobes contribute to the cascade of inflammation in the lung? Discussion about whether to leave this in the protocol…would be better to do a pilot study and not include in main protocol.
- Priorities are oropharyngeal swab for aerobic culture (Pasteurella) and Mycoplasma culture/PCR

Parasitology:

- Add ears for scabies, important in some populations, one per lesion
- Fecal floats- may be important for certain regions for GI parasites and lungworms. In the past, many fecals have been negative so not critical if no clinical signs.

Toxicology:

- CA- Se and Cu deficiency big issues, important background info for outbreaks, would be important to identify in areas where not already defined to get baseline data
- Tissue storage sites to be determined
- Gamma-globulin kits for domestic sheep reagents have been working for BHS, important to measure. Good predictor of disease before they are sick, but less informative once lambs are sick. Concern about colostrum intake but would need to be catching lambs at 2-7 days. In zoos, couldn't vaccinate lambs because handling them caused the ewe to abandon them.
- Brain cholinesterase deleted from list, only indicated if poisoning or contact with sprayed areas

Serology:

- Heart blood from shot animals usually poor sample quality
- Removed IBR- no indication of IBR in BHS, no cross reactivity with other herpes.
- Chlamydophila serology- very low on the list
- Mycoplasma- ok to use WSU lab (Tom) for research only, test validated by results but not with experimental infection samples
- OPP- low priority, removed
- Adenovirus- virus neutralization at Howard's lab for research purposes but not diagnostics, 10 adenoviruses available
- Should be thinking of the meaning of serology as exposure in the population, surveillance tool but not diagnostic for cause of disease or mortality. Limited sample sizes are an issue so serology is very important as a first step to determine exposures. For example, high adenovirus titers indicate exposure but not sure whether domestic sheep origin, probably contributes to immunosuppression.
- Lepto not an issue in BHS, is an issue in deer.
- Priority for respiratory viruses (BRSV, PI-3, BVD) and Mycoplasma. Adenovirus slightly lower priority if less sample available.

Virology:

- Add tonsil to tissues, remove brain.
- Add buffy coat- PCR for herpes viruses, valuable sample in euthanized animals
- Howard: Adenovirus can be isolated and typed out at his lab, PCR primers available, can also do serology
- Fluorescent antibody (FA)- IHC is better because easier to read and get permanent record of results.
- CE- sporadic outbreaks in the desert, do EM for CE as indicated for gross lesions

- Culture-independent techniques- sample depends on method, use mass-spectrometer to look at viruses, definitely limited to defined research project at this time.
- Priority is virus isolation using swabs in transport media, other virology as indicated. Non-culture based methods as research but not diagnostics

Long-term storage:

- Add BAL and retropharyngeal LN to list of tissues for freezing
- Add pool of abdominal and pool of respiratory organs

Spreadsheet conclusions:

- Ben: need to put together a protocol for necropsy techniques available to field people from this spreadsheet.
- At this point, these spreadsheet tissues should be collected, whether or not they are run.
- Priorities set for all categories (see spreadsheet for details)

Specific research items to be identified for funding purposes:

1. Virus isolation:
   - Concern about non-cytopathic viruses that are not being detected, collection and transport methods and available cell lines and cell strain, primary low passage cells needed. Viruses have been short changed with BHS b/c of sample preservation in the field that will be useful for isolation.
   - Concern about pooling of samples by labs (NVSL) for virus isolation and of disposing of isolation after 7 days if no growth…requires attention and time. Not enough effort has been put on virus isolation for BHS yet and needs to be a focus.
   - Case definition is important for VI if outbreak situation with early clinical signs, need to capture and sample apparently healthy lambs that may be shedding virus for this technique to be effective. VI is less of a priority with sporadic mortalities.
   - Hon from NWHC would like to get funding to establish new primary cell lines and is willing to share these, need liquid nitrogen shippers to preserve samples properly in the field.
   - Very important to gather this baseline information to examine a link between BHS and domestic sheep
2. Non-culture based methods:
   - Genomics/ proteomics to look at gene expression, need to look for a lab to collaborate with that already has an established system. Individual response stages can affect results too. Identify specific biomarkers to find correlates for disease and infection. Doesn't matter what the biomarker is as long as there is

good correlation with disease state, a form of molecular epidemiology. Compare against a healthy population to look for upregulation associated with a die-off.

- Bovine immune microarrays are available, also host immune response arrays being developed for ruminants.
- Must be in the window for detection as with virus isolation so timing is critical, should be getting live animals early in disease. Go to population with most consistent occurrence of disease to get samples.
- Longitudinal study to look at when maternal antibody is declining is the key time period
- Capture of lambs is more difficult than older age groups, 6 weeks is median age for pneumonia but mothers abandon them if handled. It will be important to collect and necropsy lambs in an outbreak. Study design is very important for appropriate sample size and statistical power. Darting lambs is difficult, would use netguns, but high risk with lambs breaking legs and under high public scrutiny with endangered species.

3. Interest in preserving Pasteurellacea collection in BHS (Lehmkuhl) Glen Weiser mentioned this as the collection is at the Caine Veterinary Center in Idaho.
   - NIH has collection of preservation but not big enough or of broad interest
   - 13,000 isolates over 20 year period of BHS, elk, deer
   - Would require 2-3 large freezer chests securely wired and protected with money for maintenance

Topics to present:

1. Ben to present why it is important to “chase the bugs” causing respiratory disease in BHS. Tom thinks it is unlikely to lead to dramatic breakthrough for management but Mike disagrees b/c knowing the agent can help target the appropriate response to that specific pathogen. If we believe the Pasteurella and Mannheimia are causes, could do interventions with vaccines experimentally but would be difficult to do with free-ranging population. When thinking of translocation issues and captive herds, need to identify a good source population by using basic principles of history of disease and serology. Must have ways to identify a “healthy herd” based on science. Translocation very important because small populations are more likely to be wiped out with outbreaks. Preventive medicine is so important to prevent outbreak because there is little that can be done once an outbreak occurs. Policy makers need the definitive science to make decisions.
2. Go through the questions
3. Go through the spreadsheet…emphasize importance of collecting live animals during an outbreak for necropsy.

	Tissue	Diagnostic Tools (existing)	Special Instructions	Priority	Where	Diagnostic Tools Wish List	Future Help Needed to Address:
Necropsy							
	carcass	gross observation of all organ systems					
Histopathology							
	Brain, heart, liver, kidney, lungs (all lobes), spleen, small and large intestines (all sections), bronchial lymph nodes, ileocecal lymph node, mesenteric lymph node, forestomachs, abomasum, thymus, bone marrow, adrenal gland, skeletal muscle (thigh), diaphragm, trachea, esophagus, nasal turbinates, reproductive organs, spinal cord, sciatic nerve, thyroid, retropharyngeal lymph node, tonsil, ear (inner, middle)		website - location of archived tissues, preservation of blocks	1			
Bacteriology							

	Lung	**Aerobic culture**	Pasteurella biotyping (stored at -70)	1	University of Idaho		nonculture-based methods
	oropharynx/tonsil	**Aerobic culture**	Pasteurella biotyping	2			
	liver	**Aerobic culture**		2			
	Lung	**Mycoplasma culture/ PCR**		1	WSVL		
	oropharynx/tonsil	**Mycoplasma culture/ PCR**		2	WSVL		
	Colon	**Salmonella culture**		Per lesion			
	lung	**Chlamydophila PCR/culture**		3	????		
	lung, BAL	see wish list	-70C/cryopreserved	3		culture-independent methods/Besser/Kelly - research	
Parasitology							
	feces feces ears	Fecal flotation for GI parasites Baermann (lungworms) scabies		1 (regional) 1 (regional) Per lesion	? ?		
Toxicology							
	As indicated in the history	Heavy metal screen	Save back fat, brain, liver, kidney for storage (future projects)	1 (regional)	Wyoming State Vet Lab/CAHFS		Need central tissue storage site
	As indicated in the history	**Other toxicology tests**					
Serology							

	Body cavity fluids (pleural, peritoneal) or blood	Anaplasmosis CARD		2	CAHFS		
		BTV ELISA	If positive, test for BlueTongue PCR/ virus isolation	2	CAHFS		
		EHD AGID		2	CAHFS		
		PI-3 HI		1			
		BRSV IFA		1			
		CE, CF		2	NVSL		
		Chlamydophila CF		3	NVSL		
		BVD Type I & II SVN		1	CAHFS		
		BT ELISA		2	CAHFS		
		Adenovirus SVN		2	Lehmkuhl (research)		
		OPP	Low priority	3	?		
		Mycoplasma HI		1	WSU (research)		
Virology							
	lung, spleen, nasal swab, tonsil, buffy coat	virus isolation	In viral transport media stored at -80	1 (early clinical time course; condition of carcass); 3 dead	Wyoming State Vet Lab, Lehmkuhl		**primary low passage cell lines, transport, storage**
	Lung	FA/IHC (BRSV, PI3, BVD)		**Per lesion**	CAHFS, WADDL		
		PCR (adenovirus, BT, EHD, MCF, PI3, BVD)		**Per lesion**	**WSVL (adeno, MCF, EHD, BVD, BT, PI3)**		

	As indicated	EM (CE)		**Per lesion**	CAHFS, WADDL		
		Non culture-based methods	Microarray-based technology	3			
Long-Term Storage							
	Liver, kidney, lung, BAL, lymphoid tissue (retropharyngeal LN), resp & abdominal organ pool		Freeze immediately at -70degrees. Ship frozen. Do not save rumen content or brain unless case justifies	1	storage??? ???		
Live Animal Protocol							

Bacteriology	oropharyngeal swab	aerobic culture	Pasteurella biotyping	1	Idaho
		Mycoplasma culture/PCR		1	WSVL
		Chlamydophila PCR		3	???
		culture-independent methods		3	WSU - Besser (research), Kelly (research)
Serology	serum	Anaplasmosis CARD		2	CAHFS - Hietala?
		BTV ELISA		2	
		EHD AGID		2	
		PI-3 HI		1	
		BRSV IFA		1	

		CE, CF			2	
		BVD Type I & II SVN			1	
		BT ELISA			2	
		Adenovirus SVN			2	
		Mycoplasma			1	
Virology	oropharyngeal swab	virus isolation			1	Lehmkuhl, Hon Ip, WSVL
		PCR (adenovirus, BT, EHD, MCF, PI3, BVD)			1	WSVL
		Culture-independent Techniques	microarrary-based techniques		3	WSU - Besser (research)
Toxicology	whole blood	heavy metals/selenium		1 (Per region)		WSVL, CAHFS, WADDL
	serum	heavy metals/selenium		1 (Per region)		
Parasitology	feces	fecal flotation		1 (Per region)		??????
	feces	Baermann		1 (Per region)		
	ear scrape	scabies		1 (Per lesion)		
Clinical pathology	whole blood	CBC				IDEXX??? ???
	serum	serum chemistry				IDEXX??? ???