

WILDLIFE CONCERNS

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Brief explanation of the study:

M. bovis, *Mycobacterium bovis* is the causative agent of bovine tuberculosis. This bacterium causes economic loss to the cattle industry from decreased production and trade restrictions. Affected animals experience chronic weight loss, variable appetite, and fluctuating fevers. Respiratory signs are common but are often subtle and mild. Abnormal lung sounds and breathing difficulties are obvious only in the terminal stages of the disease. Tuberculosis may also cause enlargement of other lymph nodes throughout the body, causing bloat, intestinal ulceration, and/or diarrhea.

Mycobacterium bovis is transmissible to humans, causing significant respiratory and/or systemic disease. The enlargement of lymph nodes causes clinical signs similar to those seen in ruminants. People that are in contact with animals in the dairy industry or in slaughter houses, are especially at risk. Hunters and animal care personnel that are in contact with infected free-ranging or captive wildlife may also be at significant risk.

The U.S. Department of Agriculture has been actively pursuing eradication of *M. bovis* for almost a century and has established a goal of total eradication in the next few years. However, eradication will be markedly delayed by slow and inaccurate identification of infected animals and the inability to differentiate strains of *M. bovis*. These problems are accentuated by the presence of *M. bovis* in captive and free-ranging wildlife.

Surveillance:

In 1990, epidemiological and diagnostic investigations revealed extensive bovine tuberculosis infection in a herd of 50 captive elk at a game ranch near Powderhorn, Colorado.⁸ These animals were subsequently depopulated and at least 60% (25/36) of the resident elk > 2 years of age were infected with bovine tuberculosis. Free-ranging mule deer were known to have inhabited the same enclosure as the captive elk and were likely to have been moving in and out of the facility. There was also a substantial amount of fence-line contact between captive and free-ranging elk. Due to the concern for potential spread of *M. bovis* to wildlife, tuberculosis surveillance was initiated. From 1990 to 1993, hunter-killed, free-ranging elk and mule deer populations in the Powderhorn vicinity were examined for evidence of *M. bovis* infection. Surveillance was accomplished by collection of lymph nodes and tonsils for culture and histopathology. Of roughly 200 elk and mule deer sampled from 1990 to 1993, none were determined to be infected with bovine tuberculosis.

Since 1993, no further investigations had been performed. An epidemic model (Miller, et al., 1990) suggested that it would be unlikely that tuberculosis would be detected so soon after introduction into free-ranging herds. This model further indicated that it was unlikely (< 20%) that tuberculosis would have become established in the wild cervid population at all. On the 1 in 5 cases where it did become established, the model predicted that prevalence would not reach detectable levels (about 1%) until 5-10 years after the introduction of the disease. These models indicated that 1997 and 1998, would be reasonable times for surveillance of free-ranging cervid populations in the Powerhorn area.

Diagnosis

Currently, diagnosis of *M. bovis* infection is accomplished by intradermal skin tests, followed by definitive diagnosis via post-mortem culture and histopathologic examination of suspected lesions. While specificity and sensitivity of these skin tests have been determined in domestic cattle and captive cervids, their utility in other species (i.e. horses, rhinos, non-domestic bovids, etc.) is questionable at best. Destruction of animals with "suspect" reactions is neither politically or ethically feasible, especially since the validity of these tests (in these species) is highly questionable. Many of these animals are endangered or threatened in the wild and it is difficult to justify their destruction based on the results of unreliable tests. Therefore, alternative technologies are being developed in order to improve diagnostic capabilities in live animals.

In some instances, composite ELISA's (enzyme-linked immunosorbent assays) have shown considerable promise for diagnosis of *M. bovis* in captive cervids and other non-domestic hoofstock. In New Zealand, an ELISA developed by Griffin, et al. (1991), utilized serum antibodies to purified protein derivative-B (PPD-B) or PPD-A on a variety of mycobacterial peptides considered to be specific for *M. bovis*. The *M. bovis* specific protein (MPB70) has also been used to improve specificity of the ELISA assay for TB diagnosis without compromising sensitivity. Using this composite ELISA, sensitivity of 86% and specificity of 98% has been obtained. At Colorado State University, a composite ELISA, using PPD, culture filtrate, two lipoarabinomannan antigens from the virulent Erdman and virulent H37Ra strains of *Mycobacterium tuberculosis* has been developed.

Detection of tuberculosis infection via lymphocyte transformation assays (LT) has also been studied. This test has been reported to be highly sensitive and specific, with some tests demonstrating a sensitivity of 95% and specificity of 92% for diagnosis in infected herds.¹⁸ However, this method is very expensive and is somewhat difficult to use under field conditions. Samples must be carefully preserved and shipped in order for diagnostic results to be obtained. This factor, along with the test's considerable expense, has limited the usefulness of this diagnostic modality. LT is of particularly limited utility in epidemiologic investigations of tuberculosis infection of free-ranging wildlife as most such investigations rely on samples from non-living animals.

The results of LT and ELISA may be combined to produce a composite laboratory assay termed the blood test for tuberculosis (BTB). This combined test has reported excellent

specificity (92%) and sensitivity (96%). A relatively high positive predictive value of 79.5% has been reported in a sample of 156 infected animals. However, once again, because of the delicate nature of the sample submission for LT, the BTB is of limited utility. This test has only been evaluated in captive cervids (specifically red deer (*Cervus elaphus*)) and has not yet been validated for other non-domestic species.

Because of the expense and limitations of other tests, ELISA analysis should continue to be investigated. Our tuberculosis research team at Colorado State University's Center of Veterinary Epidemiology and Animal Disease Surveillance Systems (CVEADSS) has studied the use of ELISA for detection of mycobacterial infection. The research team continues investigation into the utility of the ELISA for serologic detection of tuberculosis infection. This ELISA uses a protein A /protein G mixture as conjugate for antibody binding. This conjugate may be used for a broad range of host species and is not species-specific; therefore this ELISA is particularly useful and applicable to the study of tuberculosis in non-domestic species.

M. tuberculosis

Tuberculosis caused by *Mycobacterium tuberculosis* has historically been a disease primarily of humans and non-human primates. However, recent occurrences of disease in captive elephants, illustrate the importance of this disease in non-primate species. Extensive guidelines have been created for testing and surveillance of *M. tuberculosis* complex in captive elephants. These guidelines recommend the investigation of the ELISA for antemortem diagnosis of *M. tuberculosis* infection, in a general effort to improve strategies for diagnosis and management. Diagnostic tests (such as ELISA) for detection of *M. tuberculosis* infection in human beings have been developed. However, these tests are species-specific and are not uniformly applicable to non-human species. For this reason, the development and evaluation of non-species-specific tests needs to be performed. It is important to evaluate the utility of the ELISA for detection of this disease. The ELISA shows great potential for detecting *M. tuberculosis* infection in non-human species as it does not require species-specific antibodies (or other species-specific components) and can be adapted for different *M. bovis*-complex organisms.

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Projects:

Project 1:

A Two-Year Survey for Bovine Tuberculosis (*Mycobacterium bovis*) in Hunter-killed, Free-ranging Elk (*Cervus elaphus nelsoni*) and Mule Deer (*Odocoileus hemionus*) in Southwestern Colorado.

Elk and deer heads have been collected from hunters harvesting animals in game management units #66 and #67 in the Gunnison valley of Colorado. Procedures for collection and processing of samples are similar to that reported by Williams, et al. in 1996 for surveillance for tuberculosis in elk in Wyoming. The date, age, sex, subjective evaluation of the body condition, license number, and location (unit number) of the kill are noted for each animal collected. Skin of the head is dissected away; samples that are

evaluated include bilateral lateral retropharyngeal lymph nodes, bilateral medial retropharyngeal lymph nodes, bilateral parotid lymph nodes, bilateral mandibular lymph nodes, and tonsils. Collections have been made during the hunting seasons of 1997 and 1998. Limited license hunters have been contacted via letter with a request for samples. Samples have also been obtained from over-the-counter license hunters via local game processors in Gunnison and Lake City, Colorado.

Lymph nodes are examined grossly from the capsular surfaces and on longitudinal transection. Samples are then serially sectioned for examination for gross lesions. One half of each node is placed in 10% formalin, and the other half is immediately frozen. Histopathology is performed on all formalinized samples with gross lesions. Staining by the Kinyon acid-fast technique is performed on tissues with histopathologic changes consistent with *M. bovis* infection. Mycobacterial cultures are performed on frozen samples of lymph nodes with gross lesions.

Collection for the 1997 and 1998 hunting season have been completed. Usable tissue samples have been collected from the heads of 229 elk and 145 mule deer. Of these samples, 112 had at least one lymph node or tonsil with gross abnormalities.

Samples from all lymph nodes and tonsils from each of these 112 animals was submitted to National Veterinary Services Laboratory for mycobacterial culture. No *M. bovis* nor *M. tuberculosis* has been cultured from these samples. Histopathologic evaluation of these samples has begun, but has not been completed.

Histopathologic examination remains to be completed. However, currently there does not appear to be evidence of tuberculosis infection in the free-ranging herds of elk and mule deer in the area of Powderhorn, Colorado.

Project 2:

Development and evaluation of enzyme-linked immunosorbent assay (ELISA) testing for detection of *Mycobacterium bovis* and *Mycobacterium tuberculosis* infection in non-domestic species.

Serum is evaluated using composite ELISA testing. Protocols for this testing are detailed by Gaborick (1996). Relationships between results from these diagnostic tests and results of histopathology, PCR probe, and culture will be evaluated utilizing the concept of sensitivity, specificity and receiver operating curve.

Initial development and evaluation of the ELISA has been completed. Testing has been completed on over 5,000 cattle and over 1,500 captive cervids. Specificity and sensitivity for cattle, using the discriminant function, were 71% and 95% respectively. For cervids, the specificity and sensitivity were 78.6 and 70.0% respectively.

Collaboration is ongoing with zoos and other professional organizations affiliated with the National Tuberculosis Working Group. A large number of captive elephant, equine, and antelope species samples have been processed using existing ELISA protocols.

Evaluation of the sensitivity and specificity of this test, in these species, is currently being performed.

Collaboration with the University of Saskatchewan has also been instigated in order to evaluate serum from over 300 bison. Determination of ELISA sensitivity and specificity will be based on discriminant analysis and receiver operating curve.

In addition to processing samples using the above ELISA protocol, the following experimental work has been done using serum from known positive and negative cattle: comparison of culture filtrate from *M. avium* vs *M. bovis*; comparison of PPD from *M. avium* vs *M. bovis*; preabsorption of test serum using *M. phlei*; and collaboration with Hemagen, Inc. (Waltham, MA). in testing a new BCG LAM antigen that is under development. Results to-date are still preliminary, however there was no increase in specificity or sensitivity with the use of PPD from *M. avium* nor the use of *M. phlei* for preabsorption. Preliminary results suggest that the use of *M. avium* culture filtrate improves test specificity and sensitivity.

Specificity and sensitivity for *M. bovis* detection in cattle have been improved from 56% and 66% respectively, to 95% and 71% respectively. This has been accomplished by using a discriminative function with four antigens: PPD (*M. bovis*), CF (*M. bovis*), CF (*M. avium*) and NAG (*M. tuberculosis*). To date, this alternative protocol and analysis has only been performed on cattle samples for detection of *M. bovis*. Further processing and analysis is necessary to determine if this will improve specificity and sensitivity for *M. bovis* and/or *M. tuberculosis* detection in other species.

More samples need to be processed and further data analysis needs to be done. Currently, the multiple-antigen ELISA described here appears to be a useful tool in diagnosing tuberculosis infection in captive elephants.

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Future Research:

Project 1: Continued diligence needs to be maintained in surveying free-ranging and captive animals for infection with tuberculosis. Establishment of free-ranging reservoirs of infection will provide profound management difficulties. Hunters should be encouraged to submit all animals with abnormalities or signs of infection for professional examination.

Domestic animals should be diligently monitored at slaughter for signs of infection. Vigilance in monitoring of captive animals must be maintained in order to prevent introduction of tuberculosis into free-ranging wildlife.

Reexamine the Powderhorn area in another 10 years to confirm that tuberculosis was not introduced into the free-ranging herds of elk and mule deer.

Project 2: Further evaluation of alternative diagnostic tests must be done for non-domestic species. Traditional testing procedures (i.e. skin tests) have not proven to be as useful for tuberculosis detection in non-domestic animals. Diagnostic tests need to be evaluated on a species specific basis. It is likely that no single testing methodology will be applicable to all species.

Additional funding for ELISA testing and evaluation. Funding support is needed for evaluation of additional antigens and for processing of additional samples.

Interest and cooperation by individuals or organizations in possession of captive wildlife or non-traditional domestic animals. Only by increasing sample size on animals with definitive diagnosed (with or without tuberculosis infection) can we accurately define the strengths and limitations of our testing methodologies.