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# *Mycobacterium bovis* infection in North American elk *(Cervus elaphus)*

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**Abstract.** A naturally occurring outbreak of Mycobacterium bovid infection in captive wild elk (wapiti) in Montana was confirmed by mycobacteriologic examination. Twenty-eight of 143 elk responded to *M. bovis* purified protein derivative (PPD) tuberculin injected intradermally in the cervical region (SCT). The results of comparative cervical tuberculin skin tests conducted within 9 days of SCT revealed greater responses to *M. bovis* PPD tuberculin than to *M. avium* PPD tuberculin in 23 of 28 elk responding. At necropsy, several grossly visible tuberculous lesions were observed in the parenchyma of the lung, thoracic lymph nodes, and submandibular lymph nodes. Microscopic examination of appropriately stained tissue sections revealed the presence of granulomatous lesions containing acid-fast bacilli. An enzyme-linked immunosorbent assay (ELISA) was developed using a sarkosyl extract of *M. bovis* (antigen) and peroxidase-labeled protein G (conjugate); reactions were detected in the sera of 8 of 9 elk responding to *M. bovis* PPD tuberculin. Lymphocyte blastogenic assay responses were detected using *M. bovis* antigens in 7 of 9 elk positive on skin tests using *M. bovis* PPD.

Outbreaks of tuberculosis have been reported in farmed cervids in the United States and other countries, although the disease has been nearly eradicated from domestic animals and humans. [1,2,5,8,1213,16,23] Failure to eliminate tuberculosis in captive wild animals maintained in farms or animal parks is of concern to animal health regulatory officials because tuberculous animals may serve as foci of infection for cattle, other domestic animals, wild animals, and humans. [10,11,15,18]

The diagnosis of tuberculosis in farmed cervids and other captive wild animals often is based on the results of delayed-type hypersensitivity to tuberculins. [15,20] Purified protein derivative tuberculins (PPDs) prepared from *Mycobacterium bovis* or *M. avium* are widely used. Lymphocyte blastogenic assays (LBA) and other in vitro procedures have been described for detecting cervids exposed to virulent mycobacteria.[4,14] Enzyme-linked immunosorbent assays (ELISA) have been described for detecting anti-mycobacterial antibodies in sera of certain exotic species exposed to clinically significant mycobacteria.[21] Purified antigen preparations extracted form virulent strains of *M. bovis* using deoxycholate, potassium chloride, or sarkosyl provide for improved specificity in an ELISA[19] (C. O. Thoen et al., unpublished).

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## Materials and methods

*Tuberculin skin* tests. The single cervical tuberculin test (SCT) was conducted in elk *(Cervus elaphus)* by injection of 0.1 ml of *Mycobacterium bovis* PPD[a] intradermally in the midcervical region. The injection site was palpated at 72 hr for the presence of swelling. Animals with palpable responses were classified as reactors. The comparative cervical test (CCT) was conducted <9 days following the SCT using biologically balanced PPDs of *M. bovis* and *M. avium*[a] injected at separate sites on the opposite side of the neck. The skin thickness at the injection site was measured before and 72 hr following injection of tuberculin and recorded.

*Lymphocyte blastogenic assays (LBA).* Blood was obtained from elk via jugular venipuncture and placed in siliconized 20- x 150-mm sterile culture tubes containing acid-citratedextrose solution. Blood was suspended in an equal volume of 0.01 M $Na_2PO_4$ buffer containing 0.14 M NaCl, pH 7.2; the cell suspension was layered over ficoll-diatrizoate[b] and centrifuged at 400 x g for 40 min at 22 C. The lymphocyte-rich layer was removed, and the cells were resuspended and washed twice with Hanks' balanced salt solution." The mononuclear cell concentration was determined and the cells were resuspended in minimal essential medium 199 containing 25 mM HEPES,[c] 15% fetal bovine serum, penicillin (100 U/ml),[c] streptomycin (100 mg/ml), and 5 x $10^{-5}$ M 2-mercaptoethanol.[b]

Cells resuspended in this medium or in medium containing indomethacin[b] (5 µg/ml) were cultured alone or incubated with phytohemagglutinin[d] (PHA; 250 or 100 ng/well), *M. bovis* PPD[a] (10 or 20 µg/well), or *M. avium* PPD[a] (10 µg/well) in flat-bottomed microtiter plates[e] (5 x $10^5$ cells in 200

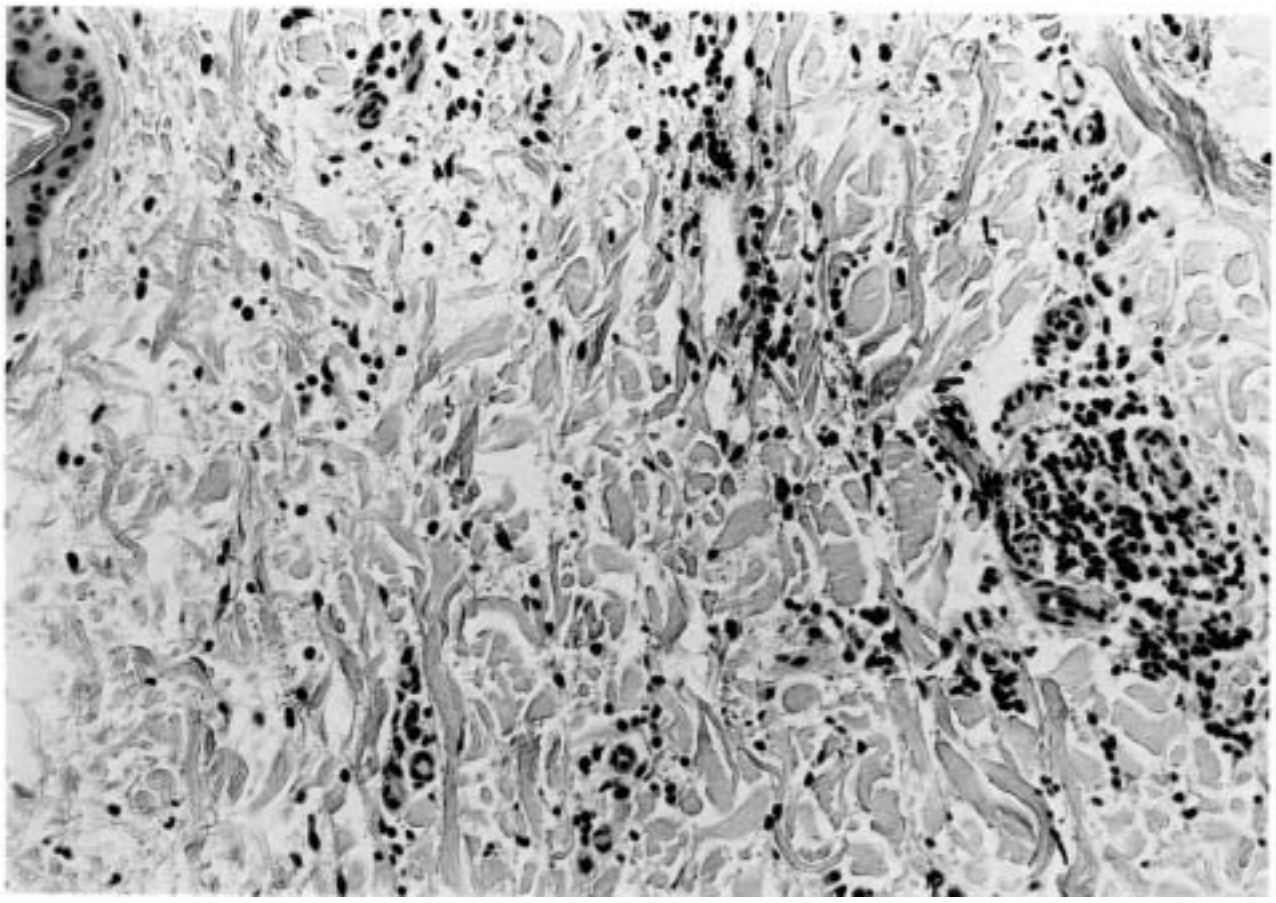

**Figure 1.** Photomicrograph of elk skin biopsy of tuberculin response collected 72 hours following injection of Mycobacterium L&s PPD tuberculin. Numerous mononuclear cells and dermal edema are present. HE, 360 x .

μ1). Cell cultures were conducted in triplicate. Cells cultured alone or incubated with PHA were harvested after 120 hr. Cultures were incubated at 37 C in a 5% CO, humidified atmosphere. Eighteen hours prior to harvest, 1 μCi of H-thymidine was added to each culture. Cells were harvested onto glass fiber filters, using a Skatron cell harvester.[f] Radioactivity was measured in a liquid scintillation spectrometer.[g] The mean counts per minute (CPM) were calculated for the triplicate cultures of the lymphocytes from each animal cultured alone or with each concentration of PHA or *M. bovis*. The PHA or *M. bovis* PPD concentration with the greatest responses from each animal was identified.

*Enzyme-linked immunosorbent assays.* An indirect ELISA was conducted by modification of procedures described previously, using a PPD of *M. bovis,* a sarkosyl (sodium N-lauroyl sarcosinate) extract of *M. bovis,* or a PPD of *M. avium* diluted in 0.1 M $Na_2CO_3$ (pH 9.6)[21] (C. O. Thoen et al., unpublished). An aliquot of antigen (0.05 ml corresponding to 500 ng, 250 ng, 150 ng, and 100 ng of protein) was added to each well of a microtiter plate. Carbodiimide[b] (50 μ1) diluted in 0.1 M $Na_2CO_3$ (1 mg/ml) was added to each well. The cuvettes were placed in plastic bags, incubated for 16 hr at 4 C, and washed 3 times with PBS. Then 0.01 M $NH_4Cl$ (0.1 ml/well) was added to each well, and the cuvettes were washed 3 times with wash solution (0.5 M NaCl containing 0.5% Tween 80, adjusted to pH 7.5 with 1 N NaOH).

Serial dilutions of sera (1:40 to 1:640) obtained from elk were made in ELISA diluent (0.5 M NaCl containing 1% bovine serum albumin and 1% Tween 80,[b] pH 7.5), and 0.1 ml was added to wells of the cuvette. Serum samples were incubated for 30 min at 22 C on a horizontal shaker.[h] The microcuvettes were then washed 8 times with wash solution and allowed to stand inverted for 30 min. Protein G[i] labeled with horseradish peroxidase was added to each well (1:4,000 dilution), and the wells were incubated for 30 min at 22 C on a horizontal shaker. Cuvettes were again washed 8 times and allowed to stand inverted for 30 min. A substrate-indicator solution of hydrogen peroxide and 2,2'-azino-di(3 ethyl benzthiazoline-6-sulfonate) in 0.05 M citric acid (pH 4.0) was added to each well (0.15 ml), and the wells were incubated for 30 min. The color intensity of the reactions was determined at a wavelength of 405 nm.[j]

*Histologic and mycobacteriologic examination.* Specimens of lymph nodes (bronchial, cervical, retropharyngeal, hepatic, and mesenteric), liver, spleen, and lungs collected at necropsy were placed in neutral buffered 10% formalin, embedded in paraffin, sectioned at 5 μm, mounted on glass slides, stained with hematoxylin and eosin (HE), and examined microscopically for lesions. Selected sections were stained for acid-fast bacteria by the Ziehl-Neelsen method.

Tissue specimens collected at necropsy also were placed in a saturated solution of sodium borate for cultural exam-

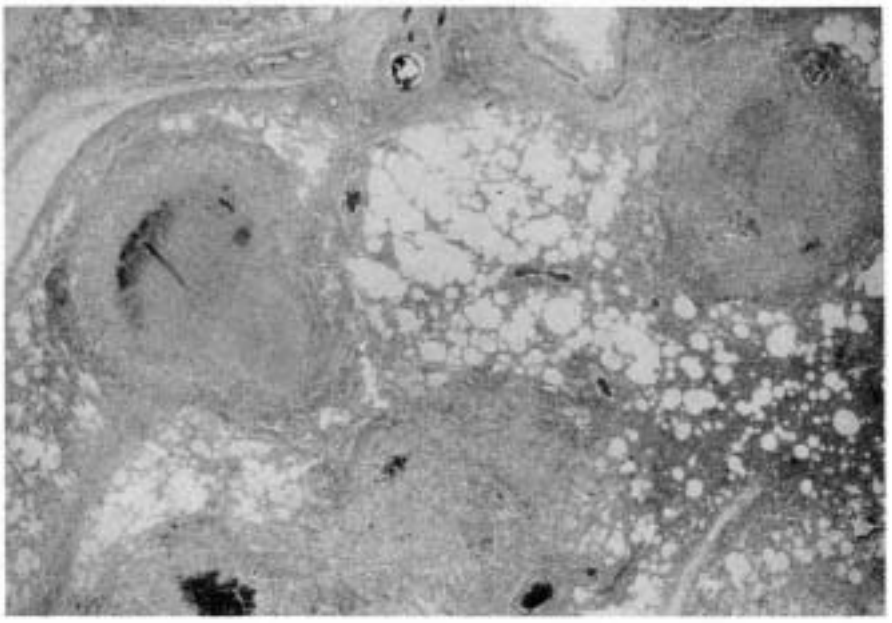

**Figure 2.** Photomicrograph of lung of an elk from which *Mycobacterium bovis* was isolated. Note multiple granulomas, some containing caseation necrosis and mineralization. One granuloma is adjacent to a bronchiole. HE, 63 x . * = ELISA units, mean value.

ination. The tissues were ground and treated with 2% NaOH and inoculated onto culture media as described previously.[17] The inoculated media were incubated at 37 C and observed at 2-wk intervals for growth. Isolates were identified by tests for nitrate reduction, susceptibility to thiophen-2-carboxylic acid hydrazide or streptomycin, and inhibition by 5% glycerol.

## Results

Twenty-eight of 143 elk responded to *M. bovis* PPD tuberculin injected intradermally in the cervical region (SCT). Twenty-three of 28 elk responding on SCT had greater responses to *M. bovis* PPD than to *M. avium* PPD on the CCT. Two elk had greater responses to *M. avium* PPD than to *M. bovis* PPD on the CCT. Responses were not detected in 2 elk, and 1 elk was not tested. NO CCT responses were observed in 6 elk previously negative on SCT.

A skin biopsy was obtained of an *M. bovis* PPD injection site for microscopic examination (Fig. 1). Edema was apparent as separation of collagen fibers and bundles in the dermis. Infiltration of leukocytes occurred as aggregates and diffusely scattered individual cells. Numerous mononuclear cells and a few neutraphils were observed in the section.

Necropsies were conducted on each of 9 elk responding on SCT. Grossly visible lesions were observed in the lungs of 1 elk. Microscopically, there were numerous granulomas observed in these lungs (Fig. 2). Caseous necrosis and mineralization were observed in some granulomas. Acid fast bacilli were found in several of the granulomas. A nonphotochromgenic slowly growing acid-fast organism was isolated from lung containing numerous tubercles. The isolate was identified as *Mycobacterium bovis* by biochemical tests.

A comparison of SCT, CCT, LBA, and ELISA in elk in a herd in which *M. bovis* was diagnosed is shown in Table 1. The results revealed that of 9 elk positive on SCT, 8 were reactors on CCT; 1 elk was not tested. Seven elk were positive on LBA using *M. bovis* antigen, and 8 were positive on ELISA using a sarkosyl extract (SK- 1) of *M. bovis*. *Mycobacterium bovis* was isolated from tissues of 1 animal positive on each of the 4 tests. Two elk positive on SCT had responses to *M. avium* PPD on CCT; however, the responses to *M. avium* PPD were <2 mm increase in skin thickness.

Six elk negative on SCT (controls) were negative on CCT and LBA. One of these 6 elk was a suspect on ELISA.

The results of CCT and LBA using *M. bovis* PPD or *M. avium* PPD in 9 elk positive on SCT are shown in

**Table 1.** Comparison of results of single cervical tuberculin test (SCT), comparative cervical tuberculin test (CCT), lymphocyte blastogenic assay (LBA), and ELISA on elk in a herd in which Mycobacterium bovis was diagnosed.

| | | CCT | | | | ELISA | | |
|---|---|---|---|---|---|---|---|---|
| SCT | *n* | B* | A† | NR‡ | LBA§ | B | A | NR |
| Positive | 28 | 23 | 2 | 3‖ | 7/9 | 8/9 | 2/9 | 2/9 |
| Negative | 6 | 0 | 0 | 6 | 0 | 1 | 0 | 5 |

* B = *M. bovis* antigen.
† A = *M. avium* antigen.
‡ NR = no response.
§ Only *M. bovis* antigen was used.
‖ One elk was not tested.

**Table 2.** Comparison of results of comparative cervical tuberculin skin tests (CCT) and lymphocyte blastogenic assays (LBA) using *Mycobacterium bovis* PPD or *M. avium* PPD in 9 elk positive on tuberculin skin test (SCT reactors) and in controls.

| | | CCT* | | LBA† | |
|---|---|---|---|---|---|
| SCT | *n* | *M. bovis* (mm) | *M. avium* (mm) | *M. bovis* | *M. avium* |
| Reactors | 9 | 5.8 | 1.0 | 3.6 | 1.0 |
| Controls | 6 | 0.2 | 0.1 | 1.1 | 0.9 |

* Increase in skin thickness, mean value.
† Stimulation index, mean value.

**Table 3.** Results of enzyme-linked immunosorbent assays conducted on sera of 9 elk responding to intradermal injection (SCT) of *Mycobacterium bovis* PPD.

| | | *M. bovis* | | *M. avium* |
|---|---|---|---|---|
| SCT | *n* | PPD | SK-1* | PPD |
| Reactors | 9 | 0.98† | 0.92 | 0.31 |
| Controls | 6 | 0.24 | 0.14 | 0.17 |

* Sarkosyl extract of *M. bovis.*
† Optical density at 405 nm.

Table 2. The increase in skin thickness (mean value) for *M. bovis* PPD was 5.8 mm (range, 2.0-12.0 mm) and for *M. avium* PPD was 1.0 mm (range, 0-3 mm). No significant increases in skin thickness were observed in controls (negative on SCT). Lymphocyte blastogenic responses (the stimulation index) for *M. bovis* PPD ranged from 1.3 to 5.9 (mean value = 3.6) and for *M. avium* PPD ranged from 0.6 to 1.3 (mean value = 1.0). In vitro LBA responses to *M. bovis* PPD were significantly greater in elk responding on SCT than in controls ($P < 0.1$). LBA responses to *M. bovis* PPD were significantly greater than responses to *M. avium* PPD in elk positive on SCT ($P < 0.5$). No stimulation index > 1.2 was observed in controls using *M. bovis* PPD or *M. avium* PPD.

Results of ELISA conducted on sera of 9 elk identified as reactors on SCT are shown in Table 3. Positive or suspicious ELISA reactions were detected in 8 of 9 elk. The results indicate greater ELISA reactions were obtained using *M. bovis* antigens (*M. bovis* PPD and SK-1 [sarkosyl extract] of *M. bovis*) than using *M. avium* antigen in elk with CCT responses than in controls ($P < 0.05$). The ELISA reactions (mean value) using the SK-l of *M. bovis* were similar to the reactions obtained using *M. bovis* PPD in sera of tuberculin test-positive elk; however, positive ELISA reactions were obtained using SK-1 in sera of 2 elk found to have suspicious ELISA reactions using *M. bovis* PPD. No important differences in ELISA reactions were detected in sera of controls using SK-l or *M. bovis* PPD.

## Discussion

The SCT was conducted as described previously. [13] Although the skin test sites were only palpated in this study, the measurement of the site 72 hours postinjection may provide useful information when conducting follow-up tests such as CCT, LBA, or ELISA. Information presented here provides further evidence that the SCT is of suitable sensitivity for identifying elk herds in which *M. bovis* infection persists.[8,12,13] However, investigations are needed to validate tuberculin test procedures for detecting tuberculosis in some species of cervids. Determination of the optimal dosage of tuberculin, appropriate test site, and most efficacious time for observing skin test responses is still necessary in certain species. The importance of immunosuppression during and following certain viral infections or vaccination with some modified-live vaccines should be investigated.[9,22]

This is a report on the first use of CCT in elk in the United States. The CCT performed well in detecting *M. bovis* sensitization in elk; however, when elk have repeatedly received tuberculin skin tests, the CCT results may be varied (M. Koller, personal communication). The application of CCT under these conditions needs further investigation. Reliable results on CCT were obtained in elk within 9 days following SCT. The purpose of CCT is to determine if delayed type hypersensitivity responses to *M. bovis* PPD are greater than those to *M. avium* PPD in the elk responding on SCT. Twenty-three of 28 elk responding on SCT were positive to *M. bovis* PPD on CCT. Similar findings have been reported in other elk herds in which *M. bovis* infection persists (D. Ferlica, personal communication). This information indicates it is not necessary to hold elk 90 days for retest, as has been previously recommended.[1,4]

The distribution of tuberculous lesions in elk is similar to that described in other animals.[3,19] Active granulomas located in close proximity to bronchioles provide an opportunity for aerosol dissemination of tubercle bacilli, which emphasizes the public health implications of *M. bovis* infection in elk. *Mycobacterium bovis* could be released from lesions, resulting in aerosol exposure of owners, caretakers, and veterinarians responsible for the care and handling of tuberculous elk.

A presumptive diagnosis of tuberculosis in elk may be made based on gross and microscopic examination of tissues and specimens collected at necropsy; however, confirmation depends on isolation and identification of the acid-fast organism. [17]

Cervids acquired for addition to a herd or animal park should come from populations with a history of being free of tuberculosis and have a negative SCT. The period of quarantine should be at least 4 months.

During quarantine, additions should receive a tuberculin skin test conducted in the cervical region, and ELISA should be conducted on serum collected at 600 day intervals. Only tuberculin test-negative caretakers should be permitted to care for animals.

When tuberculosis is diagnosed in an animal population, exposed animals should be tuberculin tested (SCT). Tuberculin skin tests can be conducted at 3-month intervals. The SCT should be conducted using double strength tuberculin prepared from *M. bovis*. Tuberculin test-positive animals should be immediately removed from the population. In such instances, the facilities, including feeders and waterers, should be thoroughly cleaned and decontaminated using a 5% cresylic compound or a derivative of phenol, such as sodium orthophenyl phenate, two times at 14-day intervals. Iodine, hypochlorites, and benzalkonium chloride, which are commonly used disinfectants, are not suitable for killing tubercle bacilli.

Tuberculosis in farmed cervids and other captive wild animals is important because these animals may serve as foci of infection for domestic and wild animals. A serum bank for captive wild species has been established at Iowa State University for use in evaluation of serologic tests. The development of DNA probes, the use of monoclonal antibodies and specific antigens in ELISA, and advances in genetic engineering techniques, such as restriction endonuclease analysis, may provide improved methods for rapid diagnosis and epidemiologic investigations of mycobacterial infections in farmed elk and other captive wild animals.[6,7]

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## Sources and manufacturers

a. National Veterinary Services Laboratories, USDA, Ames, IA.
b. Sigma Chemical Co., St. Louis, MO.
c. Grand Island Biological Co., Grand Island, NY.
d. Difco Laboratories, Detroit, MI.
e. Costar, Division of Data Packaging Corp., Cambridge, MA.
f. Flow Laboratories, McLean, VA.
g. Packard Instrument Co., Downers Grove, IL.
h. Arthur H. Thomas Co., Philadelphia, PA.
i. Kirkgaard & Perry Laboratories, Gaithersburg, MD.
j. Dynatech Laboratories, Alexandria, VA.

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