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MYCOBACTERIUM AVIUM SUBSPECIES *PARATUBERCULOSIS* AND *MYCOBACTERIUM AVIUM* SUBSP. *AVIUM* INFECTIONS IN A TULE ELK (*CERVUS ELAPHUS NANNODES*) HERD

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ABSTRACT: Between 2 August and 22 September 2000, 37 hunter-killed tule elk (*Cervus elaphus nannodes*) were evaluated at the Grizzly Island Wildlife Area, California, USA, for evidence of paratuberculosis. Elk were examined post-mortem, and tissue and fecal samples were submitted for radiometric mycobacterial culture. Acid-fast isolates were identified by a multiplex polymerase chain reaction (PCR) that discriminates among members of the *Mycobacterium avium* complex (MAC). Histopathologic evaluations were completed, and animals were tested for antibodies using a Johne's enzyme–linked immunosorbent assay (ELISA) and agar gel immunodiffusion. In addition, 104 fecal samples from tule elk remaining in the herd were collected from the ground and submitted for radiometric mycobacterial culture. No gross lesions were detected in any of the hunter-killed animals. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was cultured once from ileocecal tissue of one adult elk and was determined to be a strain (A18) found commonly in infected cattle. One or more isolates of *Mycobacterium avium* subsp. *avium* (MAA) were isolated from tissues of five additional adult elk. Gastrointestinal tract and lymph node tissues from 17 of the 37 elk (46%) examined had histopathologic lesions commonly seen with mycobacterial infection; however, acid-fast bacteria were not observed. All MAC infections were detected from adult elk ($P = 0.023$). In adult elk, a statistically significant association was found between MAA infection and ELISA sample-to-positive ratio (S/P)$\geq$0.25 (P=0.021); four of five MAA culture–positive elk tested positive by ELISA. Sensitivity and specificity of ELISA S/P$\geq$0.25 for detection of MAA in adult elk were 50% and 93%, respectively. No significant associations were found between MAC infection and sex or histopathologic lesions. Bacteriologic culture confirmed infection with MAP and MAA in this asymptomatic tule elk herd. The Johne's ELISA was useful in signaling mycobacterial infection on a population basis but could not discriminate between MAA and MAP antibodies. The multiplex PCR was useful in discriminating among the closely related species belonging to MAC.

Key words: *Cervus elaphus nannodes*, ELISA, Grizzly Island Wildlife Area, IS900, Johne's disease, *Mycobacterium avium* ss. *avium*, *Mycobacterium avium* ss. *paratuberculosis*, tule elk.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP), an obligate intracellular parasite, is the causative agent of paratuberculosis or Johne's disease, a chronic granulomatous enteritis of ruminants. The disease causes significant economic losses in domestic livestock and is an important infection of farmed cervids and other captive exotic ungulates (Manning and Collins, 2001). The likelihood that free-ranging wildlife ruminants may serve as MAP reservoirs for other wildlife, domestic livestock, and humans is not known. Clinical paratuberculosis is infrequently observed in free-ranging ungulates but has been reported in some species, including

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bighorn sheep (*Ovis canadensis*) (Williams et al., 1979), Rocky Mountain goats (*Oreamnos americanus*) (Williams et al., 1979), alpine ibex (*Capra ibex*) (Ferroglio et al., 2000), fallow deer (*Dama dama*) (Marco et al., 2002), key deer (*Odocoileus virginianaus clavium*) (Quist el al., 2002), and tule elk (*Cervus elaphus nannodes*) (Jessup et al., 1981). Fecal shedding of MAP in the absence and presence of clinical disease has been reported in tule elk (Manning et al., 2003), fallow deer (Riemann et al., 1979), axis deer (*Axis axis*) (Riemann et al., 1979), and white-tailed deer (*Odocoileus virginianus*) (Chiodini and van Kruinigen, 1983). In Scotland, MAP has been isolated from tissues and feces of numerous nonruminant wildlife species collected on farms with histories of paratuberculosis in domestic ruminants (Daniels et al., 2003).

The mycobacterial species of interest for this investigation are grouped within the *M. avium* complex (MAC), which is comprised of *Mycobacterium intracellularae* and the three currently recognized subspecies of *M. avium*: subsp. *avium* (MAA), subsp. *paratuberculosis*, and subsp. *sylvaticum* (Rastogi et al., 2001). While phenotypically different, MAP and MAA are genetically similar and share many common antigens (McIntyre and Stanford, 1986), potentially confounding immunologically based diagnostic tests (Manning and Collins, 2001).

Mycobacterium avium subsp. *avium* is considered a ubiquitous environmental saprophyte and opportunistic pathogen that rarely causes progressive disease in mammals with unimpaired immune systems. It is not considered a cause of health problems on a herd basis as it is generally not spread from animal to animal (Ashford et al., 2001; Bercovier and Vincent, 2001). In avian species, however, MAA often causes progressive disease and is commonly disseminated in feces (Tell et al., 2000). For immunosuppressed humans, MAA infection may cause serious health problems (Thorel et al., 2001).

The zoonotic potential of MAP infection is not known. A human case of systemic MAP infection has been reported (Richter et al., 2002), and some scientists have associated MAP with cases of Crohn's disease, but a causal link between MAP infection and Crohn's disease has not been proven (Naser et al., 2004).

In California, approximately 3,700 free-ranging tule elk are confined to 22 small isolated populations throughout the state (J. Fischer, unpubl. data). Clinical paratuberculosis and fecal shedding of MAP have been reported in the herd at Point Reyes National Seashore (PRNS) (38°64′N, 122°51′W). The prevalence of MAP in the other 21 herds, however, is unknown (Cook et al., 1997). In a retrospective serologic evaluation by California Department of Fish and Game using an enzyme-linked immunosorbent assay (ELISA) (IDEXX, Portland, Maine, USA), several other California elk herds, including the herd at Grizzly Island Wildlife Area (GIWA) (38°09′N, 121°58′W), yielded ELISA-positive animals (B. Gonzales, unpubl. data). This ELISA, however, had not been certified for use in elk by the United States Department of Agriculture, and the assay's specificity for MAP antibodies, as opposed to antibodies produced by MAC organisms, is also unknown. Thus, the serological results in elk were not helpful for possible management purposes (Gardner et al., 1996; Dargatz et al., 2001).

The tule elk herd at GIWA was established in 1977 with seven adult animals from the Tupman Tule Elk Reserve, California (35°17′N, 119°21′W), and one adult animal from the Owens Valley, California (37°21′N, 118°23′W). An additional adult animal from PRNS was added to the herd in 1978, prior to paratuberculosis being diagnosed in the PRNS herd. Although 13 additional adult animals have been added to the GIWA herd since 1979, most of the population growth has been a result of calf recruitment that approaches 75% in some years.

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Currently the size of this closely managed herd is maintained between 100 and 140 animals with an annual controlled hunt. Morbidity and mortality due to disease in this population are rare, and neither MAP infection nor clinical paratuberculosis has been previously reported (J. Fischer, unpubl. data).

Because of the previous ELISA data and the uncertainty regarding subclinical MAP status in tule elk herds throughout California (exclusive of the PRNS herd), the primary objective of this study was to evaluate intensively the GIWA tule elk herd for the presence of MAP infection. In addition, we aimed to evaluate the utility of the available diagnostic methods for identifying infection and screening elk herds for MAP or other mycobacterial infections.

MATERIALS AND METHODS

Subjects and study site

The GIWA habitat is a 3,450-ha tract in the San Joaquin River delta approximately 11 km south of Fairfield, California, USA, consisting of fresh to brackish wetlands and transition grasslands. Large migratory and local populations of birds, as well as a variety of mammals including black-tailed jackrabbit (*Lepus californicus*), beaver (*Castor canadensis*), cottontail rabbit (*Sylvilagus* sp.), coyote (*Canis latrans*), muskrat (*Ondatra zibethicus*), and river otter (*Lutra canadensis*) are sympatric with the elk. Although not known for certain, it is likely that cattle grazed on GIWA prior to the introduction of elk. Since the introduction of elk, cattle have grazed on the land adjacent to the wildlife area, and there have been rare reports of cattle on GIWA and elk on the cattle range (J. Fischer, unpubl. data).

In 2000, California wildlife managers determined hunt demographics from a herd estimated to contain a total of 140 animals. Hunters received permits to kill individuals within specified groups and were allowed to select individuals freely according to their permits. Between 2 August and 22 September 2000, 37 hunter-killed elk (four branched antlered males, 18 adult females, nine yearling males, two yearling females, and four calves) were available for post-mortem evaluation.

Sample collection and processing

All killed elk were transported to a central processing station within the wildlife area where a complete gross post-mortem examination was completed. Blood was collected by cardiocentesis, thoracocentesis, or abdominocentesis (depending on the location of optimal blood quality and quantity) within 130 min of death, centrifuged at 1100 × G for 10 min, and the resulting serum was chilled (~4 C) and then stored at −80 C. A fecal sample was collected from the colon of each animal. Findings from a thorough external examination and body weights were recorded. Ages of adult animals were determined in a commercial laboratory (Matson's Laboratory, Milltown, Montana, USA) by examining the cementum annuli of the extracted first incisor root tips (Hamlin et al., 2000).

Following external examination, viscera of each animal were removed and evaluated. Five-centimeter longitudinal sections of the following areas were collected: midduodenum, distal jejunum, proximal ileum, spiral colon, and terminal colon. In addition, the ileocecal valve (ICV), ileocecal lymph node (ICL), all identified mesenteric lymph nodes (MSL), and 5-cm^2 sections of cecum and liver were collected. Tissues were divided in half, with one half chilled (~4 C) in individual plastic bags (Whirl-Pak Bags, NASCO Intl., Ft. Atkinson, Wisconsin, USA) for mycobacteriological evaluation, and the other half preserved in 10% buffered formalin for histopathological evaluation. Samples of kidney, spleen, heart, gonad, skeletal muscle, rumen, reticulum, abomasum, and omasum were collected and preserved in 10% buffered formalin for histopathologic evaluation only.

After the conclusion of the hunt, 104 fresh fecal samples (as judged by moisture content and color) were collected from the ground at resting sites of two subgroups of the remaining herd. Nine samples were collected from a group of seven branched antlered male elk, and 95 samples were collected from a group consisting of approximately 10 branched antlered males, two yearling males, and 75 antlerless elk (females and calves). Samples could not be associated with individual animals, so repeated sampling from individual animals was a possibility. Fecal samples were placed in individual plastic bags (Whirl-Pak), chilled (~4 C), and shipped immediately for mycobacterial culture.

Tissue and fecal samples were processed for radiometric mycobacterial culture (Johne's Testing Center, University of Wisconsin, Madison, Wisconsin, USA) within 24 hr of

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collection. Bacterial growth characteristics, mycobactin dependency, and analysis with a multiplex PCR were used to identify acid-fast isolates (Collins et al., 1990). The PCR used primers for 16S (to confirm the mycobacterial nature of the isolate) and for IS*1311* (MAA, MAP), IS*900* (MAP only), and IS*901* (MAA only) genetic targets.

Histopathologic samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (California Animal Health and Food Safety Laboratory System, University of California, Davis, California, USA). Tissue sections that contained epithelioid macrophages and/or multinucleate giant cells were considered MAC histopathologic suspects and were further evaluated for the presence of acid-fast bacteria using the Ziehl-Neelsen's method. The pathologist was unaware of the tissue culture results.

The IDEXX ELISA was performed on serum samples from the 37 hunter-killed elk. The ELISA uses a protein G–based conjugate to detect antibody and measures optical density (OD) units on a continuous scale. Results were reported as a sample-to-positive ratio (S/P). The serum dilution was modified to optimize antibody detection based on protein G–binding data for elk sera (Kramsky et al., 2003). An agar gel immunodiffusion (AGID) (Immucell, Portland, Maine, USA) was performed on all serum samples that produced an ELISA higher than 0.25.

Statistical analysis

The relationships between MAC tissue isolation and the independent variables sex and age (adult vs. yearling and calf) were examined by means of two-way contingency comparisons: the Fisher exact test was used to compare proportions. Odds ratios (ORs) and 95% CIs were calculated as a measure of the association between MAA tissue isolation and seroreactivity (ELISA≥0.25) and MAC tissue isolation and histopathologic suspect tissue. Odds ratios were obtained using univariate logistic regression, with P values≤0.05 considered significant (Hosmer and Lemeshow, 1989). Sensitivity and specificity were calculated to detect MAA tissue infection for the ELISA≥0.25 (Sackett et al., 1985). Sparse data prevented the evaluation of factors associated with MAP infection alone. All statistical analyses were performed using commercial software (SPSS, Chicago, Illinois, USA).

RESULTS

Physical, microbiologic, serologic, and histopathologic results are detailed in Table 1. The median age of adult elk was 5.5 yr; adult ages ranged from 2.5 to 9.5 yr. All animals were in moderate to very good body condition, and there was no gross evidence of infectious or other diseases in any of these animals.

Microbiologic results

A MAP isolate was obtained from ileocecal tissue of one of the 37 hunter-killed elk, a 5.5-yr-old male. This acid-fast organism was mycobactin dependent and 16S/IS*1311*/IS*900* positive. Its fingerprint pattern categorized it as A18, a strain that predominates among MAP infections in domestic cattle in the United States (Motiwala et al., 2004). Isolates of MAA were obtained from tissue samples from five of 37 elk (14%) and from a fecal sample of an additional (3%) elk. These isolates were not mycobactin dependent, were 16S/IS*1311*/IS*901* positive, but IS*900* negative. Specifically, MAA was isolated from the MSL, ICL, or ICV of adult elk and from the feces of a calf (Table 1). All tissue isolates were from animals 4.5 yr or older. Sample contamination was minimal (3 of 344 samples) and not thought to have interfered with isolation of mycobacteria.

Mycobacterium avium subsp. *avium* was isolated from one fecal sample collected from the ground for the branched antlered male group. One of 104 fecal samples was contaminated.

Histopathologic results

Epithelioid macrophages and/or multinucleated giant cells were observed in the MSL of eight adults, the ICL of five adults, the ICV of one adult, the jejunum of one adult, and the ileum of one adult and two calves (Table 1). Acid-fast organisms were not detected within any of these tissues.

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TABLE 1. Positive microbiologic, histopathologic, and serologic results found in 22 of 37 tule elk tested. (Grizzly Island Wildlife Area, California, August to September 2000).

Demographic results			Culture results[a]		Tissue results[b]		Serologic results[c]	
Elk	Age (yr)	Sex	MAP	MAA	Histopathologic lesion site	AFB	ELISA S/P	AGID
Herd (1/103)[d]	2.5+[e]	M	—	Feces	ND	ND	ND[b]	Nd
20	9.5	F	—	—	MSL	NS	0.20	Nd
6	8.5	F	—	ICV	NS	ND	0.77	Neg
15	8.5	F	—	—	ICL	NS	0.41	Neg
3	7.5	F	—	—	MSL	NS	0.14	Nd
7	7.5	F	—	—	MSL	NS	0.08	Nd
12	7.5	F	—	MSL	Ileum	NS	1.36	Neg
22	6.5	F	—	MSL	NS	ND	0.51	Neg
23	6.5	F	—	ICL, MSL	ICL, MSL	NS	0.02	Nd
1	6.5	M	—	—	MSL	NS	0.04	Nd
21	5.5	F	—	—	Jejunum	NS	0.75	Neg
28	5.5	F	—	—	MSL	NS	0.00	Nd
2	5.5	M	—	—	MSL	NS	0.68	Neg
18	5.5	M	—	—	NS	ND	1.23	Neg
35	4.5	F	—	—	ICL	NS	0.00	Nd
36	4.5	F	—	ICL, MSL	NS	ND	1.56	Neg
25	4.5	M	ICV	—	ICL	NS	0.00	Nd
29	3.5	F	—	—	MSL	NS	0.14	Nd
11	1.5	M	—	—	ICL	NS	0.00	Nd
27	1.5	M	—	—	ICV	NS	0.00	Nd
32	0.5	F	—	—	Ileum	NS	0.00	Nd
24	0.5	M	—	—	Ileum	NS	0.07	Nd
37	0.5	M	—	Feces	NS	ND	0.00	Nd

[a] — = negative culture result; ICV = ileocecal valve; ICL = ileocecal lymph node; MAA = *Mycobacterium avium* subsp. *avium;* MAP = *Mycobacterium avium* subsp. *paratuberculosis;* MSL = mesenteric lymph node.

[b] AFB = acid-fast bacteria; NS = none seen; ND = not done.

[c] Neg = AGID negative; Nd = AGID not done.

[d] 104 herd fecal samples collected but one contaminated, leaving 103 for evaluation.

[e] Herd isolate from adult male group.

Serologic results

For the 37 serum samples tested by ELISA, S/P ratios ranged from 0.00 to 1.56 as follows: 22 adults (0–1.56), 11 yearlings (0–0.05), and four calves (0–0.17). Of the six adult elk from which species of MAC were isolated from tissue, four (67%) had S/P ratios greater than 0.25, interpreted as test-positive in cattle (Dargatz et al., 2001). Only MAA was isolated from these ELISA test-positive animals. Twenty-five of 33 samples from adult or yearling elk (75%) had S/P ratios less than 0.25, interpreted as test-negative. All samples with S/P ratios of 0.25 or greater were from animals 4.5 yr of age or older. All sera with S/P of 0.25 or higher were tested by AGID and were negative.

Statistical analysis

The proportions of detected MAC tissue infections in male and female elk were comparable. However, a significantly higher proportion of adult elk (≥2 yr) were found to have positive MAC tissue isolates (Fisher's $P=0.023$). In adult elk, a significant association existed between MAA tissue infection and ELISA≥0.25 (OR=13, 95% CI=1.1–152.4, $P=0.021$). The sensitivity and specificity of ELISA ≥0.25 to detect MAA infection in adult elk were 50% and 93%, respectively. A statistically significant association between

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MAC infection and histopathologic suspect tissues was not detected ($P=0.323$).

DISCUSSION

Mycobacterium avium subsp. *paratuberculosis* and *M. avium* subsp. *avium* infections are present in the apparently asymptomatic tule elk herd at GIWA. While infection with MAA would not be expected to cause significant morbidity in the herd without additional stressors, the identification of one adult elk with MAP infection raises at least the possibility of the herd's developing additional MAP infections in the future. However, the MAP-infected elk showed no evidence of disseminated paratuberculosis, fecal shedding, or antibody production. The likelihood that it was significantly contaminating the premises is low; as a male, two major routes that contribute to the spread of the infection in a herd (infection in utero and through contaminated milk) could not occur. Contaminated semen from an infected bull has been documented infrequently in cattle, but cases of actual transmission via semen to either dam or offspring have not been reported: nothing is known regarding this possibility in elk. As the MAP-infected animal was moved into the herd as a young adult (J. Fischer, unpubl. data), it likely acquired the infection elsewhere. It is possible that the elk could have been infected after arriving at GIWA, and, indeed, becoming infected as an adult may explain the lack of symptoms (Hines et al., 1995). Potential sources of infection of the elk on GIWA include the original founder animals or subsequent translocation. Less likely sources of infection are shared water and range contaminated by infected livestock surrounding the area and perhaps nonruminant wildlife (Daniels et al., 2003).

Contrasting the asymptomatic GIWA herd with the symptomatic PRNS herd may offer clues to the epidemiology of MAP at GIWA. Clinical MAP infections have been documented at PRNS since 1981, indicating at least some level of environmental contamination through fecal shedding of the organism. No clinical disease of any sort has been reported since the GIWA herd formation. The difference in herd density is striking: at GIWA it is approximately 0.04 elk per hectare (140 elk on 3,450 ha), while herd density at PRNS is at least 0.38 elk per hectare (>400 elk on 1,040 ha; Manning et al., 2003). In addition to a higher herd density, continued herd infection with clinically affected animals and the subsequent increased environmental fecal contamination as well as specific environmental factors at PRNS, such as lower mean daily temperature (11.4 C vs. 16.1 C), higher annual mean precipitation (95.2 cm vs. 48.9 cm), and acidic soil (pH=6.0 at PRNS), may facilitate survival of MAP in the environment (Gogan et al., 1989; Manning and Collins, 2001; Hoar, 2002; Whittington et al., 2004). Furthermore, elk at PRNS, unlike elk at GIWA, have shown clinical and biological evidence of copper deficiency, associated with decreased immunocompetency in ruminants, which may predispose the PRNS herd to clinical paratuberculosis (Gogan et al., 1989; Stabel and Spears, 1989).

Infection of GIWA elk with MAA organisms was common but appeared to have no obvious health effect on the herd as there was no gross or histopathologic evidence of disease. While MAA infection can cause clinical disease in the related red deer (*Cervus elaphus*) (De Lisle et al., 1995), infections are presumed to be from environmental sources and are self-limiting as in other mammalian species. There is evidence that some MAC species can be transmitted among cattle, but intraspecific transmission has not been documented in other species (Thorel et al., 2001). The identification of MAA in two directly collected fecal samples provides evidence that organisms can be shed in feces. However, it is possible that the MAA fecal isolates were pass-through organisms from

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its ubiquitous presence in all environments and that only those isolates from tissue represented elk with true MAA infections.

Histopathologic examination of tissues was not useful in detecting MAC-infected animals in this study; no acid-fast bacteria were detected, and no significant association was found between histopathologic lesions and MAC tissue isolates. The presence of macrophages and/or multinucleated giant cells in tissue sections in the absence of positive culture results may represent reactions to other infectious agents or antigens or may simply reflect the insensitivity of histopathology as a test for subclinical mycobacterial infections. Microgranulomas have been associated with MAP infections in cattle and bison (*Bison bison*) even in the absence of acid-fast bacteria (Buergelt et al., 1978, 2000), as well as MAA infection in clinically normal farmed red deer (De Lisle et al., 1995).

On a herd basis, the ELISA provided a useful index of suspicion for the MAC infections confirmed in this subclinical population. On an individual animal basis, the relationship between culture confirmation and positive ELISA result was poor but is comparable to what is seen in clinically healthy cattle herds with a low MAP infection prevalence. While the commercial ELISA is intended to screen herds for MAP infection, given the extremely close antigenic relationship between MAP and other MAC organisms, cross-reactivity with MAA infection was not surprising and has been reported in MAA-infected farmed red deer (Mackintosh et al., 1999). Therefore, for herds with serologic evidence of MAC infection, managers should direct resources for subsequent testing with a more specific assay (e.g., individual or pooled fecal/tissue culture followed by PCR analyses of acid-fast isolates) to confirm the identity of the mycobacterium that is responsible for the antibody response. The uniformly negative AGID results may reflect the low sensitivity and higher specificity reported for this assay in a number of species (Hope et al., 2000; Davidson et al., 2004).

Mycobacterium avium subsp. *avium* or MAP tissue infections and higher ELISA values were more likely to be detected in older animals. Indeed, all animals with tissue infection or ELISA S/P$\geq$0.25 in this study were 4.5 yr or older. From a practical perspective, these associations suggest that when elk herds are evaluated for MAC infections, older animals might be better animals for targeted sampling. Clinically affected animals should also be part of the target group to increase certainty of finding the infection.

Despite recent advances in mycobacterial diagnostics, the detection of MAP infection in asymptomatic tule elk herds (or any healthy ruminant species herd) remains challenging. The positive predictive value of testing is low for any assay in these low-prevalence herds. Nevertheless, serologic and bacteriologic analyses used in combination on older animals may increase the ability to identify infected herds. Our results should advise wildlife veterinarians, biologists, and managers that tule elk can be asymptomatic reservoirs of *M. avium* complex species.

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