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TESTING FOR *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTION IN ASYMPTOMATIC FREE-RANGING TULE ELK FROM AN INFECTED HERD

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ABSTRACT: Forty-five adult tule elk (*Cervus elaphus nannodes*) in good physical condition were translocated from a population located at Point Reyes National Seashore, Marin County (California, USA), to a holding pen 6 mo prior to release in an unfenced region of the park. Because infection with *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) had been reported in the source population, the translocated elk underwent extensive ante-mortem testing using three Johne's disease assays: enzyme linked immunosorbent assay (ELISA); agar gel immunodiffusion assay (AGID), and fecal culture. Isolation of *Mptb* was made from fecal samples in six of 45 elk (13%). All AGID results were negative while ELISA results for 18 elk (40%) were considered elevated. Elevated ELISA results or *Mptb* isolation from fecal samples were obtained for 22 of 45 elk (49%); these elk were euthanized and necropsied. *Mycobacterium avium* subsp. *paratuberculosis* was isolated from tissue in 10 of 22 euthanized elk (45%); of these 10 cases of confirmed infection, eight had elevated ELISA results (80%) and four were fecal culture positive (40%). One of 10 cases had histopathologic lesions consistent with M*ptb* infection. *Mycobacterium avium* subsp. *paratuberculosis* was also isolated from tissue from one of eight fetuses sampled. The number of tule elk found to be infected was unexpected, both because of the continued overall health of the source herd and the normal clinical status of all study animals.

Key words: California, *Cervus elaphus nannodes,* culture, ELISA, Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis,* paratuberculosis, tule elk.

INTRODUCTION

Paratuberculosis, or Johne's disease (JD), is a slowly developing and usually fatal clinical manifestation of *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) infection. Primarily affecting domestic and wild ruminants, paratuberculosis has been reported in elk, red deer (*Cervus elaphus*), and deer of other genera, e.g., *Odocoileus, Axis,* and *Dama* (Riemann et al., 1979; Jessup et al., 1981; Chiodini and Van Kruiningen,1983; Fawcett et al., 1995; Quist et al., 2002). A significant production problem for dairy cattle operations, the infection has also been reported in numerous other ungulate species (Hines et al., 1995), rabbits (*Oryctolagus cuniculus*) and scavenging carnivores in Scotland (red fox [*Vulpes vulpes*], stoats [*Mustela erminea*]), and raven (*Corvus corax*) believed to have fed on these infected rabbits (Beard et al., 2001). Primates (*Macaca arctoides, Papio sphinx*) have also been infected (McClure et al., 1987; Zwick et al., 2002).

The bacterium is environmentally hardy and disseminates within a population by a number of routes: fecal-oral, transplacental, and through milk and colostrum (Sweeney, 1996). The period of greatest susceptibility to this infection appears to be the first 6 mo of life. Infected animals can be clinically normal though infectious for months or years, thus allowing the infection to spread undetected in a herd. When clinical signs do appear, they are often vague and nonspecific: progressive weight loss despite adequate nutrition and diarrhea in some species. The organism is considered by some researchers to be

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linked to Crohn's disease, a human gastrointestinal ailment (Rubery, 2001).

The tule elk (*Cervus elaphus nannodes*) is a subspecies of *C. elaphus* and currently number some 3,700 individuals in several dozen populations in their North American native range. In 1978, 10 tule elk were released at the Point Reyes National Seashore (PRNS; California, USA) into what had for more than a century been a livestock range. An estimated 450 tule elk now live in a fenced 1,040-ha refuge at Tomales Point, an area of coastal prairie and coastal scrub in PRNS about 50 km north of San Francisco (California; Gogan and Barrett, 1987).

Paratuberculosis in tule elk was first discovered in 1980 in three animals born at Point Reyes (Jessup et al., 1981). The infection has subsequently been confirmed in additional tule elk and the organism has been isolated from dairy cattle, axis deer (*Axis axis*), and fallow deer (*Dama dama*) fecal samples in the Point Reyes area (Riemann et al., 1979; Cook et al., 1997).

Due to concern over the increasing elk population at Tomales Point and the desire to restore free-ranging elk to PRNS, management plans called for translocation of elk to an unfenced area of the park. In order to minimize the likelihood of translocating *Mptb*-infected animals, an extensive testing regime was designed for the population to be moved.

MATERIALS AND METHODS

In November and December 1998, 45 adult elk in good physical condition (no diarrhea, body condition scores >3.0 of 4; Zebarth,1995) were captured and moved from Tomales Point (38°10′N, 123°W) to a 10 ha holding pen about 25 km away. At capture, the animals were fitted with color-coded radio collars. Serum and feces were collected for *Mptb* antibody testing and culture, respectively, during the 6 mo holding period. In addition to the natural vegetation in the pen, the animals received alfalfa hay and pelleted alfalfa meal daily and water *ad libitum.* Livestock had been absent from the new range for more than 20 yr. Black-tailed (*Odocoileus hemionus columbianus*) and fallow deer were present in the surrounding area.

From December 1998 through June 1999, fecal samples from each animal were collected approximately monthly for culture. Fecal samples (ca. 10 g) were collected from the ground within 30 min of observing defecation from individuals identified by their collars. Each fecal sample was split into three aliquots, placed in tubes with a 1% decontamination solution (cetylpyridinium chloride) and sent overnight for testing. The radiometric method of organism isolation was used as previously described (Collins et al., 1990). Acid-fast organisms isolated from the samples were identified as *Mptb* by an IS*900* DNA probe and mycobactin-dependent growth patterns. Fecal samples collected during necropsy were submitted for both radiometric and conventional culture (Sockett et al., 1992).

Blood collected at initial capture, at culling, or upon recapture for release was shipped overnight the day of collection to the Johne's Testing Center (University of Wisconsin, Madison, Wisconsin, USA). Sera were tested for antibody to *Mptb* by enzyme linked immunosorbent assay (ELISA, IDEXX, Portland, Maine, USA) and agar gel immunodiffusion assay (AGID; ImmuCell, Portland, Oregon, USA). Enzyme linked immunosorbent assay optical density results were obtained following manufacturer's instructions with all samples tested in duplicate. Samples with a sample-to-positive (S/P) value of ≥0.25, using ELISA kit bovine sera controls, were considered elevated. *Cervus elaphus* positive and negative serum controls were also included on each plate.

Elk with ante-mortem indications of infection (defined as either those with *Mptb* isolated from fecal samples or with ELISA S/P ratios≥0.25) were euthanized and complete field necropsies were performed. Three widely separated sections of each of the following were placed in 10% buffered neutral formalin, sectioned, stained with hematoxylin and eosin (H&E) and Ziehl-Neelsen and examined microscopically: duodenum, jejunum, ileum, spiral colon, rectum, mesenteric lymph nodes, and cecal lymph nodes at the ileocecocolic junction. Single samples from these sites were also submitted for radiometric culture. In addition, H&E sections of forestomachs, abomasum, liver, kidney, lungs, and heart from each animal were examined microscopically.

RESULTS

Twenty-one of the 45 translocated animals were euthanized after capture based on ante-mortem test results. *Mycobacterium avium* subsp. *paratuberculosis* was isolated from pooled or individual tissues

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collected at necropsy from 10 (48%) of those elk and one fetus. *Mycobacterium avium* subsp. *paratuberculosis* was the only mycobacterial isolate obtained from any tissue sample.

Mycobacterium avium subsp. *paratuberculosis* was isolated at least once from fecal samples collected from six of the adult elk that were euthanized. For four of these, isolation was made from only one of three aliquots from a single sample. For the other two, isolations were made from more than one aliquot per sample. No mycobacterial species other than *Mptb* were isolated from fecal samples.

All serum samples were AGID negative. Serum antibody ELISA results ≥0.25 S/P ratio were obtained at capture for 18 of 45 elk (40%). Three months later, 39 elk were tested again. Six fewer elk were sampled because two were culled due to *Mptb* isolation from feces collected at capture, three died from causes unrelated to JD, and one escaped from the enclosure. The correlation between the first and second S/P values (i.e., the similarity among the individual results 3 mo apart) was high (r^2=0.949). The range of S/P ratios for both rounds of testing was 0.00−2.23. Agar gel immunodiffusion assay results for all serum samples were negative. Of 10 elk with isolations of *Mptb* from tissue, eight had ELISA S/P values≥0.25 and four were fecal culture positive.

All 21 animals were in good condition (body condition score >3) until they were culled. One of 10 elk from which *Mptb* was isolated from tissue had gross and microscopic lesions consistent with JD. The intestine was grossly thickened and associated mesenteric lymph nodes were enlarged. There was diffuse granulomatous inflammation in the intestinal mucosa and submucosa with intralesional acid-fast bacilli. Neither caseation nor calcification was noted. In two other elk from this group, several multi-nucleated giant cells were found in the cortical lymphoid tissue of the mesenteric or cecal lymph nodes. While cells of this type located in these tissues are consistent with *Mptb* infection, acid-fast bacilli were not demonstrated within them. No microscopic indications of mycobacterial infection were found for the other 18 elk. A summary of assay results for the euthanized elk is found in Table 1.

DISCUSSION

The number of tule elk found infected in the study group was unexpected, both because of the continued overall health of the source herd and the normal clinical status of the study animals themselves. If fecal shedding of the organism had been a criterion for establishing an ante-mortem diagnosis, as is the case for several state voluntary bovine JD control programs, an additional two elk for a total of 12 or 55% of the euthanized elk would have been classified as infected. If these animals are representative of the entire Point Reyes tule population, more elk may be infected than has been apparent through standard JD surveillance of the herd.

It appears that the effect of the *Mptb* infection in the herd may be less detrimental than what is reported for captive cervids (Manning,1998). Given that *Mptb* infection has been recorded in elk at PRNS for more than two decades and that the population grew rapidly and has been stable over the last 3 yr with apparently low *Mptb*-related morbidity, the infection appears to have had a minimal impact to date on the Point Reyes tule elk population.

Continued monitoring of the source population may show an increase in disease severity in the future if more calves are born to infected dams and the environmental burden of *Mptb* in the fenced Tomales Point area increases. Occurrence of clinical disease may also increase when the population approaches the carrying capacity of the enclosure and the elk experience the stressors of reduced forage, increased transmission of other diseases, heavier parasite loads, etc. Another potential outcome is the dissemination of *Mptb*

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TABLE 1. Results for 22 elk with ante-mortem indication of *Mycobacterium avium* subsp. *paratuberculosis* infection.

Elk identification	Culture		Histopathology[a]	ELISA	
	Tissue	Fecal		S/P 1	S/P 2[b]
GB[c]	Positive	Positive	Suspect	2.22	2.27
BY[c]	Positive	Positive	Negative	0.57	0.53
SG[c]	Positive	Positive	Positive	0.03	na[d]
WY[c]	Positive	Negative	Negative	1.39	0.98
RO[c]	Positive	Negative	Negative	0.82	0.38
GO[c]	Positive	Negative	Negative	0.76	0.63
OR[c]	Positive	Negative	Negative	0.46	0.25
RX[c]	Positive	Negative	Negative	0.41	0.03
WG[c]	Positive	Positive	Negative	0.00	0.02
YB[c]	Positive	Negative	Negative	0.96	1.01
RB	Negative	Positive	Negative	0.00	na
OY	Negative	Positive	Negative	0.00	na
BB	Negative	Negative	Negative	0.67	0.59
BO	Negative	Negative	Negative	0.47	0.43
OW	Negative	Negative	Negative	0.37	0.46
RW	Negative	Negative	Negative	0.36	0.44
WW	Negative	Negative	Negative	0.45	0.26
SB	Negative	Negative	Negative	0.42	0.30
SO	Negative	Negative	Negative	0.29	0.23
SR	Negative	Negative	Negative	0.34	0.28
WR	Negative	Negative	Negative	0.27	0.12

[a] Positive=microscopic lesions consistent with *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*) infection; negative=no histopathologic lesions; suspect=multinucleated giant cells in appropriate sites.
[b] Serum drawn 3 mo later.
[c] Fit the case definition for infection (i.e., *Mptb* isolation from tissue).
[d] na=not applicable.

infection to other susceptible ruminant and perhaps non-ruminant wildlife at PRNS. Should such a "spill-over" to other species occur, however, it will be difficult to attribute it to a specific infected population since strong *Mptb* infection pressure exists within other animal populations in California, especially dairy cattle; approximately 90% of Central Valley dairy herds are estimated to be infected (I. Gardner, pers. comm.).

To maximize postmortem detection sensitivity, relevant tissues (at least mesenteric lymph nodes and ileum) should be collected for both isolation of *Mptb* through culture and for histopathology. Tissues should be collected for evaluation even if no gross lesions are observed as active mycobacterial infections can exist in cervids in the absence of visible lesions (Kaneene, 2002). The insensitivity of microscopic examination of tissue for *Mptb* infection for these tule elk may simply have been due to the inherent limitations of sampling 4 μm sections from many meters of tissue to find the minute and focal lesions present in early phase infections.

To best characterize the status of this chronic and subtle infection in a wild population, more than one round of surveillance should be completed. Diagnostic tests at a single point in time cannot provide sufficient confidence that a population is free of the infection given that the negative predictive value of ante-mortem assays in free-ranging species is not known.

Concurrent use of the ELISA and fecal culture increased the number of infected elk detected beyond what would have been found using a single assay. The ELISA was most effective in identifying

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cases of confirmed infection (80%) but the potential for non-specific results must always be considered when interpreting ELISA values for mycobacterial infections. The fact that every serum sample was AGID-negative, including those from confirmed cases of infection with repeated high ELISA S/P values, does not support use of the AGID for JD diagnosis in elk. Further evaluation of diagnostic assay performance could not be done since antemortem test-negative elk were not culled and the true infection status of these animals could not be obtained. Without this information, an analysis of assay sensitivity, specificity, and predictive value was not possible.

In cattle, an elevated antibody level due to *Mptb* infection often indicates that the animal is or will soon develop signs of clinical disease (Collins, 1996). A number of possibilities may explain the ELISA's detection of elevated antibody for elk in robust condition with no post-mortem evidence of infection. The first is the use of an S/P≥0.25 to define an elevated amount of antibody; a higher S/P cut-off value may be more appropriate for this species. Studies are underway to establish optimal ELISA protocols for elk. Other possibilities include the insensitivity of postmortem methods of detecting early phase infection (i.e., the elk were truly infected, but no evidence was observed) or infection as an adult animal resulting in antibody production but a mature and fully functional immune system cleared the infection. Another possibility is the antibody was elicited by infection with another organism that cross-reacts with the *Mptb* antigens in the JD ELISA assay. Previous reports have noted the inability of an ELISA (albeit an assay with different reagents) to discriminate between serum antibodies produced by red deer infected with *Mptb* and those infected with the closely related *Mycobacterium avium* subsp. *avium* (Mackintosh,1999). In the present study however there was no evidence at necropsy of chronic infection with other organisms that might have elicited production of antibody cross-reacting with *Mptb* (e.g., *M. avium* subsp. *avium*, members of the Actinomyces family, or *Nocardia*) nor were any isolations of confounding organisms made through tissue or fecal culture.

It is relevant to distinguish clearly among the terms "test-positive," "infected," "infectious," and "diseased" when discussing policies for wild populations carrying a transmissible infection. This is especially true for paratuberculosis where the core epidemiologic information is based on husbandry and pathogen exposure patterns for domestic animals, conditions that may not prevail for wild populations. Progression to disease and capacity for transmission may not mimic what is seen for infected sheep, goats, or cattle under domestic husbandry. It is possible that *Mptb*'s pathogenic capacity in free-ranging animals is lower than what is observed for animals managed under intensive husbandry.

For tule elk, the wildlife management and health implications of this infection must be evaluated in light of the relative scarcity of this native species. Whether the elk culled for antemortem evidence of infection would have died of or contributed significantly to the transmission of *Mptb* infection cannot be known. The released ante-mortem test-negative elk are regularly monitored visually for symptoms of the infection (i.e., loss of condition or diarrhea) and samples are collected semi-annually for fecal culture.

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ture of the elk. Staff from CDFG and CAHFS conducted the necropsies.

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