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GRANULOCYTIC EHRLICHIOSIS AND TICK INFESTATION IN MOUNTAIN LIONS IN CALIFORNIA

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ABSTRACT: Forty-seven mountain lions (*Puma concolor*) collected year-round in 1996 to 1998 from the Sierra Nevada foothills, the northern coast ranges, and in Monterey County (California, USA) were examined for infestation with *Ixodes pacificus* and *Dermacentor variabilis* ticks. Ticks were found predominantly in winter and spring. The seroprevalence of granulocytic ehrlichiae (GE) antibodies (*Ehrlichia equi* or the agent of human granulocytic ehrlichiosis) was 17% and the PCR-prevalence of DNA characteristic of GE in blood was 16%. There were eight polymerase chain reaction (PCR)-positive but seronegative mountain lions, one that was PCR-positive and seropositive, and eight that were PCR-negative and seropositive. Nineteen percent of engorged tick pools from mountain lions were PCR-positive. Because mountain lions inhabit tick-infested habitat and are frequently bitten by *I. pacificus,* surveillance for GE antibodies and DNA in mountain lions and other vertebrate hosts may be useful as indicators for geographical regions in which humans are at risk of GE infection.

Key words: Disease ecology, *Ehrlichia equi,* granulocytic ehrlichiae, *Ixodes pacificus,* mountain lion, *Puma concolor,* survey.

INTRODUCTION

Human granulocytic ehrlichiosis (HGE) is an emerging tick-transmitted rickettsial disease in the northeastern and upper midwestern United States (Dumler, 1997). Four cases of HGE have been identified in human patients in California (Foley et al., 1999a; Gewirtz et al., 1996). The agent of HGE is either *Ehrlichia equi*, the agent of equine granulocytic ehrlichiosis, or a very closely related organism, based on serological (Dumler et al., 1995), genetic (Madigan et al., 1996), and clinical characteristics (Barlough et al., 1995). The human and equine granulocytic ehrlichiae share tick vectors: *Ixodes scapularis* occurs east of the Rocky Mountains (Telford et al., 1996), *I. pacificus* is found in California (Richter et al., 1996), and probably *I. ricinus* is the vector in Europe (Petrovec et al., 1997).

The ecology of granulocytic ehrlichiae (GE) is complex, with cosmopolitan host ranges of both the rickettsiae and the tick vector. Natural infections with granulocytic ehrlichiae have been reported in white-tailed, mule, and black-tailed deer (*Odocoileus virginianus*) (Bakken et al., 1996a), (*Odocoileus hemionus hemionus* and *O. hemionus columbianus*) (Foley et al., 1998), and elk (*Cervus elaphus nannodes*) (Foley et al., 1998), deer mice (*Peromyscus leucopus*) (Walls et al., 1997), voles (*Microtus microti*) (Tyzzer, 1938), woodrats (*Neotoma fuscipes*) (V. Kramer and W. Nicholson, pers. comm.), black bears (*Ursus americanus*) (J. Walls, unpubl. data), domestic dogs (Madewell and Gribble, 1982), horses (Madigan and Gribble, 1987), and cats, skunks (*Mephitis mephitis*) and coyotes (*Canis latrans*) (J. Foley and J. Madigan, unpub. data). The adult *Ixodes pacificus* feed on cervids, domestic animals, coyotes, black bears, bobcats (*Lynx rufus*), and humans (Furman and Loomis, 1984). Ticks transmit *E. equi* transtadially but not transovarially (Lewis, 1979; Munderloh and Kurtti, 1995).

Carnivores may make good sentinels for granulocytic ehrlichiae because they are

hosts for both the ehrlichiae and the tick vector and because they may have more widely distributed activity than some other hosts of the tick, such as rodents. The duration of seropositivity following infection is not known for nondomestic carnivores, but experimental studies in dogs and cats (J. Madigan and J. Foley, unpubl. data) and non-carnivores (horses) (Madigan, 1993; Nyindo et al., 1978) reveal that animals may be seropositive on the order of 12 mo. The mountain lion (*Puma concolor*) has a range in California (USA) including much of the Sierra Nevada, coast ranges, and transverse ranges (Mansfield and Weaver, 1989; Torres et al., 1996), and is present throughout the range of *I. pacificus*. Extrapolating from experimental infections of *E. equi* in domestic cats (Lewis et al., 1975), mountain lions may experience minimal clinical signs of ehrlichiosis, reducing possible biases that could arise if the host were severely or fatally affected. This study examined mountain lions collected year-round from 1996–1998 in California for antibodies reactive to *Ehrlichia equi* and infestation with *I. pacificus*. Blood samples from mountain lions were tested for antibodies to GE and for DNA characteristic of GE using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Counties grouped as Sierra included Calaveras, Amador, El Dorado, Butte, Yuba, and Nevada Counties. The habitat was blue oak/foothill pine in the northern Sierra Nevada foothill geographic subdivision (Hickman, 1993). North coastal counties represented were Mendocino, Napa, Sonoma, and Lake within the outer and inner north Coast Range geographic subdivisions. The habitat ranges from redwood/mixed evergreen and hardwood to chaparral/pine/oak woodland. All mountain lions listed under Monterey were from Monterey County per se in the outer south Coast Range subdivision. This habitat is redwood and mixed hardwood to oak, blue oak, foothill pine woodland and chaparral.

Forty-seven mountain lions were sampled from the Sierra Nevada foothills (38°20′ to 39°45′N, 119°00′ to 121°30′W), northern coast ranges (38°15′ to 40°00′N, 122°00′ to 123°7′W), and Monterey County (35°50′ to 36°55′N, 120°30′ to 121°58′W) in California after being killed under California Department of Fish and Game (CDFG; Sacramento, California, USA) depredation permits as threats to livestock. Typically, mountain lions were killed by gunshot, then transported to regional CDFG headquarters and maintained for up to 6 mo in a freezer. Approximate ages of lions were determined by body size and tooth wear. At the time of necropsy, carcasses were thawed and clotted blood was removed from the heart for serological analysis and polymerase chain reaction.

Ectoparasites were collected from the carcasses using a flea comb and were stored in glass tubes containing 70% ethanol in water. Approximately 10 to 20 min of effort were expended per carcass, with greatest attention given to the head, ears, axillae, and inguinal regions. Ticks were identified using a key (Furman and Loomis, 1984) and voucher specimens were retained in the laboratory.

Clotted blood from the heart was centrifuged at 1800 × g for 10 min and serum harvested for serology. Anti-GE IgG was assayed by immunofluorescent antibody assay (IFA) as previously described (Barlough et al., 1995) using *E. equi*-infected horse neutrophils as substrate. Samples were tested starting at dilutions of 1:10 to endpoint, and positive and negative control sera were included on each run. The titer was considered the highest dilution at which bright green fluorescence could be detected on morulae on the slide, including 10.

Extraction of DNA was performed from clot blood by QiaAmp Tissue Extraction kit (Qiagen, Chatsworth, Massachusetts, USA) using the manufacturer's protocol for tissue. Specimens of *I. pacificus* for DNA extraction were cut into small pieces using sterile scissors and finely ground in a 1:1 wt:vol. ratio of sterile phosphate-buffered saline using a mortar and pestle. After a 5 min low speed (300 × g) centrifugation to remove debris, supernatant from each individual tick preparation was pooled for all ticks from each lion (omitting one tick per lion as a voucher), up to 200 μl, and the pooled sample incubated with 50 μl proteinase K and 200 μl buffer "AL" (from the QiaAmp Extraction kit) at 70 C for 20 min. The subsequent steps of extraction were as described by in the manufacturer's protocol.

Nested polymerase chain reaction (PCR) was performed in a thermal cycler (MJ Research, Watertown, Massachusetts, USA) as described in Barlough et al. (1996) using primers EE-1 and EE-2 for the outer product and EE-3 and EE-4 for the inner product. Verification of extraction of DNA from ticks was performed by PCR using tick-specific primers 16S+2 and

TABLE 1. Age, sex, and location reported for mountain lions collected on depredation permits in California (USA).

	Age (years)				Sex			Location			
	1–2	3–6	>6	U[a]	M	F	U	SNF[b]	NCR[c]	Monte-rey	U[a]
Tick-infested	12	14	2	7	22	8	5	2	25	4	4
No ticks	4	6	2	0	10	2	0	6	6	0	0

[a] U indicates data not specified.
[b] SNF = Sierra Nevada foothills.
[c] NCR = North Coast Range.

16S−1 (Black and Piesman, 1994) following the PCR protocol described in Barlough et al. (1997). The PCR products were visualized by transillumination in 1.5% agarose gels stained with ethidium bromide. Negative controls (water) were included for extraction and PCR and a positive control was included for PCR. Additional steps to prevent contamination included performing extraction in a UV hood and using pipettors dedicated to extraction of DNA from blood and PCR in a separate room. Plugged pipette tips were utilized for all steps, and water negative controls were included as the last sample in a batch, to test for cross-contamination.

The seasonal distribution of data (tick infestation, serological test results, and PCR test results in lion blood and ticks) was assessed after grouping data from summer and fall (corresponding to nymphal tick distribution), compared to grouped winter and spring (adult tick distribution) in order to increase individual cell size. Homogeneity of tick infestation over season and location was tested with chi-square contingency tests, with a cut-off of $P < 0.05$ for significance (S-Plus Version 3.3, Mathsoft, Inc., Seattle, Washington, USA). Similarly, the seasonal distribution of seropositive tests was assessed with a chi-square contingency test. The mountain lion ages were divided into 4 age classes: ≤1 yr, 1–2 yr, 2–4 yr, and ≥4 yr. Age-related PCR and seroprevalence were reported for the age groups but not analyzed statistically.

RESULTS

The age, sex, and approximate location of mountain lions are given in Table 1. Thirty-five mountain lions were tick-infested and 12 appeared to be free of ticks. All ticks on mountain lions were either *I. pacificus* or *D. variabilis,* mostly adults and a few nymphs. Most ticks were observed in lion pinnae and ear canals, with some along the top of the head and muzzle. Other sites of tick attachment included inguinal and axillary regions where the attachment sites were typically erythematous and inflamed.

Two pools of ticks were observed on mountain lions from the Sierra Nevada, both during the fall. The three tick pools from Monterey County were from spring and summer. In contrast, the north coast region mountain lions had 25 tick pools, distributed across all seasons, but more commonly from winter and spring ($P = 0.03$).

The seroprevalence of anti-GE IgG was 18% for the tick-infested mountain lions and 17% for the non-infested mountain lions, for an overall seroprevalence of 17%. Both mountain lions from Monterey were serologically positive, while the seroprevalence was approximately 12% in mountain lions from north coastal areas and the Sierra foothills. Seasonal seroprevalence rates ranged from 0% in winter to 19% in spring, 29% in summer, and 29% in fall. These differences were not statistically significant ($P = 0.52$). The PCR-prevalence for GE in mountain lions was 16%; the prevalence was 15% in mountain lions with ticks and 17% in mountain lions without ticks. The prevalence in north coastal mountain lions (with and without ticks) was 19%, compared with 25% in the Sierra Nevada and 0 from Monterey. There were eight PCR-positive, seronegative mountain lions, one PCR-positive, seropositive, and eight PCR-negative, seropositive. There was no statistically significant seasonal effect evident in PCR-test results ($P = 0.68$).

Male mountain lions had a PCR and seroprevalence of 19%, compared with rates of 20% and 20%, respectively, in females. PCR-prevalence declined over age from 20% in juveniles, 25% in yearlings down to 13% in ≥4-yr old mountain lions. In contrast, seroprevalence increased slightly over age, starting at 20% in juveniles, to 23% in mountain lions ≥4 yr.

Nineteen percent of engorged tick pools from mountain lions were PCR-positive. Three of these were from PCR-positive mountain lions. Three PCR-positive tick pools were from PCR-negative mountain lions. There was no significant seasonal distribution of PCR-positive tick pools ($P = 0.36$). Of the five PCR-positive tick pools, three were detected in spring and two in summer. Five pools of 25 (20%) of the ticks from mountain lions from the north coast region were PCR-positive, but sample sizes from Monterey and Sierra sites were too small to analyze statistically.

DISCUSSION

Mountain lions from California were frequently infested with *I. pacificus,* the tick which transmits HGE and *E. equi,* and DNA characteristic of GE was detected in mountain lion blood and ticks attached to mountain lions. The PCR-prevalence of GE, 16%, was probably an underestimate because of sub-optimal handling of blood samples before running the PCR test. Nevertheless the data demonstrate that GE DNA is generally hardy and PCR can be performed on even poorly handled samples with reasonable success. The estimates of tick distributions on mountain lions were likewise rough because many ticks may have been lost during carcass storage and transport and because low intensity infestations may not have been detected. In particular, the examination format would be more likely to overlook smaller ticks such as larvae, nymphs and unengorged adults. However, larval *I. pacificus* and *D. variabilis* would probably not have been present, because they prefer rodents and reptiles as hosts (Furman and Loomis, 1984).

The species and seasonal distribution of ticks collected from mountain lions were expected. Both species of the ticks are cosmopolitan in host preference and both have the greatest adult densities during the cooler, wet months of the California winter and spring (Clover and Lane, 1995; Furman and Loomis, 1984; Lane, 1990). No evidence of seasonality was evident in serological or PCR test results. However, mountain lions could be seropositive yearround based on the duration of antibodies in other species (horses and humans) which has been shown to be months to 3 yrs (Bakken et al., 1996b; Nyindo et al., 1978). In contrast, PCR test results would be expected to be positive only during acute infection (Walker and Dumler, 1997), i.e. during the season of the primary tick stage that transmits the *Ehrlichia* sp. It is unlikely that mountain lions experience chronic infection, extrapolating from lack of chronicity in human cases and all other animal models of equine and human granulocytic ehrlichiosis, including horses (Nyindo et al., 1978), mice (Hodzic et al., 1998), monkeys (Foley et al., 1999b), cats (J. Foley and J. Madigan, unpubl. data), and dogs (Madewell and Gribble, 1982). There was one reported human case of persistent HGE (Dumler and Bakken, 1996) and a horse with apparent recrudescent equine ehrlichiosis and underlying Cushing's disease (J. Madigan, unpubl. data), but such reports are extremely rare. Human cases of HGE have been detected in late summer or fall (Foley et al., 1999a; Gewirtz et al., 1996), corresponding to the nymphal tick distribution (Lane, 1990), while veterinary cases frequently occur during winter and spring during the peak adult tick season (Madigan and Gribble, 1987). PCR-based surveillance detected one naturally infected pool of five nymphal *I. pacificus* from 47 individuals tested in Sonoma County (Barlough et al., 1997) and a PCR-prevalence ranging from 0.8% to 5% in adult ticks (Barlough et al.,

1997; Kramer et al., 1999). Our data revealed that adult ticks had DNA typical of GE; the data do not rule out nymphal transmission as well. It is possible that horses acquire GE predominantly from adult *I. pacificus* because they are an attractive host to the adult tick and cannot groom effectively, while humans attract both nymphal and adult ticks but easily groom the adult ticks. The present data do not clarify what stages of ticks transmit *Ehrlichia* to mountain lions.

There were seropositive and PCR-positive mountain lions in each of the geographical regions examined. Human cases of GE have only occurred in the coastal regions of Humboldt and Santa Cruz counties, but equine and canine cases are commonly observed in the Sierra Nevada foothills as well (Gribble, 1969) and PCR-positive ticks have been found in Alameda, El Dorado, Napa, Orange, and Santa Cruz Counties (Barlough et al., 1996; Kramer et al., 1999). However, seroprevalence data of horses and dogs from throughout northern California (Madigan et al., 1990; J. Foley and J. Madigan, unpubl. data) suggest that antibodies to GE are more common in north coastal counties including Marin, Sonoma, Mendocino, and Humboldt than in Sierra Nevada foothill counties. Mountain lions cover large areas of land and likely encounter hundreds of individual ticks from which they might acquire GE. In comparison to mountain lions, Madigan et al. (1990) found seroprevalence to GE in horses ranged from 10.4% to up to 50% in one enzootic focus in Sonoma and Mendocino County, and Nicholson et al. (1998) found GE seroprevalence in woodrats of 9%. Studies from GE-endemic foci in the upper midwest and greater New England areas have estimated seroprevalence to GE to be >9% in dogs (Magnarelli et al., 1997), and to range from 2% (Bunnell et al., 1998) to 10% (Walls et al., 1997) to 23% in deer mice (Nicholson et al., 1998).

Results of serological and PCR tests were not concordant in individual mountain lions, which was expected given previously published results of these tests in people (Dumler, 1997) and animals, including dogs (Greig et al., 1996), rhesus macaques (Foley et al., 1999b), horses (Madigan et al., 1995), and deer mice (Walls et al., 1997). After completion of tick feeding, animals develop IgG antibodies in 12–16 days, but have transient ehrlichemia and detectable *Ehrlichia* DNA as early as 4 days (Foley et al., 1999; Madigan et al., 1995). The duration of positive PCR results varies according to the sensitivity of the test and host (Hodzic et al., 1998), but was found to be ≤5 days in peripheral blood in experimentally infected cats, using the same nested PCR used here (J. Foley, N. Pedersen, and J. Madigan, unpubl. data).

The prevalence of PCR-positive mountain lions was comparable to reports in other species. For example, the prevalence of GE in black-tailed deer was 5%, 31% in elk from Sonoma County (Foley et al., 1998), and 11% in wild rodents from an HGE-endemic region of Wisconsin (USA)(Nicholson et al., 1998). The mountain lions in this study were sampled from heavily tick-infested and GE-endemic areas; mountain lions from other parts of the state would be expected to be less frequently infected. The frequency of active infection in mountain lions suggests that they could function as reservoirs. However, many other species are susceptible to GE and multiple mammalian reservoirs may exist. Additionally, the overwintering infected tick bank may provide a source of *Ehrlichia.*

The frequency of positive PCR tests in ticks was high compared to previously published frequencies in host-seeking ticks of 0.8% (Barlough et al., 1997) and 2% (Kramer et al., 1999) and 4% for Alameda County (Kramer et al., 1999). The results for ticks from mountain lion may have been so high partly because of passive acquisition of GE DNA by feeding on infected lion blood. Richter et al. (1996) found 50% of engorged ticks removed from an experimentally infected horse to be PCR-positive. It is possible that the PCR test in engorged ticks is more com-

monly positive than in questing ticks because tick feeding may allow for replication of ehrlichiae from either the tick or the host originally in quantities below the threshold of detection of PCR. Similar responses after tick feeding have been described for *Borrelia burgdorferi* (Ribeiro et al., 1987), *Theileria parva* (Purnell and Joyner, 1968), and *Rickettsia rickettsii* (Spencer and Parker, 1923).

In summary, the data presented in the study reveal that mountain lions are frequently exposed to granulocytic ehrlichiae in the ecosystems studied. Because mountain lions inhabit tick-infested habitat and are frequently bitten by *I. pacificus*, surveillance for GE antibodies and DNA in mountain lions and other vertebrate hosts may be useful as indicators for geographical regions in which humans are at risk of GE infection.

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