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SURVEY FOR ZOONOTIC RICKETTSIAL PATHOGENS IN NORTHERN FLYING SQUIRRELS, *GLAUCOMYS SABRINUS,* IN CALIFORNIA

Janet E. Foley,[1,5] Nathan C. Nieto,[1] S. Bernadette Clueit,[2] Patrick Foley,[3] William N. Nicholson,[4] and Richard N. Brown[2]

[1] Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616, USA
[2] Department of Wildlife, Humboldt State University, Arcata, California 95521, USA
[3] Department of Biological Sciences, California State University, Sacramento, California 95819, USA
[4] Viral and Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Atlanta, Georgia 30333, USA
[5] Corresponding author (email: jefoley@ucdavis.edu)

ABSTRACT: Epidemic typhus, caused by *Rickettsia prowazekii*, is maintained in a southern flying squirrel (*Glaucomys volans*) sylvatic cycle in the southeastern United States. The northern flying squirrel (*Glaucomys sabrinus*) has not been previously associated with *R. prowazekii* transmission. A second rickettsial pathogen, *Anaplasma phagocytophilum,* infects dusky-footed woodrats (*Neotoma fuscipes*) and tree squirrels in northern California. Because northern flying squirrels or their ectoparasites have not been tested for these rickettsial pathogens, serology and polymerase chain reaction (PCR) were used to test 24 northern flying squirrels for *R. prowazekii* and *A. phagocytophilum* infection or antibodies. Although there was no evidence of exposure to *R. prowazekii*, we provide molecular evidence of *A. phagocytophilum* infection in one flying squirrel; two flying squirrels also were seropositive for this pathogen. Fleas and ticks removed from the squirrels included *Ceratophyllus ciliatus mononis*, *Opisodasys vesperalis*, *Ixodes hearlei*, *Ixodes pacificus*, and *Dermacentor paramapertus*.

Key words: *Anaplasma phagocytophilum*, epidemic typhus, granulocytic anaplasmosis, *Rickettsia prowazekii*, rodents, sylvatic typhus, vectorborne disease.

INTRODUCTION

Epidemic typhus, caused by *Rickettsia prowazekii* infection, is characterized by fever, headache, rash, arthralgia, central nervous system dysfunction, pulmonary edema, shock, and sometimes death (Raoult et al., 2004). The body louse *Pedicularis humanus humanus* is the vector, and it inoculates the bacterium via contaminated fecal matter scratched into the skin. Recent epidemics have been reported from Burundian refugee camps (WHO, 1997), Andean South America (Raoult et al., 1999), and Russia (Tarasevich et al., 1998), particularly among impoverished and displaced people. Sporadic cases have also been observed among homeless people in France (Brouqui et al., 2005) and in rural residents of the southeastern United States (Reynolds et al., 2003).

Infection with *R. prowazekii* is rare in the United States, although human cases are occasionally reported in the eastern United States that are not associated with louse infestations (CDC, 1983; Reynolds et al., 2003). Most such cases have been associated with contact with southern flying squirrels (*Glaucomys volans*) or flying squirrel nests (Reynolds et al., 2003). *Rickettsia prowazekii* has been isolated from the blood of southern flying squirrels (Bozeman et al., 1975), but the arthropod vectors have not been confirmed. Experimental infection in *G. volans* individuals has been associated with rickettsemia and death (Bozeman et al., 1981), but no survey for *R. prowazekii* in northern flying squirrels (*Gluacomys sabrinus*) has been reported. The clinical consequences of *R. prowazekii* infection in northern flying squirrels are not known.

Northern flying squirrels may also be exposed to other zoonotic tick-borne rickettsial pathogens such as *Anaplasma phagocytophilum.* Granulocytic anaplasmosis (GA) has variable clinical signs in humans, including pyrexia, headache, myalgia, nausea, ataxia, organ failure, suscep-

tibility to opportunistic infections, neuritis, respiratory dysfunction, and death (Foley, 2000). Although evidence of infection has been found in mountain lions, bears, coyotes, mustelids, and other wildlife species, the clinical significance of this infection is not known (Foley et al., 2004). Wild rodents (together with *Ixodes* spp. ticks) constitute the natural reservoir, and disease lesions have been reported from some species of rodents. Histopathologic lesions in experimentally infected mice closely mimic those observed in humans, horses, and dogs with GA, in which the presence of organisms together with induction of interferon-γ (IFNγ) may lead to severe hepatic inflammatory lesions with numerous apoptotic hepatocytes (Martin et al., 2001). The prevalence of *A. phagocytophilum* among rodents in far northwestern California is very high: in a study in the Hoopa Valley of Humboldt County, 88% of woodrats (*Neotoma fuscipes*), the best characterized mammalian reservoir in California, were found to be seropositive, and 71% were polymerase chain reaction (PCR) positive (Drazenovich et al., 2006). This suggests that other, less well-studied rodents such as flying squirrels could also be at risk.

Ixodes pacificus, the western blacklegged tick, is a known bridging vector for the transmission of *A. phagocytophilum* from rodents to humans, dogs, horses, and other large mammals in the western United States (Richter et al., 1996). *Ixodes spinipalpis,* a nidicolous tick that primarily infests woodrats, functions as an important vector in enzootic cycles (Zeidner et al., 2000). The ectoparasite fauna of northern flying squirrels in the Pacific Northwest has been poorly identified, in part because the animals are difficult to observe, difficult to capture, and rarely examined. The purpose of this report is to describe the ectoparasite fauna from a small series of *G. sabrinus* from California and to evaluate these animals for exposure to, and infection with, *A. phagocytophilum* and *R. prowazekii.*

MATERIALS AND METHODS

Animals

Twenty-four northern flying squirrels were live-trapped from six locations in northern California from 2003 to 2007: eight from the Hoopa Valley (HV), Humboldt County (41°10′5″N, −123°43′43″W); eight from Yosemite National Park (YNP), Mariposa County (45°24′29″N, −117°35′19″W); three from Humboldt Redwoods State Park (HRSP), Humboldt County (40°11′17″N, −123°35′19″W); two from Sagehen Creek Field Station (SH), Nevada County (39°26′23″N, −122°46′12″W); two from the Plumas National Forest (PNF), Plumas County (40°0′59″N, −121°0′1″W); and one from Teakettle Experimental Area (TEA), Fresno County (36°58′0″N, −119°2′0″W). Animals were baited with different combinations of corn, oats, barley, peanut butter, and molasses into Tomahawk wire-mesh live-traps (Tomahawk, Tomahawk, Wisconsin, USA; TEA and HV) or Sherman live-traps (HB Sherman, Tallahassee, Florida, USA; HRSP and YNP). The TEA animal was found dead in the trap. Remaining animals were anesthetized with 20 to 40 mg/kg ketamine and 4 mg/kg xylazine. Whole blood was collected via venipuncture of the femoral vein (HV), by abrasion of the retroorbital sinus, or by contact with skin bleeds after ear tissue samples were collected for another project; blood was collected into ethylene diamine tetraacetic acid and saved at –20 C. Ectoparasites were removed with forceps and stored in 70% ethanol.

Ectoparasite identification

Fleas were washed in 70% ethanol, cleared by incubation in dilute KOH for 24 hr, dehydrated in an ethanol series (75%, 85%, 95%, and 100% for 30 min each), and then mounted in Euparal (BioQuip, Rancho Dominguez, California, USA). Fleas were identified using multiple references including Stark (1958), Hubbard (1968), Holland (1985), and Lewis et al. (1988). Ticks were identified using keys in Keirans and Clifford (1978), Furman and Loomis (1984), Webb et al. (1990), and Durden and Keirans (1996).

DNA extraction and PCR

DNA was extracted from rodent whole blood using the Qiagen DNA extraction kit (Qiagen, Valencia, California, USA) following manufacturer's recommendations. TaqMan real-time PCR for the *A. phagocytophilum* p44 gene was performed to identify active infection as pre-

viously described (Drazenovich et al., 2006). In order to obtain a product for DNA sequencing, primers HS1a and HS6a for round one and HS43 and HSVR for the second round were used in a nested PCR reaction targeting the 1054 bases of the GroESL gene, as reported previously (Liz et al., 2000). The product was visualized under ultraviolet (UV) transillumination, extracted from a 1% agarose gel using a kit (QiaQuick Gel Extraction Kit, Valencia, California, USA), and sequenced forward and reverse with PCR primers at Davis Sequencing (Davis, California, USA). The resulting sequence was compared with sequences available on GenBank (National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) using the BLAST algorithm searching nr/nt nucleotide databases.

For *R. prowazekii,* a nested PCR protocol designed by the Centers for Disease Control and Prevention (CDC) targeting the *htr*A (17 kD) gene was utilized, with Ready-to-Go beads (Amersham, Piscataway, New Jersey, USA), 460 nM primer mix, and 2 µl of sample DNA. For the nested reaction, 1 µl of first-round DNA was added to beads with 480 nM primer mix. First-round primers were R17-122 (5′-CAGAGTGCTATGAACAAACAAGG) and R17-500 (5′-CTTGCCATTGCCCATCAGGTTG). Second-round primers were RP2 (5′-TTCACGGCAATATTGACCTGTACTGTTCC) and RPID (5′-CGGTACACTTCTTGGTGGCGCAGGAGGT). Cycling conditions for both rounds were 95 C denaturation for 5 min, 40 cycles of 95 C for 30 sec, 55 C for 30 sec, and 72 C for 60 sec, followed by extension at 72 C for 5 min. Amplicons were evaluated in Gelstar-stained (Cambrex, East Rutherford, New Jersey) 1% agarose gels by UV transillumination.

Serology

Antibodies against *A. phagocytophilum* and *R. prowazekii* were assessed by immunofluorescent antibody assays (IFA). Plasma was separated by centrifugation at 3000 rpm (rotations per min) for 10 min, diluted in phosphate buffered saline (PBS) from an initial dilution of 1:25 to 1:400, applied to commercial *A. phagocytophilum* antigen slides (Protatek International, Saint Paul, Minnesota, USA), and incubated at 37 C with moisture for 30 min. Slides were then washed three times in PBS and incubated with fluorescein isothyocyanate (FITC)-conjugated goat anti–flying squirrel IgG, diluted 1:30 in PBS (courtesy CDC, Atlanta, Georgia, USA). Slides were washed three additional times and, during the third wash, they were incubated with two drops of iriochrome black (Sigma, St. Louis, Missouri, USA) for 2 min. Positive (an experimentally infected positive woodrat sample) and negative controls were included in each run. For *R. prowazekii*, IFA was performed using *R. prowazekii*–infected vero cells in lyophilized suspension as substrate, prepared according to CDC protocols. The IFA was performed as for *A. phagocytophilum*, except the dilution buffer was PBS-1% bovine serum albumin solution at a pH of 7.4. The positive control was a previously reported human sample reacted with a goat–anti human secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA).

RESULTS

No mites or lice were recovered from the flying squirrels. Fleas were recovered from 16 flying squirrels, including nine from YNP and seven from HV. Twenty-eight fleas were identified in two species. Male and female *Opisodasys vesperalis* were recovered from seven flying squirrels from HV and six YNP animals. A single *Ceratophyllus ciliatus mononis* (1 male and 1 female) was found on each of two YNP animals. Four of seven flying squirrels from HV had one tick each. The tick species found on flying squirrels were one adult *Ixodes hearlei*, two larval *I. pacificus*, and one nymphal *Dermacentor parumapertus*. No ticks were found on the remaining flying squirrels.

Antibodies to *A. phagocytophilum* were detected in two flying squirrels, both from HV, for an overall site prevalence of 25% (95% confidence interval 4.5–64.4%). Antibody titers were 100 and 200 in these two flying squirrels. One squirrel from HRSP tested PCR-positive for the *A. phagocytophilum* p44 gene. Sequencing of the *A. phagocytophilum* GroESL gene indicated 98% similarity to seven reported *A. phagocytophilum* sequences. The best match, differing in seven nucleotides, was from a sample reported in 2000 from a human patient in Humboldt County, California (Chae et al., 2000). All samples were PCR-negative and seronegative for *R. prowazekii*.

DISCUSSION

Little is known about zoonotic pathogens in northern flying squirrels. This paper reports *A. phagocytophilum* infection for the first time in any flying squirrel species. Evidence of exposure and infection has previously been reported for *Peromyscus* spp., woodrats, and western gray squirrels (*Sciurus griseus*; Nicholson et al., 1998, 1999; Zeidner et al., 2000; Castro et al., 2001; Foley et al., 2002; Lane et al., 2005; Drazenovich et al., 2006). These data, together with data from the present study, suggest that sciurids could be important hosts in the ecology of GA.

The ticks identified here and earlier on *G. sabrinus* include *Ixodes pacificus*, *Ixodes angustus*, *I. hearlei*, *Ixodes marxi*, and *D. paramapertus* (Wells-Gosling and Heaney, 1984; Murrell et al., 2003). The role of *I. angustus* in GA epidemiology is not known, but vector competence for *Borrelia burgdorferi* has been established (Peavey et al., 2000). Interaction of woodrats with *Ixodes spinipalpis* may help maintain *A. phagocytophilum* infection in nature (Zeidner et al., 2000). Flying squirrels will feed on the ground but have minimal exposure to *I. spinipalpis*. Further research will be necessary to define the ecology of *A. phagocytophilum* in northern flying squirrels and any possible deleterious effects *A. phagocytophilum* might have on this species.

The relatively small sample size precluded definitive evaluation of susceptibility of northern flying squirrels to *R. prowazekii* infection. The seroprevalence of *R. prowazekii* in *G. volans* from Maryland and Virginia ranged from 25% to 75% (Sonenshine et al., 1978), and experimentally infected southern flying squirrels retained infection for 40 days or longer (Bozeman et al., 1981). Transmission was successful among captive flying squirrels using the southern flying squirrel louse *Neohaematopinus sciuropteri* (Bozeman et al., 1981). Additionally, *Ctenocephalides felis*, *Orchopeas howardi*, and *Xenopsylla cheopis* could be infected via feeding on infected flying squirrels. Ticks and mites were considered to be unlikely vectors (Bozeman et al., 1981), although successful inoculation of *R. prowazekii* into the soft tick, *Ornithodorus papillipes*, has been reported (Kesarev and Prodan, 1963), and *R. prowazekii* has been isolated from ticks in Ethiopia (Philip et al., 1966).

The two species of fleas obtained in the present study have been reported from flying squirrels previously (Lewis et al., 1988). It would have been useful to identify mites and lice as well; however, smaller ectoparasites were not removed from animals at the time of capture. The northern flying squirrel flea fauna includes at least 20 species in 15 genera, with some generalist species, such as *Aetheca wagneri* and *C. ciliatus*, and much more specialized species, such as *O. vesperalis* (Hubbard, 1968; Wells-Gosling and Heaney, 1984; Lewis et al., 1988). *Ceratophyllus* spp., which primarily infests chipmunks, also will reportedly bite humans (Hubbard, 1968). Individual flying squirrels may be heavily infested, facilitated by their social system and use of tree hollows and constructed nests (Hubbard, 1968). Breeding females occupy separate nests with only their own young. Males may share nests together (Wells-Gosling and Heaney, 1984; Carey, 1991). In cold months, up to 19 individuals of both sexes may nest communally, although adults abandon nests as they become fouled and flea-infested (Carey, 1991).

Flying squirrels, together with other tree squirrels and semi-arboreal rodents such as woodrats may participate in enzootic cycles of rickettsial disease maintenance. Understanding potential negative repercussions of infection for the flying squirrels and any role of flying squirrels in maintenance of human disease would be an important focus of future studies.

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