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Evidence of Multiple Zoonotic Agents in a Wild Rodent Community in the Eastern Sierra Nevada

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ABSTRACT: This study aimed to describe the occurrence of *Yersinia pestis*, *Rickettsia rickettsii*, *Anaplasma phagocytophilum*, and ectoparasites in a wild rodent community in the eastern Sierra Nevada. From May to September 2006, rodents were live-trapped, examined for ectoparasites, and blood was collected. All rodents were serologically tested for antibodies to *Y. pestis*, *R. rickettsii*, and *A. phagocytophilum*; in addition, blood samples and ectoparasites were tested by PCR to detect the presence of these zoonotic agents. Overall, 89 rodents, 46 fleas, and four ticks were collected. Antibody prevalence rates observed for rodents were 14% for *R. rickettsii* or antigenically related spotted-fever group rickettsiae, and 8% for *A. phagocytophilum*. No samples were positive for antibodies to *Y. pestis*. Positive PCR results included one yellow-pine chipmunk for *Y. pestis* (C_T=32.8), one golden-mantled ground squirrel for *R. rickettsii* (C_T=33), and one flea found to be co-infected with both *R. rickettsii* (C_T=17) and *A. phagocytophilum* (C_T=36). The results of this study provide evidence of multiple zoonoses overlapping within a single, located rodent community.

Key words: *Anaplasma phagocytophilum*, California, fleas, *Rickettsia rickettsii*, rodents, ticks, *Yersinia pestis*.

Multiple bacterial zoonoses overlap in their geographic distribution within wild rodent communities in California, including *Yersinia pestis*, the causative agent of plague; *Rickettsia rickettsii*, which causes Rocky Mountain spotted fever (RMSF); and *Anaplasma phagocytophilum*, the etiologic agent of granulocytic anaplasmosis (GA; Hoar et al., 2003; Harrus and Baneth, 2005; Holden et al., 2006). The complex dynamics that maintain these bacteria are determined by interactions of flea or tick vectors with mammalian hosts, and are modified by climatic factors such as temperature, precipitation, and relative humidity (Harrus and Baneth, 2005). However, the dynamics of how these agents persist, and overlap enzootically, remain largely unknown (Hoar et al., 2003; Holden et al., 2006). Here we aim to describe the prevalence, incidence, and co-infection rates of *Y. pestis*, *R. rickettsii*, and *A. phagocytophilum* within a single wild rodent community, as well as their ectoparasites, in the higher elevations of the eastern Sierra Nevada.

From May to September in 2006, trapping was conducted at three 180 m^2 sites (located at approximately 39.44 to 39.45 decimal-degrees latitude and 120.22 to 120.23 decimal-degrees longitude). Each site was set approximately 2.5 km from the next one along a road that runs through Sagehen Creek Field Reserve, a field station in the eastern Sierra Nevada managed by the University of California, Berkeley, USA. Trapping sites included yellow pine, mixed conifer, and red fir forests; and brush fields, scattered mountain meadows, and fens. During a trapping event, 20 Sherman live-traps (HB Sherman, Tallahassee, Florida, USA) and 10 Tomahawk live-traps (Tomahawk Live Trap, Tomahawk, Wisconsin, USA) were baited with rolled oats and peanut butter, set, and evenly distributed throughout each site (30 traps set, in grids, per 180 m^2 study site; 90 traps total in the reserve per trapping event). Sherman live-traps, used to collect nocturnal rodents, were set in the evening and checked the morning after a trapping night. Tomahawk live-traps were set during daylight hours and checked every 2–3 hr.

Trapping was conducted for 3–4 days monthly at each site.

Trapped rodents were injected with a 1:4 dilution of ketamine/xylazine, identified, and ear-tagged. Blood samples of 0.2–1.0 ml were collected by retro-orbital bleeding into 1.5 ml ethylendiaminetetraacetic acid tubes; samples were stored on ice in the field and then transported to the University of California, Davis and stored at −20 C. Ectoparasites were collected using fine forceps and stored in 70% ethanol. Trapped rodents were allowed to recover from the anesthesia and then released. Recaptured rodents were identified; if more than 2 months had elapsed since initial capture, blood was collected. All traps were sterilized with bleach between trapping events.

Fleas were washed in 70% ethanol, and ventro-dorsal incisions were made across the dorsal abdominal tergites using sterile #40 scalpel blades. Fleas were incubated overnight with 0.4 mg proteinase K and 180 µl buffer ATL (Qiagen, Valencia, California, USA) at 55 C. Exoskeletons were removed for mounting and DNA extracted from the remaining material using the Qiagen DNA extraction kit. Exoskeletons were cleared via 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 to species using North American taxonomic keys (Hubbard, 1968; Lewis et al., 1988). If one rodent yielded multiple fleas, a random sample of three was processed, with the remainder saved in 70% ethanol. Ticks were identified to species using keys (Furman and Loomis, 1984; Webb et al., 1990) and DNA was then extracted using the Qiagen DNA extraction kit.

Whole blood samples were analyzed for *A. phagocytophilum* and *R. rickettsii* using indirect fluorescent assay. Blood was diluted in phosphate buffered saline (PBS) at 1:25, applied to Webster-strain *A. phagocytophilum* antigen slides (Barlough et al., 1995) and commercially purchased *R. rickettsii* antigen slides from VMRD (Pullman, Washington, USA), and incubated at 37 C with moisture for 40 min. Slides were then washed three times in PBS and incubated with fluorescein isothiocyanate-labeled rabbit anti-rat IgG heavy- and light-chain antibodies (Kirkegaard and Perry, Gaithersburg, Maryland, USA) diluted in PBS at 1:30. Slides were washed three additional times and, during the third wash, stained with two drops of Evans Blue for 2 min. All slides included a positive and negative control. Samples were considered positive if strong fluorescence was detected at dilutions of at least 25. For plague serology, anti-*Yersinia* spp. V-antigen IgG was detected in an ELISA format (Anderson et al., 1996). Only 33 specimens were analyzed for *Y. pestis* due to the amount of blood available.

DNA was extracted from each blood sample using a kit (Dneasy Tissue kit, Qiagen) according to manufacturer's instructions. PCR assays were performed on all extracted blood and flea DNA samples using the TaqMan real-time PCR system in a combined thermocycler/fluorometer (ABI Prism 7700, Applied Biosystems, Foster City, California, USA). Each 12 µl reaction contained 1 µl DNA, 1× TaqMan Universal Master Mix (Applied Biosystems), 2 nmol each primer, and 400 pmol probe. The thermocycling conditions consisted of 50 C for 2 min, 95 C for 10 min, and 40 cycles at 95 C for 15 sec, followed by 60 C for 1 min. Samples were considered positive if they had a cycle threshold (C_T) value <40 and characteristic amplification plots. Real-time PCR reactions for the *msp2* gene of *A. phagocytophilum* were performed as reported previously (Drazenovich et al., 2006; Leutenegger et al., 1999). A TaqMan system was developed for amplification of the *Y. pestis* pla gene and the *Rickettsia* sp. APGEA35 citrate synthase (gltA) gene. The primers and probe for *Y. pestis* are: YPpla-109F-GGAGGTACTC-AGACCATTGATAAGA

AT, YPpla-209R-AGACC CGCCGTCAC AGTATAA, and YPpla-159P-CGGAGAT GCTGCCGGTATTTC CAATAAAA. The primers and probe for *Rickettsia* sp. are: 5′ AATGCTTCTACTTCAACAGTCC GAA T 3′, 5′ GTG AGGCAATACCCGTGCT AA 3′, and 5′ CTCATCCGGAGCTAACC CTTTTGCTTGT 3′ (courtesy of Christian Leutenegger, Lucy Whittier TaqMan Service, University of California, Davis, California, USA).

Data were maintained in Excel (Microsoft, Redmond, Washington, USA). Descriptive statistical analyses were conducted to describe the antibody prevalence rates observed for each pathogen and for the distribution of antibody positive rodents and ectoparasites by month, collection site, and species. PCR results were also summarized and described.

Overall, 114 rodent captures (22% recaptures) representing 89 unique rodents occurred, including 45 yellow-pine chipmunks (*Tamias amoenus*), 31 deer mice (*Peromyscus maniculatus*), six lodgepole chipmunks (*Tamias speciosus*), four golden-mantled ground squirrels (*Spermophilus lateralis*), and three California ground squirrels (*Spermophilus beecheyi*). Approximately 29% of the rodents were captured on study site one, 50% from study site two, and 21% from study site three. Blood samples were collected from all rodents, with a second sample taken from one recaptured lodgepole chipmunk (90 blood samples total). Four ticks were collected from three rodents, all *Dermacentor andersoni* nymphs, and 48 fleas from 25 rodents, of which 39 fleas were analyzed and identified. These included six *Aetheca wagneri*, 20 *Ceratophyllus ciliatus mononis*, five *Eumolpianus eumolpi*, five *Oropsylla montana*, and three *Peromyscopsylla hesperomys adelpha*. Approximately 24% of the infested rodents came from study site one, 34% from study site two, and 28% from study site three. A summary of the rodents and their ectoparasites collected, by month, is shown in Table 1.

All rodent samples were analyzed for *R. rickettsia* and *A. phagocytophilum*; 33 were analyzed for *Y. pestis* (Table 1). Overall, 14% were positive for antibodies to *R. rickettsii* or antigenically related spotted-fever group rickettsiae (Marshall et al. 2003), and 8% tested seropositive for *A. phagocytophilum*. Antibodies to *Y. pestis* were not detected. Additionally, 2% were positive for antibodies to both spotted-fever group rickettsiae and *A. phagocytophilum*.

All samples were tested by PCR for *R. rickettsii*, *Y. pestis*, and *A. phagocytophilum*. Of these, one male yellow-pine chipmunk sampled in May was positive for *Y. pestis* (C_T=32.8) and one male golden-mantled ground squirrel sampled in June was positive for *R. rickettsii* (C_T=33.0). Additionally, a *C. ciliatus mononis* taken off of a female yellow-pine chipmunk in July was co-infected with *R. rickettsii* (C_T=17.0) and *A. phagocytophilum* (C_T=36.0).

The antibody prevalence rates for *A. phagocytophilum* observed in our study are similar to those reported in previous studies, though the rodent species differ. Nicholson et al. (1998) reported serologic evidence of exposure, in several *Peromyscus* species, ranging from 9% to 10% in northern California, though no PCR positives were found. We found an exposure rate of 9% in chipmunks, with the only PCR positive sample for *A. phagocytophilum* in our study coming from a *C. ciliatus mononis* taken off of an infested female chipmunk. Infection in chipmunks with *A. phagocytophilum* has been previously reported, though all were in the eastern US (Foley et al., 2004).

All four ticks collected directly from rodents were *D. andersoni*, the primary vector of RMSF in California; RMSF is the most severe human rickettsial disease in the US (Warner and Marsh, 2002). However, even though both transovarial and transstadial transmission of *R. rickettsii* are reported to occur in *D. andersoni* ticks, only 1% to 5% harbor the pathogen,

TABLE 1. Rodent and ectoparasite species, numbers, and antibody prevalence at Sagehen Creek Field Reserve from May to September 2006.

Rodent species (*n*)	Ecoparasite species (*n*)	*Rickettsii rickettsii* (% antibody positive)	*Anaplasma phagocytophilum* (% antibody positive)
Peromyscus maniculatus (31)	*Aetheca wagneri* (4) *Peromyscopsylla hesperomys adelpha* (3)	13%	0%
Tamias amoenus (45)	*Dermacentor andersoni* (4) *Ceratophyllus ciliatus mononis* (18) *Eumolpianus eumolpi* (5)	16%	7%
Spermophilus lateralis (4)	*Oropsylla montana* (1)	25%	25%
T. speciosus (6)	*C. ciliatus mononis* (2)	17%	33%
S. beecheyi (3)	*A. wagneri* (2) *O. montana* (4)	33%	33%

even in the most endemic areas (Warner and Marsh, 2002), thus severely limiting our ability to detect the pathogen with only four ticks. Even so, over 14% of all rodents sampled were positive for *R. rickettsii* antibodies, including 14% of chipmunks, 13% of deer mice, and 33% of California ground squirrels. Additionally, one PCR-positive chipmunk flea was detected that was also co-infected with *A. phagocytophilum*. While fleas do not transmit these diseases, these results demonstrate that the host was actively infected with both pathogens.

While no rodents were antibody positive for *Y. pestis*, we detected a PCR-positive chipmunk in early May prior to the active flea and plague season; this may suggest that *Y. pestis* was transmitted by a flea maintaining the bacteria over winter. Of the 30 mammalian species suspected to be involved in plague transmission in California, none have been identified as a classical reservoir (Gage and Kosoy, 2005). Instead, the 20 flea species reported to transmit *Y. pestis* in California (Adjemian et al., 2006) may contribute significantly to its maintenance. Eskey and Haas (1940) noted that fleas may function as reservoirs because they can retain infection for several months in cold climates. Many *Y. pestis*-infected fleas can maintain the bacterium in their gut for over 6 mo when held at 14–27 C, and some species maintained infection for over 15 mo at 0–15 C (Gage and Kosoy, 2005). Infected fleas have also been shown to survive in burrows for up to 1 yr after rodent die-offs (Gage and Kosoy, 2005; Girard et al., 2004), enabling them to persist in off-host environments between typical plague-transmission seasons. Several fleas identified in this study are potential vectors of plague; *Eumolpianus* spp., *A. wagneri*, and *O. montana* are relatively efficient vectors of plague, and *O. montana* readily feeds on humans when typical hosts are unavailable (Gage and Kosoy, 2005). This is especially important at our study site, because all *O. montana* were found on ground squirrels, which exhibit high plague-induced mortality rates (Lang, 1993).

This study provides evidence that the etiologic agents of plague, RMSF, and GA coexist within a single wild rodent community. More studies are needed in order to determine how this overlap may increase the likelihood for co-infection, which may complicate diagnosis and treatment in people and animals. Increasing our awareness of how these bacterial

agents interact enzootically will facilitate better management of the risks present to both human health and sensitive wildlife species.

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