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Coinfection of Western Gray Squirrel (*Sciurus griseus*) and other Sciurid Rodents with *Borrelia burgdorferi* sensu stricto and *Anaplasma phagocytophilum* in California

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ABSTRACT: Overlapping geographic distributions of tick-borne disease agents utilizing the same tick vectors are common, and coinfection of humans, domestic animals, wildlife, and ticks with both *Borrelia burgdorferi* and *Anaplasma phagocytophilum* has been frequently reported. This study was undertaken in order to evaluate the prevalence of both *B. burgdorferi* sensu stricto (hereinafter referred to as *B. burgdorferi*) and *A. phagocytophilum* in several species of sciurid rodents from northern California, USA. Rodents were either collected dead as road-kills or live-trapped in four state parks from 13 counties. Thirty-seven western gray squirrels (*Sciurus griseus*), nine nonnative eastern gray squirrels (*S. carolinensis*) and an eastern fox squirrel (*S. niger*), four Douglas squirrels (*Tamiasciurus douglasii*), and two northern flying squirrels (*Glaucomys sabrinus*) were tested by polymerase chain reaction (PCR) and serology for evidence of coinfection. Of the 14 individual *S. griseus* that were PCR-positive for *B. burgdorferi*, two (14%) also were PCR-positive for *A. phagocytophilum* and 11 (79%) had serologic evidence of *A. phagocytophilum* exposure. Two of the four Douglas squirrels were PCR positive for *B. burgdorferi* and seropositive to *A. phagocytophilum*. Evidence of coinfection with these zoonotic pathogens in western gray squirrels suggests that both bacteria may be maintained in a similar transmission cycle involving this sciurid and the western black-legged tick *Ixodes pacificus*, the primary bridging vector to humans in the far-western US.

Key words: Coinfection, granulocytic anaplasmosis, *Ixodes pacificus*, Lyme borreliosis.

In recent years, both Lyme borreliosis (LB), caused by several genospecies in the *Borrelia burgdorferi* sensu lato (s.l.) complex and granulocytic anaplasmosis (GA), caused by *Anaplasma phagocytophilum*, have emerged in many areas throughout the Holarctic (Bakken et al., 1994; Ostfeld and Keesing, 2000). In the Northern Hemisphere, *B. burgdorferi* s.l. and *A. phagocytophilum* are transmitted by four members of the *Ixodes ricinus* tick-complex in sylvatic ecologic cycles involving small mammals, birds, or lizards (Brown et al., 2005). Overlapping geographic distributions of these diseases are common, and co-infection of humans, domestic animals, wildlife, and ticks with both *B. burgdorferi* and *A. phagocytophilum* has been frequently reported (Swanson et al., 2006). The ecologic maintenance and subsequent risk of coinfecting pathogens to humans, domestic animals, and wildlife remain poorly understood.

Worldwide, 15 genospecies of *B. burgdorferi* s.l. have been described (Postic et al., 2007). *Borrelia burgdorferi*, *B. afzelii*, and *B. garinii* are the only genospecies that commonly cause clinical disease in humans, and *B. burgdorferi* is the only one known to infect people in North America. In California alone, five genospecies have been detected in or isolated from ticks or vertebrates (Burgdorfer et al., 1985; Postic et al., 1998, 2007), and recent molecular evidence suggests that others await description (Brown et al., 2006).

The evolutionary and ecologic complexity of *A. phagocytophilum* in northern California shares some features with *B. burgdorferi* (Foley et al., 2004). For instance, there are locations in Placer County, Santa Cruz County, and parts of Sonoma County where equine and canine cases are abundant yet dusky-footed wood rats (*Neotoma fuscipes*), putative primary reservoir host, manifest no evidence of

infection (Madigan et al., 1990; Nicholson et al., 1999; Foley et al., 2001). Sequences from the *msp2* gene of isolates from a hyperendemic area in Humboldt County were similar to each other, but distinctly different from eastern US human isolates (Barbet et al., 2006; Drazenovich et al., 2006).

In the eastern US, the primary reservoir host for *B. burgdorferi* and *A. phagocytophilum* is the white-footed mouse, *Peromyscus leucopus,* and the tick vector is the blacklegged tick, *Ixodes scapularis* (Telford and Spielman, 1989; Burgdorfer, 1991). In the western US, both *B. burgdorferi* and *A. phagocytophilum* were initially described as being maintained in cycles involving dusky-footed wood rats and *I. pacificus* ticks (Brown and Lane, 1992; Nicholson et al., 1999; Foley et al., 2002). However, there is now mounting evidence that additional maintenance hosts (*S. griseus* and *Dipodomys californicus* for LB, *S. griseus* for GA) also may be important reservoirs (Lane et al., 2005; Nieto and Foley, 2008).

To evaluate the prevalence of both *B. burgdorferi* and *A. phagocytophilum* in sciurids from northern California, animals were either collected dead as road-kills or live-trapped from sites where both LB and GA reportedly are of high risk to humans, dogs, or wildlife. Fresh, road-killed animals were placed in zip-lock bags and then transported to the laboratory. All animals were identified to species, age, and sex and then dissected. Coagulated heart blood, spleen, and ticks were removed. Blood and spleen tissue were frozen (−20 C) prior to serologic and genetic analyses, whereas ticks were placed in 70% ethanol and identified to species, stage, and sex using taxonomic keys (Furman and Loomis, 1984). Animals that did not retain any significant internal morphology due to trauma or gross autolysis were discarded.

Additional sciurids were captured in four California State Parks (Big Basin SP, Samuel P. Taylor SP, Hendy Woods SP, and Humboldt Redwoods SP) during spring and autumn over a period of 2 yr (2006–07). Sciurids were trapped using 15×15×47.5-cm wire-mesh Tomahawk live-traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) in areas where sciurids were observed. One trap was attached at breast height (ca. 1.5 m) to the trunk of the largest tree in the area and one placed at the base of the same tree. Trapped animals were anesthetized using ketamine (20–40 mg/kg) and xylazine (4 mg/kg), identified to species, weighed, marked with an identifying numbered ear tag, and bled via venipuncture of the femoral vein. Blood was placed into tubes containing ethylenediamine tetraacetic acid (EDTA) and kept cold until transported to University of California–Davis and frozen (−20 C) prior to serologic and DNA analyses. Moreover, a small piece (1–2 mm^2) of ear tissue was removed from one pinna of all rodents using sterile scissors. The tissue was placed on ice and then frozen at −20 C until processing.

Total DNA was extracted for *A. phagocytophilum* from whole blood, if available, or spleen using a DNAeasy Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions. DNA extraction of sciurid ear tissue for *B. burgdorferi* used the same kit and followed the procedures for animal tissue extraction. Polymerase chain reaction (PCR) was performed for the *A. phagocytophilum msp2/p44* gene using real-time TaqMan PCR (Drazenovich et al., 2006). Polymerase chain reaction for *B. burgdorferi* was performed using traditional PCR of the *rrf-rrl* gene (Lane et al., 2007). All *B. burgdorferi* positive samples were then sequenced and compared to known *B. burgdorferi* isolates using neighbor joining and distance matrix comparison methods (Postic et al., 2007).

Serology was performed on plasma specimens by indirect flluorescent assay to determine exposure to *A. phagocytophilum*. Plasma was diluted in phosphate-buffered saline at 1:25, applied to Web-

ster-strain *A. phagocytophilum* antigen slides (Dumler et al., 1995), and incubated at 37 C at 100% humidity for 30 min. A secondary, antirat heavy and light chain IgG-FITC (KPL Inc., Gaithersburg, Maryland, USA) was applied at a 1:25 dilution and slides further incubated at 37 C for an additional 30 min. Slides were stained using erichrome black and viewed using a compound fluorescent microscope. Positive and negative controls were included in each run.

For each squirrel species that was coinfected, the odds of coinfection were calculated as a cross-product ratio using numbers of *B. burgdorferi*-only PCR positive individuals, *A. phagocytophilum*-only PCR positive individuals, coinfected individuals, and uninfected individuals, *B. burgdorferi*-only PCR positive individuals, *A. phagocytophilum* serologically positive, coinfected and uninfected individuals. This information provides us with the relative risk of either being actively coinfected with both pathogens and of being infected with *B. burgdorferi* and exposed to *A. phagocytophilum*. Data were maintained in Excel (Microsoft, Redmond, Washington, USA) and analyzed in R (R-Development Core Team, http://www.r-project.org).

Five species of sciurids were tested consisting of 37 western gray squirrels (*S. griseus*), nine nonnative eastern gray squirrels (*S. carolinensis*) and a nonnative eastern fox squirrel (*S. niger*), four Douglas squirrels (*Tamiasciurus douglasii*), and two northern flying squirrels (*Glaucomys sabrinus*) from 13 counties in northern California. Most *S. griseus* were collected as road-kill (32 of 37, 86%), while *S. carolinensis* (seven of nine, 78%), *S. niger* (one of one, 100%), *T. douglasii* (four of four, 100%), and *G. sabrinus* (two of two, 100%) were mostly live-caught. Serologic prevalence of *A. phagocytophilum* varied by species with only the two northern flying squirrels having no evidence of infection (Table 1). However, both sero- and PCR-positive flying squirrels were identified previously (Foley et al., 2007). *Sciurus griseus* and *S. carolinensis* were PCR-positive for *A. phagocytophilum* (Table 1). Three species were PCR-positive for *B. burgdorferi* including *S. carolinensis* (11%), *S. griseus* (38%), and *T. douglasii* (50%; Table 1). All *B. burgdorferi* PCR products sequenced from the squirrels in this study had a high sequence similarity to *B. burgdorferi*.

Coinfection with *B. burgdorferi* and *A. phagocytophilum* occurred in *S. griseus* and *T. douglasii* (Table 1). Of the 14 individual *S. griseus* that were PCR-positive for *B. burgdorferi*, two (14%) also were PCR positive for *A. phagocytophilum* and 11 (79%) had serologic evidence of *A. phagocytophilum* exposure (Table 1). Both of the gray squirrels coinfected (PCR-positive) were from Humboldt County, California. Of the gray squirrels that were PCR-positive for *B. burgdorferi* and were seropositive for *A. phagocytophilum*, exposed squirrels were identified from five counties (Humboldt Co., Mendocino Co., Napa Co., Trinity Co., and Siskyou Co.) all located in the northern coast range of California. Both *T. douglasii* individuals infected with *B. burgdorferi* had serologic evidence of exposure to *A. phagocytophilum* but neither was PCR-positive to the latter bacterium. Both of these animals were found in Mendocino County. All odds ratios (ORs) calculated were >1 although none was statistically significant (Table 1).

This study provides evidence of coinfection with *B. burgdorferi* and *A. phagocytophilum* in two out of three *Sciurus* species for which at least nine animals were tested, as well as in just two of four *T. douglasii*. In Mendocino County, the western gray squirrel was implicated as a primary reservoir host of the LB spirochete, because eight of 10 squirrels and 47% of the *I. pacificus* larvae attached to them were PCR positive for *B. burgdorferi* (Lane et al., 2005). In the same series of squirrels, blood or a skin biopsy from two squirrels was PCR-positive for *A.*

TABLE 1. Numbers of squirrels infected with *Borrelia burgdorferi* (Bb) and/or *Anaplasma phagocytophilum* (Ap) throughout northern California, USA.

Species	*n*	Bb positive	Ap PCR positive[a]	Ap seropositive	Bb PCR+/Ap PCR+ (coinfection)[a]	Bb PCR+/Ap seropositive (coinfection)[a]
G. sabrinus	2	0	0	0	0	0
S. carolinensis	9	1	1	5	0	0
S. griseus[b]	37	14	4	24	2	11
S. niger	1	0	0	1	0	0
T. douglasii[c]	4	2	0	3	0	2
Total	53	17	5	33	2	13

[a] PCR = polymerase chain reaction.

[b] Odds ratio for PCR coinfection=1.72, CI=0.11–26.6, P=0.62, Odds ratio for serologic coinfection=2.74, CI=0.51–19.45, P=0.28.

[c] Odds ratio for serologic coinfection=2.60, CI=0.07–234, P=0.22.

phagocytophilum (Lane et al., 2005). Also, a northern flying squirrel was PCR-positive with *A. phagocytophilum* in a study evaluating rickettsial diseases in California (Foley et al., 2007). In another study, PCR and sero-positive squirrels were identified in all regions where various squirrels were collected, and logistic regression identified being a western gray squirrel (odds ratio [OR]=20.5, $P=2.95\times10^{-8}$) and from the north coastal region of California (OR=9.052, $P=1.41\times10^{-6}$) as having the highest risk of exposure to *A. phagocytophilum* (Nieto and Foley, 2008). In both the Lane et al. (2005) and Nieto and Foley (2008) studies, *S. griseus* was infested by *I. pacificus*. *Sciurus vulgaris* in Europe and *S. carolinensis* in eastern North America are both able to infect *Ixodes* spp. ticks with *B. burgdorferi* sensu lato (Humair and Gern, 1998; Ostfeld et al., 2002) and *S. carolinensis* was experimentally shown to be reservoir competent for *A. phagocytophilum* when fed on by *I. scapularis* (Levin et al., 2002).

Extensive exposure in multiple counties in northern California suggests that sciurid rodents are important in maintaining the agents of both LB and GA, and indicates that studies of reservoir competence of these species are warranted. In particular, co-infection with both zoonotic agents in the western gray squirrel confirms recent evidence (Lane et al., 2005, Nieto and Foley, 2008) suggesting that both bacteria are maintained in a similar transmission cycle involving this sciurid and the western black-legged tick *Ixodes pacificus*, the primary bridging vector to humans in the Far West. Due to the immunomanipulative nature of *A. phagocytophilum*, coinfection with *B. burgdorferi* may be likely, increasing risk for humans, domestic animals, and wildlife.

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