JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2007, p. 2411–2418 Vol. 45, No. 8
0095-1137/07/$08.00+0 doi:10.1128/JCM.02539-06

Gray Foxes (*Urocyon cinereoargenteus*) as a Potential Reservoir of a *Bartonella clarridgeiae*-Like Bacterium and Domestic Dogs as Part of a Sentinel System for Surveillance of Zoonotic Arthropod-Borne Pathogens in Northern California▽

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Received 19 December 2006/Returned for modification 6 March 2007/Accepted 30 May 2007

Two species of *Bartonella*, a novel *Bartonella clarridgeiae*-like bacterium and *B. vinsonii* subsp. *berkhoffii*, were isolated from rural dogs and gray foxes in northern California. A novel *B. clarridgeiae*-like species was isolated from 3 (1.7%) of 182 dogs and 22 (42%) of 53 gray foxes, while *B. vinsonii* subsp. *berkhoffii* was isolated from 1 dog (0.5%) and 5 gray foxes (9.4%). PCR and DNA sequence analyses of the citrate synthase (*gltA*) gene and the 16S-23S intergenic spacer region suggested that strains infecting dogs and gray foxes were identical. Fifty-four dogs (29%) and 48 gray foxes (89%) had reciprocal titers of antibodies against *Bartonella* spp. of ≥64. The high prevalence of bacteremia and seroreactivity to *Bartonella* spp. in gray foxes suggests that they may act as a reservoir species for the *B. clarridgeiae*-like species in this region. Domestic dogs were also tested for other arthropod-borne infectious agents. Fifty-one dogs (28%) were positive for *Dirofilaria immitis* antigen, seventy-four (40%) were seroreactive to *Anaplasma phagocytophilum*, and five (2.7%) were seropositive for *Yersinia pestis*. Fourteen dogs (7.6%) were PCR positive for *A. phagocytophilum*. Polytomous logistic regression models were used to assess the association of *Bartonella* antibody titer categories with potential risk factors and the presence of other vector-borne agents in domestic dogs. Older dogs were more likely to be seroreactive to *Bartonella* spp. There was no association between the exposure of dogs to *Bartonella* and the exposure of dogs to *A. phagocytophilum* in this study.

Bartonella spp. are fastidious gram-negative bacteria that infect the erythrocytes and endothelial cells of their hosts (3). More than 20 species or subspecies of *Bartonella* have been described, and 12 of these are recognized as human pathogens. Among the 12 zoonotic *Bartonella* spp., 6 have been identified in domestic dogs (9, 29), suggesting that dogs may have potential as part of an important sentinel system for the surveillance of *Bartonella* spp. that infect humans. *Bartonella* spp. are usually vector borne (9), although the vectors involved in the transmission of *Bartonella* to dogs have not been definitively identified. Ticks are suspected as a vector for the transmission of *Bartonella vinsonii* subsp. *berkhoffii* to dogs. A survey in a Walker hound kennel in North Carolina found that 18 of the 19 dogs that were *Bartonella* PCR positive were also PCR positive for *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Babesia canis*, or *Rickettsia rickettsii* (28). In addition, several studies have reported antibodies against one or more tick-borne infectious agents in dogs that are *Bartonella* seroreactive (25, 34, 42). Coyotes (*Canis latrans*) are suspected of serving as a wildlife reservoir of *B. vinsonii* subsp. *berkhoffii* in some areas of California, as 28% of coyotes (31 of 109) in Santa Clara County that were tested were found to be bacteremic (8). Fleas have been implicated in the transmission of *B. henselae* among domestic cats, which are the principal reservoir of this bacterium (12). Infection in cats appears to occur through the multiplication of *B. henselae* bacteria in the gut of the cat flea and subsequent intradermal inoculation of the infectious flea feces when the cat scratches itself (16, 17). The role of fleas in the transmission of *Bartonella* spp. to dogs is unknown, although DNA of *B. vinsonii* subsp. *berkhoffii* and a species closely related to *B. clarridgeiae* was previously identified among DNA extracted from *Pulex* sp. fleas collected in Peru (36).

The identification of vector-borne diseases in domestic dogs is important to both veterinary and human medicine. Clinical illness in dogs due to infection with *Bartonella* spp. (9), *A. phagocytophilum* (2, 31), and *Dirofilaria immitis* (13), among various vector-borne pathogens, has been reported previously. The identification of infectious agents such as *Bartonella* spp., *A. phagocytophilum*, *Yersinia pestis*, and *D. immitis* in domestic dogs can also provide important information about the risk of human exposure to these zoonotic pathogens within defined geographic areas, as dogs may serve as sentinel systems indicating the presence of an infectious agent within local arthropod and wildlife populations (9, 11, 20, 43).

A. phagocytophilum, the agent of granulocytic anaplasmosis,

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▽ Published ahead of print on 6 June 2007.

is associated with the vector *Ixodes pacificus* in California (20). Dogs often have mild or subclinical symptoms from *A. phagocytophilum* infection and have been suggested as a possible a sentinel system for the surveillance of *A. phagocytophilum*. Two human cases of granulocytic anaplasmosis were diagnosed in Humboldt County, CA, in 1998 (18), and a 2001 study reported a canine seroprevalence of 47% in this region (19).

Plague is caused by the bacterium *Y. pestis* and is endemic in the western half of the United States, where it is maintained in nature through flea-rodent transmission cycles. Domestic dogs are usually exposed through contact with infected rodents or their fleas (11). Clinical signs appear to be rare in dogs naturally infected with *Y. pestis*, and experimentally infected dogs experience a mild febrile illness (40). Public health prevention programs in California have typically used rodent and wild carnivore samples for plague surveillance; however, the surveillance of plague antibody in domestic carnivores has also been suggested as a supplement for defining plague activity. A survey of serum samples collected from 4,115 dogs in California between 1979 and 1991 found that 2.1% had *Y. pestis* antibodies, with the highest seroprevalences observed among samples from Native American reservations (4.0%), military bases (3.3%), and areas where an outbreak investigation or suspected human cases had occurred (15.8%) (11).

D. immitis, the cause of canine heartworm, has been detected in all 50 states of the United States and is regionally endemic in California (13, 32). *D. immitis* is the most important zoonotic species in the genus *Dirofilaria* in the United States (45), and transmission to dogs and humans occurs through the bite of a mosquito infected with third-stage larvae (13). Infection with *D. immitis* in humans may result in a "coin lesion" in the lungs, which is a health care concern primarily because of the diagnostic workup required to rule out other serious conditions that this finding may imply (45).

Our primary purpose was to detect and characterize infection with *Bartonella* spp. in domestic dogs and gray foxes in a rural area of northern California, as well as to investigate factors associated with *Bartonella* exposures. Gray foxes in this study had been previously tested for *A. phagocytophilum* as part of another study (21). However, as dogs living in rural areas often have intense exposure to arthropod vectors, domestic dogs in this study were also tested for *A. phagocytophilum*, *D. immitis*, and *Y. pestis* in order to assess their importance as part of a sentinel system for the surveillance of these zoonotic arthropod-borne infections.

MATERIALS AND METHODS

Sample collection. Blood samples were collected from dogs and gray foxes in a rural area of Humboldt County along the Trinity River corridor near the town of Hoopa in northern California. Blood samples were collected from 184 rural dogs at three separate spay/neuter and vaccination clinics in 2004 and 2005. On a questionnaire, owners were asked to supply information on age, gender, breed, the amount of time the dog spent outdoors, and whether or not flea control was used routinely. Owners and veterinary staff were also asked to indicate whether any clinical signs of disease were present at the time the dog was visiting the clinic. Blood samples were then sent to the University of California, Davis, for testing. In the laboratory, plasma from the EDTA tubes was transferred into 1.5-ml sterile plastic vials; blood and plasma samples were frozen at −70°C.

Fifty-four gray foxes (*Urocyon cinereoargenteus*) were live trapped in the same rural area of Humboldt County between June 2003 and October 2004 in 81-by-25-by-31-cm traps (Tomahawk Live Trap Company, Tomahawk, WI) with attached modified nest boxes (22). Each captured fox was weighed and then anesthetized with 20 mg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA)/kg of body weight and 4 mg of xylazine (Wildlife Pharmaceuticals, Inc., Fort Collins, CO)/kg via intramuscular injection. Blood was collected from a femoral, jugular, or cephalic vein and stored in 4-ml nonheparinized sterile tubes for the removal of serum and 3-ml sterile heparinized tubes for the isolation of pathogens. Blood was kept at 4 to 12°C until centrifuged later the same day. The whole blood was then frozen at −70°C. All foxes were released at their capture location once they exhibited normal responses to stimuli and regained locomotor capacity. Each fox received a uniquely numbered modified Roto ear tag (Premier 1 Supplies, Washington, IA) in each ear for future identification. Blood samples were collected from foxes recaptured ≥1 month from the most recent capture date.

Culture of blood samples for *Bartonella* spp. Blood samples from dogs and gray foxes were cultured on heart infusion agar containing 5% rabbit blood and incubated in 5% CO_2 at 35°C for up to 4 weeks (7, 10). Morphological characteristics and the time of growth on the plates were used for the initial identification of the isolates, as previously described (27).

***Bartonella* PCR procedures.** DNA extraction from putative *Bartonella* isolates, amplification using PCR analysis, and DNA sequencing of the 16S-23S intergenic spacer (ITS) region (38) and the citrate synthase *gltA* (33) gene allowed the specific identification of the isolates. The DNA was extracted as previously described (7). PCR vials were set up as previously described (10) except that DNA was diluted 1:10 in buffer (10 mM Tris, 1 mM EDTA) and 0.25 μM (each) primer was used. The primers used for the ITS region were 5′-CTTCGTTTCT CTTTCTTCA-3′ and 5′-CTTCTCTTCACAATTTCAAT-3′. Run conditions were 94°C for 10 min and then 44 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 0.75 min, followed by a final step at 72°C for 10 min. The primers used for the *gltA* gene were 5′-GGGGACCAGCTCATGGTGG-3′ and 5′-AA TGCAAAAAGAACAGTAAACA-3′. Run conditions were 94°C for 10 min and then 40 cycles of 94°C for 0.5 min, 57°C for 1 min, and 72°C for 2 min, followed by a final step at 72°C for 5 min. An approximately 400-bp fragment of the *gltA* gene and a 670-bp fragment of the ITS region were amplified and then verified by gel electrophoresis. Amplified PCR products were identified by ethidium bromide fluorescence after electrophoresis on 2% agarose gels (SeaKem LE agarose; Cambrex Bio Science Rockland Inc., Rockland, ME).

***Bartonella* DNA sequencing and alignment.** PCR products from the *gltA* gene and ITS region were used for DNA sequencing. Products were purified with the QIAquick PCR purification kit (QIAGEN Sciences, MD), and the sequencing of both DNA strands was done using a fluorescence-based automated sequencing system (Davis Sequencing, Davis, CA).

A consensus sequence for each amplification product was obtained after raw sequences were imported into Vector NTI Suite 9.0 software (Invitrogen Co., Carlsbad, CA). Blastn (http://ncbi.nih.gov/BLAST/) was utilized to compare sequences with entries in GenBank. AlignX in the Vector NTI package was then used to align sequence variants of each gene with one another and with relevant sequences available through GenBank.

***A. phagocytophilum* PCR procedures.** DNA was extracted from 200 μl of whole blood from the 184 rural dogs by using the commercial DNeasy tissue kit according to the instructions of the manufacturer (QIAGEN, Valencia, CA). A real-time PCR system for *A. phagocytophilum* was run using primers and probes, as described previously (14, 37), in a combined thermocycler and fluorometer (ABI Prism 7700; Applied Biosystems, Foster City, CA).

Serology. Antibodies against *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and *B. henselae* isolates in both domestic dog plasma samples and gray fox serum samples were detected using an indirect immunofluorescent-antibody (IFA) assay. The IFA procedure was similar to the procedure previously described (8), with modifications. A 90% confluent tissue culture flask (containing MDCK cells) was inoculated with a 4-day-old culture of *B. vinsonii* subsp. *berkhoffii* (ATCC 51672) resuspended in 0.5 ml of saline. Similarly, flasks containing Vero tissue cultures were inoculated with either *B. clarridgeiae* ATCC 51734 or a mixture of *B. henselae* ATCC 49882 and *B. henselae* U4 (University of California, Davis, strain). The flasks were incubated for 2 days at 37°C with 5% CO_2. After incubation, the tissue culture was washed two times with calcium- and magnesium-free 10× Hanks' balanced salt solution (GIBCO-BRL, Gaithersburg, MD) and trypsinized (GIBCO-BRL) for 40 min (MDCK cells) or 10 min (Vero cells) at 37°C. The suspended tissue culture was centrifuged at 200 × *g* for 10 min. The supernatant was discarded, and the cells were resuspended in 30 ml of tissue culture growth medium. Forty microliters of the cell culture was spotted onto heavy Teflon-coated supercured glass slides (12-well slides; Cell-Line/Erie Scientific Co., Newfield, NJ), and the slides were incubated overnight at 37°C with 5% CO_2. The slides were then washed twice in phosphate-buffered saline (PBS [pH 7.4]; Sigma Chemical, St. Louis, MO), set for 20 min in acetone at room temperature, air dried, and then stored at −20°C until they were used. Plasma or

serum samples added to the test wells were initially screened at dilutions of 1:32 and 1:64 in PBS with 5% milk. Slides were incubated at 37°C for 30 min, followed by three washes in PBS. Fluorescein-conjugated goat anti-dog immunoglobulin G (IgG; ICN Biomedicals Inc., Irvine, CA) was diluted in PBS (1:1,400 for *B. vinsonii* subsp. *berkhoffii*, 1:3,600 for *B. clarridgeiae*, and 1:2,800 for *B. henselae*) with 5% milk containing 0.001% Evans blue, and 20 μl of the dilution was applied to each well. The slides were incubated at 37°C for 30 min and again washed in PBS three times. Two microliters of antifade reagent in glycerol buffer (Molecular Probes, Eugene, OR) and glass coverslips were applied to the slides prior to evaluation. The intensity of bacillus-specific fluorescence was scored subjectively from 1 to 4. Samples with a fluorescence score of ≥2 at a dilution of 1:64 were titrated in serial twofold dilutions to the end point. The same two readers performed a double-blind reading of each slide. Negative and positive control samples were included on each slide.

Anti-*A. phagocytophilum* IgG in the 184 rural dogs was assayed by the IFA procedure (15) using *A. phagocytophilum*-infected HL-60 cells as a substrate and fluorescein isothiocyanate-labeled goat anti-dog IgG (Kirkegaard & Perry, Gaithersburg, MD). Samples starting at dilutions of 1:20 were tested, and canine positive and negative control sera were included on each run. Samples were considered positive if strong fluorescence at dilutions of >1:80 was detected (15). Samples from gray foxes were not tested for *A. phagocytophilum* as part of this study.

Plasma from the rural dogs was tested for antibodies to *Y. pestis* by using a passive hemagglutination assay (PHA) with hemagglutination inhibition (PHI) controls as described by the World Health Organization Expert Committee on Plague (47). Samples were considered positive at titers of ≥1:16 (11, 44). The PHA and the PHI tests were run simultaneously, and samples showing strong reactivity in both the PHA and the PHI tests were considered to be nonspecific and classified as negative. Gray foxes were not tested for other arthropod-borne pathogens as part of this study. Dogs of ≥6 months of age were tested for adult female *D. immitis* cuticular antigen by using a commercially available enzyme-linked immunosorbent assay kit (Dirochek; Symbiotics Corp., San Diego, CA) as previously described (46).

Variable definitions and statistical analysis. Polytomous logistic regression, using the proportional odds model, was performed in LogXact-6 (Cytel Software Co., Cambridge, MA) to assess the relationships between domestic dog *Bartonella* IFA titers and several covariates. The *Bartonella* IFA titer was the ordered polytomous response, and four categories of dog serology results were created. Negative titers were defined as reciprocal titers of ≤32, as previous studies of *Bartonella* in dogs and coyotes have used a reciprocal IFA titer of ≥64 to define a positive reaction (8, 34, 42, 43). The small size of the study prevented examining each IFA titer individually as the response. Therefore, for dogs, reciprocal titers of 64 and 128 were classified as low, reciprocal titers of 256 and 512 were classified as moderate, and reciprocal titers of ≥1,024 were classified as high. IFA testing for three *Bartonella* antigens was done, and as there was often reactivity to more than one antigen, results for the dogs were classified according to the highest reciprocal titer observed among those corresponding to the three antigens. Covariates examined included age, sex, indoor versus outdoor management, the use of flea control, and the presence or absence of antibodies to *A. phagocytophilum* and *D. immitis* antigen. Dogs were grouped into three age categories: <1 year, 1 to 3 years, and ≥4 years. Dogs were classified as being primarily indoor dogs, indoor/outdoor dogs, or primarily outdoor dogs. Due to the low prevalence of *Bartonella* bacteremia and antibodies against *Y. pestis* in dogs, these variables were not analyzed in the statistical model. A sensitivity analysis was performed using two other polytomous logistic regression models, the adjacent categories and the continuation ratio models, to check for consistency in results. Results were expressed as prevalence odds ratios (OR) and 95% confidence intervals (95% CI).

Results from the gray foxes were analyzed using polytomous logistic regression as described above. *Bartonella* IFA titers were grouped into three categories, with moderate and high titers (those of ≥256) now defined as one group. Covariates examined included whether the fox was *Bartonella* culture positive (bacteremic), the gender of the fox, and the location and season in which the fox was trapped. In the analysis of IFA titers and bacteremia in the gray foxes, foxes for which blood cultures were incubated for less than 2 weeks (eight samples) due to problems with contaminants growing on the plates were excluded from the analysis. Data from recaptured foxes were not included in the statistical analyses. In addition, a chi-square test for differences between culture-positive and culture-negative foxes with respect to each variable was performed.

RESULTS

Dogs and gray foxes. Blood samples were collected from 89 dogs in March 2004, 41 dogs in October 2004, and 54 dogs in March 2005. Seventy-four dogs were male, and 109 dogs were female. Information on the sex of one dog was not recorded. The ages of 168 dogs (91%) were known and ranged from 4 months to 13 years, with a median age of 2 years. Only 19 dogs were ≥6 years old. A variety of dog breeds were represented in the sample, with the most common being pit bull and pit bull crosses (29 dogs), German shepherd and shepherd crosses (15 dogs), and Australian cattle dogs (13 dogs). Overall, 111 of the dogs (60%) were of mixed breeds. Ten dogs were indicated to have clinical signs of illness at the time a blood sample was collected. Clinical symptoms included cough or shortness of breath (six dogs), nasal discharge (one dog), and congestive heart failure (one dog). Diagnoses included a heart murmur (two dogs) and mange (two dogs).

Of the 54 gray foxes, 33 were male and 21 were female. Thirty-one foxes were captured in a peridomestic area near the town of Hoopa, and the remaining 23 foxes were captured in uninhabited areas surrounding the town. Ten gray foxes were recaptured during the course of the study, including five foxes recaptured one additional time, four foxes recaptured twice, and one fox recaptured three times. No clinical signs of disease in gray foxes at the time of capture were observed.

Identification of *Bartonella* spp. Some findings on the characterization of gray fox *Bartonella* isolates have been presented previously (30). *Bartonella* spp. were isolated from four dogs (2.2%; 4 of 182) and from 26 gray foxes (49%; 26 of 53). There was insufficient blood from two dogs and one fox to perform culture. For blood culture isolates, PCR amplification using primers for the ITS region and the *gltA* gene produced fragments strongly suggestive of *Bartonella*. Partial sequences from the two genes suggested that one dog (0.5%; 1 of 182) and five gray foxes (9.4%; 5 of 53) were infected with *B. vinsonii* subsp. *berkhoffii*. As part of a separate study, a typing scheme based on 16S-23S ITS and *pap31* sequences (30) identified these gray fox isolates as a type III strain of *B. vinsonii* subsp. *berkhoffii*, which has been associated with a human endocarditis case (39). The *B. vinsonii* subsp. *berkhoffii* isolate from the infected dog was identical to the gray fox isolates in comparisons based on partial fragments of the genes sequenced in this study. The dog was a 1-year-old Jack Russell terrier that was outdoors during the day and indoors at night. There were no clinical symptoms described on the questionnaire, and the owner stated that flea control products were not used on the dog.

A novel *Bartonella* sp., closely related to *B. clarridgeiae*, was isolated from three rural dogs (1.7%; 3 of 182) and 22 gray foxes (42%; 22 of 53). Two of the dogs with these novel *B. clarridgeiae*-like isolates were from the same household, and all three dogs were outdoor dogs. Flea control products were not used on these three dogs, and no clinical symptoms were indicated on the questionnaires. Ages were known for two of the three dogs and were given as 8 and 9 months.

Five out of the 10 foxes that were recaptured during the study gave a recapture sample that was culture positive for *Bartonella*. *Bartonella* spp. had been isolated from the initial samples taken from all five of these foxes; the culture-positive recapture samples were obtained between 2.5 and 9 months

TABLE 1. Results from blood culture and the IFA assay for antibodies against *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and *B. henselae* antigens for 10 gray foxes (*Urocyon cinereoargenteus*) recaptured one time or more during the study[a]

Fox no.	Date (mo/day/yr) of initial capture	IFA titers	Culture result	Date (mo/day/yr) of second capture	IFA titers	Culture result	Date (mo/day/yr) of third capture	IFA titers	Culture result	Date (mo/day/yr) of fourth capture	IFA titers	Culture result
1	6/22/2003	Bvb, 64; Bc, <32; Bh, <32	–	4/29/2004	Bvb, 128; Bc, <32; Bh, <32	–	5/14/2004	Bvb, 256; Bc, <32; Bh, 128	+ (Bvb)	ND	ND	ND
6	7/3/2003	Bvb, 256; Bc, 128; Bh, 256	+ (Bvb)	2/8/2004	Bvb, 512; Bc, 128; Bh, 128	–	4/9/2004	Bvb, 128; Bc, <32; Bh, 64	+ (Bc-like)	5/15/2004	Bvb, 256; Bc, <32; Bh, 64	–
7	7/6/2003	Bvb, 512; Bc, 256; Bh, 256	–	10/15/2003	Bvb, 128; Bc, <32; Bh, 128	+ (Bc-like)	4/9/2004	Bvb, 64; Bc, 64; Bh, 64	+ (Bc-like)	ND	ND	ND
8	7/10/2003	Bvb, 512; Bc, 128; Bh, 128	+ (Bc-like)	10/15/2003	Bvb, 128; Bc, <32; Bh, 64	+ (Bc-like)	ND	ND	ND	ND	ND	ND
9	7/10/2003	Bvb, 128; Bc, 64; Bh, 128	–	4/28/2004	Bvb, 256; Bc, 64; Bh, 256	–	ND	ND	ND	ND	ND	ND
12	7/30/2003	Bvb, 256; Bc, 128; Bh, 128	+ (Bc-like)	5/22/2004	Bvb, 512; Bc, 256; Bh, 256	–	7/3/2004	Bvb, 512; Bc, 256; Bh, 128	–	ND	ND	ND
13	8/22/2003	Bvb, 128; Bc, <32; Bh, 128	+ (Bc-like)	5/20/2004	Bvb, 128; Bc, <32; Bh, <32	+ (Bc-like)	10/10/2004	Bvb, 128; Bc, <32; Bh, <32	+ (Bc-like)	ND	ND	ND
17	9/27/2003	Bvb, <32; Bc, <32; Bh, <32	+ (Bc-like)	12/9/2003	Bvb, 128; Bc, <32; Bh, 64	+ (Bc-like)	ND	ND	ND	ND	ND	ND
18	10/10/2003	Bvb, 128; Bc, 64; Bh, 64	–	3/5/2004	Bvb, <32; Bc, <32; Bh, <32	–	ND	ND	ND	ND	ND	ND
34	9/28/2003	Bvb, 256; Bc, 128; Bh, 256	–	2/22/2004	Bvb, 128; Bc, 64; Bh, 128	+ (Bc-like)	ND	ND	ND	ND	ND	ND

[a] Reciprocal IFA titers are given. Bvb, *B. vinsonii* subsp. *berkhoffii*; Bc, *B. clarridgeiae*; Bh, *B. henselae*; Bc-like, *B. clarridgeiae*-like species; ND, not done.

following the collection of the initial culture-positive sample. The *B. clarridgeiae*-like species was isolated again from four of the five foxes. One fox that was captured a total of four times during the study was initially culture positive for *B. vinsonii* subsp. *berkhoffii* and was then culture positive for the *B. clarridgeiae*-like species when recaptured 9 months following the collection of the initial sample. We were unable to isolate *Bartonella* from blood collected when this fox was recaptured at 7 and 10 months following the initial isolation of *B. vinsonii* subsp. *berkhoffii* (Table 1).

***A. phagocytophilum* PCR.** Fourteen dogs (7.6%) were positive for *A. phagocytophilum* by real-time TaqMan PCR. *A. phagocytophilum* DNA was amplified from seven dog samples obtained in March 2004, no dog samples obtained in October 2004, and seven dog samples collected in March 2005. None of the four dogs that were *Bartonella* culture positive were PCR positive for *A. phagocytophilum*. Thirteen of the 14 dogs that were PCR positive for *A. phagocytophilum* had reciprocal IFA titers of <32 for all three *Bartonella* antigens tested. One *A. phagocytophilum* PCR-positive dog showed seroreactivity to all three *Bartonella* antigens, with a reciprocal titer of 1,024 for antibody against *B. vinsonii* subsp. *berkhoffii*.

Serology for arthropod-borne pathogens. Fifty-four of the 184 rural dogs (29%) were seroreactive to *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and/or *B. henselae* as determined by the IFA assay, with a reciprocal antibody titer of ≥64 (Table 2). Twenty-eight dogs had reciprocal titers of ≥64 for at least two of the three *Bartonella* antigens. Titers ranged from <1:32 to 1:32,768, with 25 dogs (14%) having titers of ≥256 for at least one antigen (Fig. 1). Seventy-four dogs (40%) had antibodies against *A. phagocytophilum*, and 51 (28%) out of 182 dogs tested were positive for *D. immitis* antigen (Table 2). Of the 14 dogs that were PCR positive for *A. phagocytophilum*, 10 were also seroreactive to *A. phagocytophilum* as assessed by the IFA test. Two dogs were not tested for heartworm because they were <6 months old. Five of the six dogs that had respiratory symptoms such as coughing and shortness of breath as indicated on the questionnaires were heartworm positive. One dog with both respiratory symptoms and congestive heart failure had antibodies against *Bartonella*, *A. phagocytophilum*, and *Y. pestis* in addition to being heartworm positive. Blood culture for *Bartonella* was not done for this dog due to a limited sample volume. The dog was a 5-year-old spayed female and a primarily outdoor dog. The owner stated on the questionnaire that flea control had been started only within the past 6 months. Five dogs out of 183 tested (2.7%) were seropositive for *Y. pestis* (Table 2), with antibody titers ranging from 1:16 to 1:512. No *Bartonella* culture-positive dogs had antibodies against *Y. pestis*. Three of the five dogs that had antibodies against *Y. pestis* also had antibodies against at least one of the three *Bartonella* antigens that were tested.

Among the gray foxes, 48 out of 54 (89%) had reciprocal IFA titers of ≥64 for one or more of the three *Bartonella* antigens, and the majority of these foxes (79%; 38 of 48) were reactive to more than one antigen. Titers ranged from <1:32 to 1:1,024. Nine out of the 10 recaptured gray foxes had reciprocal IFA titers of ≥64, with little observed variation between titers of the initial samples and those of the recapture samples. One fox that was *Bartonella* culture-positive on the initial capture date was observed to have low IFA titers (<1:32) for all

TABLE 2. Seroprevalence of antibodies to *Bartonella* spp., *A. phagocytophilum*, *Y. pestis*, and *D. immitis* antigen in 184 domestic dogs in Humboldt County, CA[a]

Date of sample collection	% Of samples seropositive (no. positive/total) for antibodies to:			
	Bartonella spp.	*A. phagocytophilum*	*D. immitis*	*Y. pestis*
March 2004	34 (30/89)	34 (30/89)	25 (22/87)	2.3 (2/88)
October 2004	32 (13/41)	46 (19/41)	32 (13/41)	2.4 (1/41)
March 2005	20 (11/54)	46 (25/54)	30 (16/54)	3.7 (2/54)
Total	29 (54/184)	40 (74/184)	28 (51/182)	2.7 (5/183)

[a] Cutoff titers for seropositivity were ≥1:64 for antibodies to any of the tested *Bartonella* antigens, >1:80 for *A. phagocytophilum* antibodies, and ≥1:16 for *Y. pestis* antibodies.

three antigens at this time. When recaptured 2 months later, this fox had elevated IFA titers (1:128 for *B. vinsonii* subsp. *berkhoffii*, 1:32 for *B. clarridgeiae*, and 1:64 for *B. henselae*) and was again culture positive for the *B. clarridgeiae*-like species (Table 1).

Statistical analysis. The odds of having results below one of the *Bartonella* IFA titer category cutoffs were decreased by approximately 77% for dogs aged 1 to 3 years (OR, 0.23; 95% CI, 0.081 to 0.64; $P < 0.01$) and by approximately 75% for dogs ≥4 years old (OR, 0.25; 95% CI, 0.079 to 0.77; $P = 0.02$) compared to those for dogs of <1 year of age according to the proportional odds model. The odds of having results below one of the *Bartonella* IFA titer category cutoffs were also decreased for dogs positive for *A. phagocytophilum* (OR, 0.63; 95% CI, 0.34 to 1.2; $P = 0.2$) and *D. immitis* (OR, 0.62; 95% CI, 0.32 to 1.2; $P = 0.2$), although the 95% CI included the null value. The proportional odds model assumes that the cumulative effects (OR) can be interpreted as constant across all possible cutoff points for the IFA titers.

A sensitivity analysis using the continuation ratio and adjacent categories models also found that dogs in higher age categories were more likely to have elevated *Bartonella* IFA titers (Table 3). In the adjacent categories model, dogs positive for *A. phagocytophilum* and *D. immitis* had OR of 1.26 (95% CI, 0.90 to 1.8; $P = 0.2$) and 1.4 (95% CI, 0.96 to 1.92; $P = 0.09$), respectively, corresponding to elevated IFA titers. In the continuation ratio model, dogs that were positive for *D. immitis* antigen had nearly four times higher odds (OR, 3.9; 95% CI, 1.1 to 15; $P = 0.03$) of having a reciprocal *Bartonella* IFA titer of ≥1,024 than dogs that tested negative for heartworm. However, the 95% CI for the OR corresponding to *A. phagocytophilum* antibodies and a reciprocal *Bartonella* IFA titer of ≥1,024 did not suggest a strong association between exposures of dogs to these two organisms (OR, 2.1; 95% CI, 0.61 to 7.7; $P = 0.3$) (Table 3).

Polytomous logistic regression analysis of data from the gray foxes did not reveal any clear associations between *Bartonella* IFA titers and bacteremia, sex, capture location, or season of capture. No significant associations between *Bartonella* bacteremia and sex, capture location, or season of capture for the gray foxes were observed using chi-square tests.

DISCUSSION

Two species of *Bartonella* were identified in both rural dogs and gray foxes from Humboldt County, CA. DNA sequence analysis of partial fragments from two genes suggested that the strains of a novel *B. clarridgeiae*-like species and those of *B. vinsonii* subsp. *berkhoffii* infecting dogs were identical to those infecting gray foxes. A *B. clarridgeiae*-like species has previously been associated with a fatal case of endocarditis in a dog from northern California (29). As isolates of this *Bartonella* species are now available, further work is needed to characterize this species. *B. vinsonii* subsp. *berkhoffii* is recognized as a cause of infective endocarditis in dogs (5, 29) and has been associated with at least one human case of endocarditis (39). In addition, myocarditis, cardiac arrhythmias, granulomatous lymphadenitis, granulomatous rhinitis, and epistaxis have been attributed to infection with *B. vinsonii* subsp. *berkhoffii* in dogs

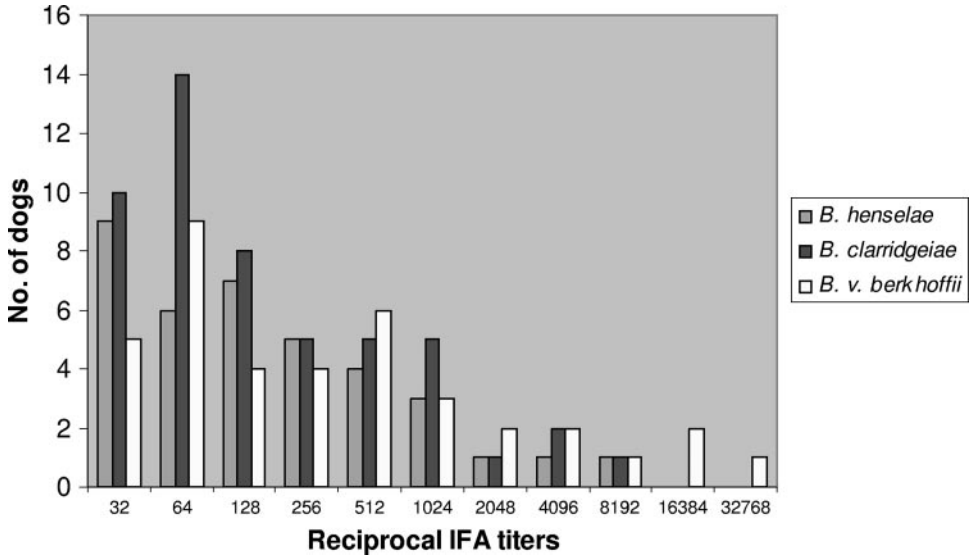

FIG. 1. Reciprocal IFA titers of ≥32 for *B. vinsonii* subsp. *berkhoffii* (*B. v. berkhoffii*), *B. clarridgeiae*, and *B. henselae* among samples from 184 rural dogs from Humboldt County, CA, collected from 2004 to 2005. Reciprocal IFA titers of <32 are not shown.

TABLE 3. Results from three different polytomous logistic regression models[a]

Model	Covariate	OR	95% CI	*P* value
Proportional odds	Age of 1 to 3 yr	0.23	0.08–0.64	<0.01
	Age of ≥4 yr	0.25	0.08–0.77	0.02
	A. phagocytophilum exposure (IFA titer, >1:80)	0.63	0.34–1.2	0.2
	D. immitis infection	0.62	0.32–1.2	0.2
Adjacent categories	Age of 1 to 3 yr	2.4	1.3–5.7	<0.01
	Age of ≥4 yr	2.2	1.1–5.4	0.02
	A. phagocytophilum exposure (IFA titer, >1:80)	1.3	0.90–1.8	0.2
	D. immitis infection	1.4	0.96–1.9	0.09
Continuation ratio				
IFA titer, 1:64 or 1:128	Age of 1 to 3 yr	4.1	1.4–15	<0.01
	Age of ≥4 yr	4.1	1.2–17	0.02
IFA titer, 1:256 or 1:512	Age of 1 to 3 yr	10	1.5–430	0.01
	Age of ≥4 yr	6.0	0.63–300	0.2
IFA titer, ≥1,024	*A. phagocytophilum* exposure (IFA titer, >1:80)	2.1	0.61–7.7	0.3
IFA titer, ≥1,024	*D. immitis* infection	3.9	1.1–15	0.03

[a] The *Bartonella* IFA titer was the ordinal response variable.

(9). The low prevalence of culture-positive domestic dogs may be due to the lack of sensitivity of blood culture for detecting low levels of bacteremia or, alternatively, may indicate a short duration of bacteremia in these dogs more typical of infections in nonreservoir hosts (4).

One fox was culture positive for *B. vinsonii* subsp. *berkhoffii* when initially captured and then culture positive for the *B. clarridgeiae*-like species when recaptured 9 months later, suggesting that infection with *B. vinsonii* subsp. *berkhoffii* does not provide protection against infection with the *B. clarridgeiae*-like species or that coinfection may occur. Four recaptured foxes were repeatedly culture positive for the *B. clarridgeiae*-like species, suggesting that they may have had persistent or relapsing bacteremia. The high prevalence of *B. clarridgeiae*-like species bacteremia in gray foxes, combined with the data from the small number of recaptured foxes, may indicate that gray foxes are acting as a reservoir for this *B. clarridegiae*-like species in this area of Humboldt County. Foxes sampled for this study were also tested for *A. phagocytophilum* as part of another recent study (21). In that study, 52% of foxes (28 of 54) were *A. phagocytophilum* seropositive and 11% of foxes (6 of 54) were *A. phagocytophilum* PCR positive. The prevalence of antibodies to *A. phagocytophilum* was highest in the spring, and foxes captured outside of human residential boundaries were more likely to have antibodies than those captured in a peridomestic area. The findings appeared to correspond to the variation in the documented abundance of *I. pacificus* ticks on these foxes in relation to season and capture location (21). In the present study, no differences in *Bartonella* bacteremia in relation to season or capture location in the same group of foxes were found.

Humboldt County was selected for the investigation of *Bartonella* exposure among dogs and gray foxes because it is an area in which vector-borne disease risk, particularly the risk of tick-borne disease, appears to be important (6, 19, 20). Seroreactivity at a reciprocal titer of ≥64 against *B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, and/or *B. henselae* antigens in 29% of the dogs and 89% of the gray foxes tested was observed. An IFA titer of 1:64 is commonly used as the cutoff to differentiate positive and negative animals (34, 43), although a definitive cutoff was not selected in the present study as the sensitivity and specificity of the IFA test for canines have not been evaluated and the validity of applying the 1:64 cutoff to different species of *Bartonella* is not known. The seroprevalence of *A. phagocytophilum* in this study (40%) was comparable to the 47% seroprevalence observed in dogs tested in Humboldt County in 1997 and 1998 (19). In addition, 14 dogs tested during clinics held in late winter were PCR positive for *A. phagocytophilum*. Infection with *D. immitis* was also prevalent in dogs in this study, with 28% having a positive antigen test. *D. immitis* infection in both domestic dogs and coyotes in the North Coast Range foothills of California has been documented previously. A recent study found a *D. immitis* prevalence ranging from 44 to 72% in coyotes from foothill regions of three north coastal counties (41). The seroprevalence of *Y. pestis* in dogs from Humboldt County was low (2.7%), which is consistent with previously reported results for domestic dog populations surveyed in California in situations not involving an outbreak investigation (11).

Analysis using different polytomous logistic regression models to assess correlations between *Bartonella* IFA titers and factors associated with exposure in dogs indicated that dogs in older age categories were more likely to have elevated (>1:32) *Bartonella* IFA titers. This association was consistent across all polytomous logistic regression models and was expected, as older dogs would have had more opportunities for exposure to vector-borne diseases. The CI around the OR for *Bartonella* IFA titers and age categories are wide in the continuation ratio model as a result of small sample sizes. The continuation ratio model differs from the other models in that it compares exposure in dogs in a given *Bartonella* IFA titer category with exposure in dogs in the next lower IFA titer category, as opposed to comparing each category to one baseline category. Because the continuation ratio model allows for the calculation of OR corresponding with each titer category, the OR reported for this model are based on smaller sample sizes. Results from proportional odds and adjacent categories models suggested that dogs with either *D. immitis* infection or *A.*

phagocytophilum exposure may have slightly higher odds of *Bartonella* exposure. By the continuation ratio model, which has fewer assumptions than the other polytomous regression models, dogs infected with *D. immitis* were more likely to have reciprocal *Bartonella* IFA titers of ≥1,024 than dogs negative for *D. immitis*. The association between high *Bartonella* IFA titers and *D. immitis* infection is likely due to increased exposure to multiple vectors and is not an indication that these organisms have a common vector. As infection with *B. vinsonii* subsp. *berkhoffii* has been found to result in immune suppression in dogs (35), it is also possible that *D. immitis* infection may occur with greater frequency in dogs with active or recent *Bartonella* infection. Overall, however, the lack of consistent results in the assessment of the statistical association between *Bartonella* and either *D. immitis* or *A. phagocytophilum* across the three polytomous regression models suggests that there is not a strong association between *Bartonella* and these vector-borne agents in this population of dogs. A recent study of 239 coyotes from 15 different counties in California found that coyotes that were seropositive for *B. vinsonii* subsp. *berkhoffii* were three times more likely to be seropositive for *A. phagocytophilum* and two times more likely to be infected with *D. immitis* than *Bartonella*-seronegative coyotes; there was also a positive association between *D. immitis* infection and antibodies against *A. phagocytophilum* (OR, 4.3; 95% CI, 2.5 to 7.6) in these coyotes (1).

Interestingly, primarily outdoor dogs were not found to be more likely to have elevated *Bartonella* IFA titers than other dogs, and flea control was not protective against *Bartonella* exposure. The lack of a strong association between *Bartonella* infection and these covariates may be a result of low statistical power. Alternatively, the risk factors for exposure to the vector responsible for the transmission of *Bartonella* to these dogs may be different from the observed risk factors for exposure to *Bartonella* in other populations of dogs (34). Gray foxes are a potential reservoir of the *B. clarridgeiae*-like species in this area of Humboldt County and have been observed in close contact with domestic dogs in and around the town of Hoopa (M. W. Gabriel, personal communication). Furthermore, *B. clarridgeiae*-like species and *B. vinsonii* subsp. *berkhoffii* DNA has been identified in fleas (*Pulex* spp.) collected from the foxes tested as part of the present study (M. W. Gabriel, unpublished data). Flea control in these dogs, which may involve only the use of an insecticide collar or an occasional flea bath, may not be adequate to completely eliminate flea infestation if there is frequent exposure to fleas from gray foxes.

In the present study, zoonotic *Bartonella* species were identified in domestic dogs and gray foxes from Humboldt County, CA (23). Domestic dogs also had high levels of exposure to two other arthropod-borne zoonotic agents. The level of exposure of the dog population to *Bartonella* spp. within this area of Humboldt County appears to be substantially higher than that encountered in other domestic dog populations tested within California (24, 26). This finding is likely due to complex interactions between wildlife reservoirs, arthropod vectors, and domestic dogs that are influenced by both ecological factors (e.g., humidity and vegetation) and characteristics of this dog population (e.g., intense exposure to arthropod vectors and contact with wildlife). The unique characteristics of this region make it difficult to generalize the results for other domestic dog populations. However, studies conducted on a finer scale can aid in the understanding of the epidemiology and transmission dynamics of *Bartonella* infection among domestic dogs. The reservoir and vectors involved in the transmission of *Bartonella* to dogs, as well as the infecting species, are likely to vary with geographic location and the characteristics of the dog population. Further studies in this area of Humboldt County are needed to better understand the mechanisms by which the transmission of *Bartonella* spp. is occurring and to assess human exposure to the zoonotic agents identified in the dog population.

ACKNOWLEDGMENTS

This work was supported by a grant from the Center for Companion Animal Health at the University of California, Davis, and by the American Kennel Club Canine Health Foundation.

We thank Niki Drazenovich, the Hoopa Tribe, and the Rural Area Veterinary Service for their contributions to this research. This work was performed at the University of California, Davis.

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