Spatial analysis of the exposure of dogs in rural north-coastal California to vectorborne pathogens

J. E. FOLEY, R. N. BROWN, M. W. GABRIEL, J. HENN, N. DRAZENOVICH, R. KASTEN, S. L. GREEN, B. B. CHOMEL

Between 0 and 50 per cent of the dogs in eight rural villages in far northern California with a high risk of tickborne diseases were seropositive for *Anaplasma phagocytophilum* and *Bartonella vinsonii* subspecies *berkhoffii*, and between 0 and 10 per cent were seropositive for *Borrelia burgdorferi.* The odds ratio for the co-exposure of individual dogs to *B vinsonii berkhoffii* and *A phagocytophilum* was 18·2. None of the diseases was associated with the sex of the dogs, whether they slept out of doors, or whether tick-preventive measures were taken. When the villages were assessed for landscape risk factors, a particularly high seroprevalence for *B vinsonii berkhoffii* and *A phagocytophilum* was observed in a village at a relatively high altitude and greater distance from the Pacific coast, and montane hardwood conifer woodland was most associated with a high seroprevalence for these two pathogens.

Borrelia burgdorferi and *Anaplasma phagocytophilum* are important tick-transmitted pathogens of human beings and dogs in California, causing borreliosis and granulocytic anaplasmosis (GA) (formerly ehrlichiosis), respectively. In the western USA both pathogens are transmitted to people and dogs by *Ixodes pacificus* (Brown and others 2005) and likely reservoirs include the dusky-footed woodrat (*Neotoma fuscipes*) and the western grey squirrel (*Sciurus griseus*) (Brown and Lane 1992, Nicholson and others 1999, Foley and others 2004, Lane and others 2005). There is a higher risk of both diseases in the northern coastal mountain ranges and the foothills of the Sierra Nevada. The coastal low mountain region of California, extending north of the San Francisco Bay area, is classified on Centers for Disease Control and Prevention (CDC) risk maps as risk 2 (moderate) for borreliosis in people (CDC 1999). Human cases of GA have been reported from coastal counties from Santa Cruz (south of San Francisco Bay) to far northern Humboldt County, where a small cluster of cases was reported as a result of prospective surveillance (Foley and others 1999). Humboldt County also has the highest seroprevalence to *A phagocytophilum* among dogs, estimated at 47 per cent (Foley and others 2001). Despite reports of the statewide or countywide distributions of these diseases, fine spatial scale differences in incidence and environmental risk factors are poorly understood, particularly in dogs, even in highly enzootic areas.

Even less is known about the ecology and landscape risk of bartonellosis, a newly described, apparently vectorborne disease of dogs caused by *Bartonella vinsonii* subspecies *berkhoffii.* Coyotes (*Canis latrans*) may be a western reservoir of *B vinsonii berkhoffii* (Chang and others 1999, 2000), and *I pacificus* is a possible vector (Chang and others 2001). Infection in coyotes appears to be concentrated in the coastal ranges and foothills of the Sierra Nevada (Chang and others 1999, Hoar and others 2003).

This study was undertaken in an area where both GA and borreliosis are highly enzootic, in order to evaluate the risk to dogs of these two diseases and bartonellosis, to determine whether the risk varied over a relatively fine spatial scale, and to examine such environmental variables as climate, location (altitude and proximity to the coast), and vegetation for their association with their prevalence. Serological methods and PCR for all three pathogens were used to assess dogs in eight rural villages in far northern Humboldt and Del Norte Counties. It was hypothesised that the findings could be valuable for the identification of infection 'hot spots', the management of the diseases in dogs, and the development of dogs as sentinels for human disease.

MATERIALS AND METHODS

Samples of whole blood were collected from 97 dogs in eight rural villages in Humboldt and Del Norte Counties, California, during a month in summer 2003. The dogs were being examined, vaccinated and neutered. Information about the dogs' sex, whether they slept inside or outside, their travel history, and whether tick-preventive treatment was used routinely, were obtained from their owners. The blood was collected by cephalic venepuncture into EDTA tubes and kept cool until the plasma could be separated by centrifugation and frozen at –20°C.

Serology

Plasma anti-*A phagocytophilum* immunoglobulin G (IgG) was assayed by an indirect immunofluorescent antibody (IFA) assay (Dumler and others 1995), using *A phagocytophilum*-infected HL-60 cells as the substrate and fluorescein isothiocyanate-labelled goat anti-dog IgG (Kierkegaard and Perry). The samples were tested starting at a dilution of 1:20, and positive and negative canine control sera were included on each run. Samples were considered positive if strong fluorescence was detected at dilutions of at least 80, consistent with previously published cut-off values (Dumler and others 1995).

Antibodies against *B burgdorferi* were evaluated by Western blot; proteins were extracted from *B burgdorferi* strain PLA68 (*N fuscipes,* Placer County, 1998) (Sambrook and others 1989), and 4·4 µg of protein per lane were electrophoresed on a 12 per cent acrylamide 16 cm gel at 200 V for 4·5 hours. The proteins were transferred on to a polyvinylidene fluoride membrane (Amersham Biosciences) and electrophoresed overnight at room temperature at 10 V. The blots were incubated with 500 µl of dog plasma and 4·5 ml of phosphate-buffered saline (PBS)-Tween for one hour, washed, incubated with goat anti-dog IgG alkaline phosphate conjugate diluted 1:3000 (Antibodies) and then developed in 100 µl of NBT-BCIP (Roche Applied Science). The interpretation of the results was consistent with CDC guidelines (CDC 1995), except that three or more diagnostic bands at 18, 21, 28, 30 39, 41, 45, 58, 66, or 93 kDa were considered positive.

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J. E. Foley, DVM, PhD, **N. Drazenovich,** MS, Department of Medicine and Epidemiology, **J. Henn,** PhD, **R. Kasten,** PhD, **B. B. Chomel,** DVM, PhD, Department of Population, Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA
R. N. Brown, DVM, PhD, **M. W. Gabriel,** MS, Department of Wildlife Biology, Humboldt State University, Arcata, CA 95521, USA
S. L. Green, DVM, Veterinary Housecalls, Arcata, CA 95521, USA

TABLE 1: Percentage seroprevalence (95 per cent confidence interval [CI]) of three tickborne pathogens in dogs from eight rural northern California villages in summer 2003, and the annual precipitation, distance from the coast and vegetation type of the villages

Village	County	Number of dogs	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Bartonella vinsonii berkhoffii*	Precipitation (cm/year)	Distance from coast (km)	Altitude (m)	Vegetation type
Crescent City	Del Norte	8	0 (0-40)	0 (0-40)	0 (0-40)	65	1	17·4	Douglas fir
Klamath	Del Norte	8	12·5 (1-53)	0 (0-40)	0 (0-40)	75	4	8·5	Douglas fir, coast redwood, agricultural
Smith River	Del Norte	13	15 (3-46)	0 (0-28)	8 (0-38)	75	0	12·2	Douglas fir
Trinidad	Humboldt	15	0 (0-25)	0 (0-25)	7 (0·4-34)	55	0	51·8	Coast redwood
Rohnerville	Humboldt	13	8 (0·4-38)	0 (0-28)	0 (0-28)	37·5	15	18·9	Montane hardwood/conifer
Table Bluff	Humboldt	10	10 (0·5-46)	0 (0-34)	0 (0-34)	37·5	4	97·5	Montane hardwood/conifer
Blue Lake	Humboldt	10	20 (4-56)	10 (1-46)	0 (0-34)	45	12	13·4	Coast redwood, annual grassland
Pecwan/ Weitchpec	Humboldt	20	50 (30-70)	15 (4-39)	50 (30-70)	80	26	182·9	Montane hardwood/conifer, coast redwood
Overall		97	17 (11-27)	4 (1-11)	12 (7-21)				

The IFA for *B vinsonii berkhoffii* was carried out as described by Chang and others (2000), with modifications: 90 per cent confluent MDCK cells were inoculated with four-day-old *B vinsonii berkhoffii* (ATCC 51672) culture, and incubated for two days at 37°C in an atmosphere containing 5 per cent carbon dioxide. The cells were then washed twice with 10 X Hanks' Balanced Salt Solution (Gibco-BRL) and trypsinised (Gibco-BRL) for 40 minutes at 37°C. The tissue culture was centrifuged at 200 *g* for 10 minutes, the supernatant was discarded, and the cells were resuspended in 30 ml of tissue culture growth medium. Forty microlitres of cell culture was spotted on to HTC glass slides (Cell-Line/Erie Scientific) and incubated overnight at 37°C with 5 per cent carbon dioxide. The slides were washed twice in PBS (pH 7·4) (Sigma Chemical), set for 20 minutes in acetone and stored at –20°C. Plasma samples were diluted at 1:32 and 1:64 in PBS containing 5 per cent milk. The slides were incubated at 37°C for 30 minutes, followed by three PBS washes. Fluorescein-conjugated goat anti-dog IgG (ICN Biomedicals) was diluted 1:1400 in PBS containing 5 per cent milk and 0·001 per cent Evans' blue and 20 µl was applied to each well. The slides were incubated at 37°C for 30 minutes and again washed in PBS three times. The intensity of bacillus-specific fluorescence was scored from 1 to 4, with a score of at least 2 at a dilution of 1:64 considered a positive result (Chang and others 2000). The same two readers made a double-blind reading of each slide. Negative and positive plasma control samples were included on each slide.

Nucleic acid extraction and real-time TaqMan PCR

DNA was extracted from 200 µl of whole blood with the DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. Real-time PCR systems for *A phagocytophilum* and *B burgdorferi* were run in separate wells using the primers and probes described by Leutenegger and others (1999), Pusterla and others (1999) and Drazenovich and others (2007) in a combined thermocycler/fluorometer (ABI Prism 7700; Applied Biosystems). The PCR for *B vinsonii berkhoffii* was carried out as described by Chang and others (2000).

Data management and analysis

The data were maintained in Excel (Microsoft) and analysed with the statistical package 'R' (www.r-project.org; R-Development Core Team). For all the tests, the cut-off for statistical significance was P=0·05. The location of each village was obtained by a global positioning device and displayed using a geographic information system (GIS) (ArcMap 8.0; ESRI). Exact 95 per cent confidence intervals (CIs) for seroprevalence, assuming binomial distributions, were calculated in 'R' by the proportions test. Differences in seroprevalence between the villages were assessed by chi-squared analysis. The associations between the demographic and management factors and individual dog's risk of exposure to tickborne disease were assessed by calculating odds ratios (ORs) and 95 per cent CIs. The mean ages of the dogs that had or had not been exposed to the pathogens were compared by *t* tests.

The potential associations between the seroprevalence in each village and landscape factors was evaluated. Vegetation and precipitation information was obtained from the spatial vegetation GIS coverage CALVEG 2000 (California Forestry and Fire Protection) and the precipitation isohyetal polygons in the layer 'Precipitation' (Teale GIS Solutions). The latitude and longitude of each village were associated with the precipitation and vegetation polygons using ArcMap. Because of the relatively small numbers of dogs and villages, the associations between the seroprevalence of *B burgdorferi* and *A phagocytophilum* in each village and their precipitation, distance to the coast, altitude, and vegetation were assessed visually but not statistically.

RESULTS

The overall seroprevalences of the three pathogens were 17·5 per cent for *A phagocytophilum,* 12 per cent for *B vinsonii berkhoffii* and 4 per cent for *B burgdorferi* (Table 1). None of the dogs was PCR positive for any of the three pathogens. The sex ratio of the 97 dogs was 1·4 male to 1 female; the ratios of males to females for the seropositive dogs were 1·8 to 1 for *A phagocytophilum,* 2 to 1 for *B vinsonii berkhoffii,* and 0·33 to 1 for *B burgdorferi,* but the dogs' sex was not significantly associated with the seroprevalence of any of the infections. The serological evidence of exposure was not significantly associated with the dogs' sleeping outside or the use of tick preventives. The dogs travelled outside their home village only very rarely (M. Gabriel, unpublished observations). The mean age of the dogs seropositive for *A phagocytophilum* was 6·0 years compared with 4·3 years for the seronegative dogs; for *B vinsonii berkhoffii* the comparable ages were 4·2 and 4·7 years, and for *B burgdorferi* they were 3·0 and 4·7 years. Only the difference in age for *A phagocytophilum* was statistically significant (P=0·05). However, coexposure to *A phagocytophilum* and *B vinsonii berkhoffii* was statistically significant (OR 18·2, 95 per cent confidence interval [CI] 8·9 to 37·4) (P<0·0001).

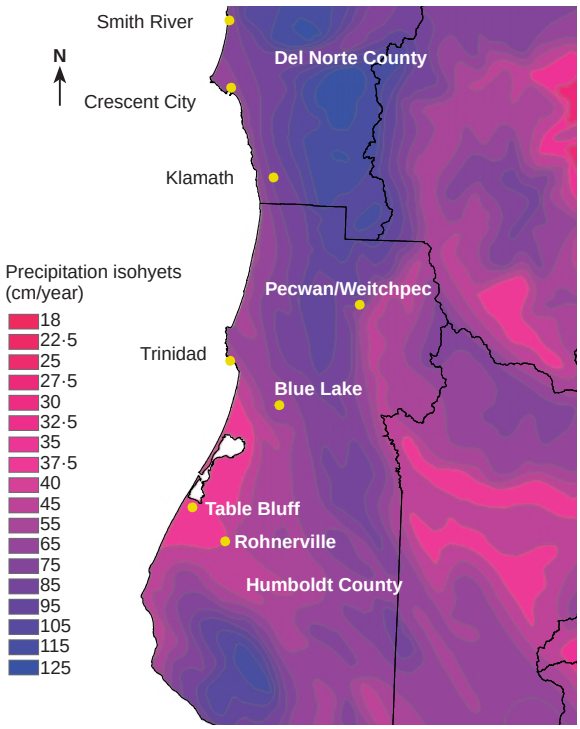

FIG 1: Map of far northern California, generated in ArcMap, showing the eight rural villages where the dogs were sampled to determine the prevalence of three tickborne pathogens, and the precipitation isohyets for the region

The spatial relationship of the villages to the precipitation isohyets is shown in Fig 1. The seroprevalence varied between the villages from 0 to 50 per cent for *A phagocytophilum* and *B vinsonii berkhoffii,* and from 0 to 15 per cent for *B burgdorferi* (Table 1). The differences in prevalence among the villages were statistically significant. In particular, the inland village Pecwan/Weitchpec had a considerably higher seroprevalence than the other villages for *A phagocytophilum* (P=0·0002), *B vinsonii berkhoffii* (P<0·0001), and *B burgdorferi* (P=0·04). The villages ranged in altitude from approximately 8 to 180 m (Table 1), and their distances from the coast ranged from 0 to 26 km. Precipitation throughout the region ranged from 37·5 cm per year in two villages with a moderately low seroprevalence to *A phagocytophilum*, to 55 to 80 cm per year in the other villages. The vegetation in the area included predominantly coast redwood communities in two villages, drier Douglas fir-dominant communities in two, mixed redwood and Douglas fir in one, and mixed hardwood conifer (a mixture of tanoak, madrone and Douglas fir) in the other three (Table 1). Pecwan/Weitchpec, the village with a higher seroprevalence of all three organisms, was further inland and at a higher altitude than the other villages, and essentially within mixed hardwood conifer forest.

DISCUSSION

Even within the relatively restricted area of Humboldt and Del Norte Counties in northern California, there were spatially distinct patterns of exposure of dogs to these three vectorborne pathogens, with some areas having virtually no exposure and others, separated by only a few dozen kilometres, having very high levels of exposure. In particular, there were high levels of exposure in the dogs from villages that were at higher altitude, further inland, and in montane hardwood conifer forests.

These three diseases pose important threats to dogs, and possibly to people, although there is no published information about their prevalence in the study area. Lyme borreliosis is often mild and non-specific in infected people and dogs, but can be severe, causing arthritis, neurological or cardiac dysfunction or, in dogs, potentially fatal nephritis (Shadick and others 1994, Dambach and others 1997). In dogs, the infection may persist lifelong (Appel and others 1993). In California, it has been shown by DNA sequencing that the disease in dogs is caused by *B burgdorferi* sensu stricto, the same genospecies that causes disease in people (J. Foley, unpublished observations). The clinical effects of GA are also variable, but in human beings they may include pyrexia, headache, myalgia, nausea, ataxia, organ failure, susceptibility to opportunistic infections, neuritis or respiratory complications with a case fatality rate up to 5 per cent (Foley 2000). Clinical GA in dogs is mild or associated with fever and potentially severe thrombocytopenia (Greig and others 1996, Foley and others 2001). DNA sequencing suggests that human cases from Humboldt County may be caused by strains with very close homology to equine strains (Foley and others 1999, Chae and others 2000). In dogs, *B vinsonii berkhoffii* is an important cause of endocarditis, nasal discharge, epistaxis, splenomegaly, vomiting, hepatomegaly, eosinophilia and lymphadenopathy (Pappalardo and others 2000, Breitschwerdt and others 2004, MacDonald and others 2004, Henn and others 2005). There has also been a human case of endocarditis associated with *B vinsonii berkhoffii* (Roux and others 2000).

None of the dogs in this study was PCR positive, which was not surprising given the relatively short duration of bacteraemia for each of these agents (Burgess 1986, Straubinger and others 2000). The optimal tissue for detecting *B burgdorferi* in dogs is synovium (Straubinger and others 1997). For *B vinsonii berkhoffii,* the tissue of choice for dogs with endocarditis appears to be cardiac valve, but it is not known for cases of mild or preclinical infection (MacDonald and others 2004). Neither cardiac valve nor synovium was available for the present study.

Demographic associations of seropositivity were limited to greater age being a risk factor for exposure to *A phagocytophilum.* Sleeping out of doors was not a risk factor, but virtually all the dogs were outdoors during the day, and the rural nature of the area would have increased the likelihood of their exposure to ticks. The use of tick preventives was not protective, but some of the methods reported by the owners may have been ineffective, including the manual removal of ticks and the infrequent use of insecticides, commonly without acaricidal properties (M. Gabriel, personal communication). Pappalardo and others (1997) reported that *B vinsonii berkhoffii*-seropositive dogs in North Carolina were more likely to live in a rural environment, roam freely, and have a history of heavy exposure to ticks and fleas. There was significant co-exposure to *A phagocytophilum* and *B vinsonii berkhoffii,* which has also been reported for coyotes and dogs with endocarditis (Beldomenico and others 2005). Co-infection with *A phagocytophilum* and *Bartonella henselae* was detected in *I pacificus* ticks (Holden and others 2006). The results therefore suggest that there may be common risk factors for these diseases in dogs, including possibly a common tick vector. *A phagocytophilum* infection is immunosuppressive in some hosts (Woldehiwet 1987, Dumler and others 2005), and prior exposure to *A phagocytophilum* could therefore increase the risk of bartonellosis. The lack of significant co-exposure to *A phagocytophilum* and *B burgdorferi* was probably due to the small numbers of *B burgdorferi*-exposed dogs.

There was a wide range of prevalence for each of the three pathogens among the eight villages. In each village, the prevalence ranged from 0 to as high as 50 per cent for *A phagocytophilum* and *B vinsonii berkhoffii,* and from 0 to 15 per cent for *B burgdorferi.* Humboldt and Del Norte Counties are highly enzootic for tickborne diseases in dogs, and Foley and others (2007) recorded a seroprevalence of 58 per cent for *A phagocytophilum* and 20 per cent for *B burgdorferi* in coyotes in nearby Mendocino County. The prevalence of bor-

reliosis in the present study was lower than in dogs in the hyperendemic areas of the eastern USA, where rates of up to 76 per cent have been reported (Magnarelli and others 1985, 1987, Eng and others 1988, Falco and others 1993). In contrast, the prevalence of exposure to *A phagocytophilum* and *B vinsonii berkhoffii* was very high in comparison with dogs from the eastern USA. In Connecticut and New York, the seroprevalence among dogs with clinical signs of GA was only 9·4 per cent (Magnarelli and others 1997), whereas 33 per cent of dogs with clinical signs of GA in Oklahoma were seropositive (Rodgers and others 1989). Earlier serological surveys for *B vinsonii berkhoffii* in wild and domestic populations of dogs revealed 8·7 per cent seropositive working dogs, 3·6 per cent seropositive sick dogs from North Carolina and Virginia, 2·2 per cent seropositive dogs from Rhode Island, and 1 per cent seropositive dogs from a northern California veterinary hospital (Breitschwerdt and others 1999, Hinrichsen and others 2001, Honadel and others 2001, Henn and others 2005).

A rigorous statistical evaluation of the ecological determinants of seroprevalence was not possible because of the lack of an adequate distribution of villages with high and low prevalences at different altitudes and distances from the coast, and with different levels of precipitation and types of vegetation. However, the prevalence of *A phagocytophilum* and *B vinsonii berkhoffii* was lower in the villages directly on the coast than in Pecwan/Weitchpec, which is further inland and at higher altitude. Foley and others (2007) observed a higher risk of *A phagocytophilum* in redwood, montane hardwood and blue oak/foothill pine, and areas with higher precipitation, and Hoar and others (2003) observed a higher risk of exposure to *B vinsonii berkhoffii* at low altitudes, and in areas close to the coast and with higher precipitation. There are several ecological factors that could account for the observed pattern of exposures, including the density and survival of the tick vectors and the density of the reservoir hosts. *I pacificus* is a common tick in northern California, and requires at least 85 to 90 per cent humidity to survive (Arthur and Snow 1968); as a result, its density and survival in the study area are optimal for the transmission of the infections. Moreover, the tick's questing activity increases with increases in humidity and decreases with increases in temperature (Loye and Lane 1988). The main reservoirs, dusky-footed woodrats and western grey squirrels, are common in Humboldt County. Woodrats, which prefer oak woodlands, participate in an important rodent-nidicolous tick enzootic cycle of Lyme disease and possibly anaplasmosis (Linsdale and Tevis 1951, Brown and Lane 1992, Zeidner and others 2000). Oak leaf litter provides an ideal microhabitat for *I pacificus*, providing year-round moisture and a safe location for moulting (Furman and Loomis 1984). Eisen and others (2003) recorded high risks of nymphal *I pacificus* activity and *I pacificus*-transmitted diseases in oak habitats, and in some redwood/tan oak-dominated habitats in the northern coast ranges. The risks associated with different habitats should be further investigated, possibly by randomised spatial sampling of ticks or woodrats to evaluate the infections across these landscape determinants systematically.

These rural, often free-roaming dogs were commonly exposed to *A phagocytophilum* and *B vinsonii berkhoffii* suggesting that there may be a risk to people in the region. The significant co-exposure of the dogs to both parasites warrants further investigation, including an evaluation of the infections in *Ixodes* species ticks.

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