Epidemiology and Genetic Diversity of *Anaplasma phagocytophilum* in the San Francisco Bay Area, California

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Abstract. In California, the agent of human granulocytic anaplasmosis (HGA), *Anaplasma phagocytophilum*, is transmitted by western black-legged ticks (*Ixodes pacificus*). Cases of HGA are infrequent in California but do occur annually. We investigated nymphal and adult western black-legged tick populations in 20 recreational areas in California's San Francisco Bay Area (Marin, Napa, San Mateo, Santa Clara, Santa Cruz, and Sonoma counties). Overall, prevalence of *A. phagocytophilum* in adult ticks was 0.8% (11/1,465), and in nymphal ticks was 4.2% (24/568), though presence was patchy and prevalence varied locally. We detected significant sequence variation in our quantitative polymerase chain reaction (qPCR)–positive samples. This included four sequences that grouped within a clade that contains clinical human and veterinary isolates as well as four others that grouped with sequences from PCR-positive lizards from northern California. Tick populations in our study sites harbor genetically diverse strains of *A. phagocytophilum*, which may influence potential risk in the region.

INTRODUCTION

Tick-borne pathogens are maintained in highly focal patches where the right mixture of competent vectors and hosts coexist within an appropriate habitat and climate. Consequently, risk of human exposure to disease agents transmitted by tick populations varies seasonally and geographically. In California, high habitat heterogeneity can result in idiosyncratic distributions of tick-borne pathogens, resulting in incomplete knowledge of local disease risk and consequent uncertainties in diagnoses of disease in human or animal populations.^{1–3} One example is *Anaplasma phagocytophilum*—a gram-negative, obligate intracellular bacterium in the family Anaplasmataceae, order Rickettsiales—which causes human granulocytic anaplasmosis (HGA).⁴ HGA often presents as a nonspecific febrile illness, including clinical manifestations such as pyrexia, headache, myalgia, nausea, ataxia, organ failure, susceptibility to opportunistic infections, neuritis, or respiratory complications.⁵ Incidence of HGA increases with age, though case-fatality rate (which is approximately 0.6–1.2%) is highest among persons 20–39 years of age (1.2%).^{6,7}

The distribution of *A. phagocytophilum* and HGA reflects the range of its tick vectors: the black-legged tick (*Ixodes scapularis*) in the eastern and upper midwestern United States, and the western black-legged tick (*Ixodes pacificus*) on the west coast.^{7,8} Annual incidence of anaplasmosis has increased in North America in recent years, most likely due to the increased recognition of the disease by clinicians, but also the potential expansion of distribution ranges of competent vectors (*Ixodes* spp. ticks) that subsequently coincide with the distribution ranges of competent reservoir hosts (small rodents).⁷

In California, ecology of *A. phagocytophilum* is influenced by habitat–reservoir host assemblies, for example, higher seroprevalence in chipmunks (*Tamias* spp.) in redwood habitat at some sites and higher seroprevalence in woodrats (*Neotoma* spp.) in oak habitat at other locales.⁹ Furthermore, distinct

California strains of *A. phagocytophilum* appear to vary in the propensity to cause clinical disease in hosts, and these strains originate from different suites of reservoir hosts: dusky-footed woodrats (*Neotoma fuscipes*) appear to host a strain that is distinct from the strain that infects and causes disease in horses, dogs, and sciurid mammals (chipmunks and squirrels).^{9–12} In addition, *A. phagocytophilum* infection has been detected in lizards and snakes, though the ability of these strains to cause clinical disease in humans has not been sufficiently evaluated.¹³ The high genetic diversity of *A. phagocytophilum* in California complicates our understanding of the epidemiology of HGA.

Incidence of HGA in California is low and sporadic, but also widespread (<http://www.cdph.ca.gov/programs/vbds/pages/vbdsannualreports.aspx>; Figure 1)^{14–18}, so it is difficult to determine how human activity and host–pathogen ecology overlap leading to human infection risk in California. Prevalence of *A. phagocytophilum* in its primary California vector, the western black-legged tick (*I. pacificus*), is generally low, and when present has been observed at levels of 0.3–10.6% in adult ticks and 1.9–8.3% in nymphal ticks (Figure 1). Herein, we extend the research in California and report on the prevalence of *A. phagocytophilum* in western black-legged ticks collected from recreational areas near San Francisco, an area of comparatively high human population density, significant urban–wildland interface, and where the primary vector and vertebrate hosts are present. In addition, we show that the tick populations harbor genetically diverse pathogen strains of *A. phagocytophilum* with recognized and unknown pathological consequences.

METHODS

Study sites and tick collection. Ticks were collected by dragging a 1-m² white flannel blanket along vegetation and/or leaf litter, and all ticks were removed from sites. Tick collection occurred from January to May 2012 and in May 2013. All sites were recreational or natural areas (e.g., state parks or open space preserves) in the San Francisco Bay Area (Table 1).³ Habitat varied from chaparral (*Baccharis* spp. dominated) and grassland to coastal live oak (*Quercus agrifolia*) woodland and redwood (*Sequoia sempervirens*) habitats. Ticks

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FIGURE 1. (A) Counties with confirmed or probable cases of human granulocytic anaplasmosis (HGA) (data from <http://www.cdph.ca.gov/programs/vbds/pages/vbdsannualreports.aspx>). (B) Counties where *Anaplasma phagocytophilum* has been confirmed in western black-legged tick populations (red), and where *A. phagocytophilum* has not been observed (gray) (data obtained from this study and from previous published studies).^{14–18} (C) Number of HGA cases reported in California from 1998 to 2014. HGA is a reportable disease in California, and cases are reported by physicians to local health departments for review, which in turn report cases to the California Department of Public Health (CDPH). We used data from CDPH spanning 1998–2014, with updated data and exposure locations courtesy of CDPH (personal communication). Probable cases involve clinically compatible case (meets clinical evidence criteria) that has supportive laboratory results, and confirmed cases are clinically compatible cases that are laboratory confirmed (<http://www.cdc.gov/nndss/conditions/ehrlichiosis-and-anaplasmosis/case-definition/2008/>, accessed November 9, 2015). Cases were differentiated into probable and confirmed from 2000 onward.

were stored in 70% ethanol, and then examined with a dissection microscope to identify species, sex, and stage using a dichotomous key.¹⁹ Herein, we analyzed western black-legged ticks only.

Pathogen detection. Adults collected in 2012 were pooled, with 1–5 ticks/pool, and we interpreted pathogen prevalence from positive pools as the minimum infection prevalence, that is, assuming one positive tick per pool. All nymphs and

TABLE 1
Prevalence of *Anaplasma phagocytophilum* in western black-legged ticks (*Ixodes pacificus*) collected from recreational areas in the San Francisco Bay Area (2012–2013)

Site	Adult <i>I. pacificus</i>		Nymphal <i>I. pacificus</i>	
	2012	2013	2012	2013
Marin County				
China Camp SP	1/143 (0.7)*	0/2	1/43 (2.3)	0/10
Napa County				
Bothe-Napa Valley SP	—	0/26	—	0/38
San Mateo County				
Jasper Ridge Biological Preserve	0/32	0/28	0/21	0/3
Pulgas Ridge OSP	0/110	0/11	3/13 (23.1)	0/6
Edgewood Park	0/1	—	2/6 (33.3)	—
Huddart Park	—	—	1/10 (10.0)	—
Corte de Madera	—	0/3	—	0/6
Thornwood OSP (woodland)	0/156	0/26	2/25 (8.0)	0/19
Thornwood OSP (redwood)	0/9	0/3	110/9	1/9 (11.1)
Windy Hill OSP (woodland)	0/120	0/3	0/17	0/51
Windy Hill OSP (chaparral/grassland)	0/122	0/10	—	—
Wunderlich County Park	0/15	0/22	0/15	0/27
Santa Clara County				
Foothills Park	2/13 (15.4)	2/51 (3.9)	11/37 (29.7)	0/27
Hidden Villa	—	0/9	—	0/28
Henry Coe SP	0/132	—	—	—
Monte Bello OSP	1/77 (1.3)	0/37	0/9	1/20 (5.0)
Sanborn County Park	0/53	—	—	—
Sierra Azul OSP	1/112 (0.9)	0/1	—	0/2
Los Trancos OSP†	2/58 (3.5)	0/10	0/18	2/27 (7.4)
Santa Cruz County				
Castle Rock SP	0/51	—	—	0/2
Sonoma County				
Annadel SP	—	1/4 (25.0)	—	0/41
Jack London SP	—	1/15 (6.7)	—	0/29
Total	7/1,204 (0.58)	4/261 (1.5)	20/223 (9.0)	4/345 (1.2)

SP = State Park; OSP = Open Space Preserve.

*Number positive/number tested (percentage positive).

†Los Trancos straddles the San Mateo and Santa Clara County border.

adults collected in 2013 were screened individually to improve the accuracy of our measure of infection prevalence. DNA was extracted from ticks following manufacture's protocols (DNeasy Blood and Tissue Kit; Qiagen, Valencia, CA) and stored at -20°C until molecular analysis. We performed quantitative polymerase chain reaction (qPCR) on all samples acquired during the study period utilizing primers and fluorescent hybridization probes developed previously to specifically identify *A. phagocytophilum*.²⁰ All assays were performed using qPCR SsoFast Supermix 1X (Life Science Research, Bio-Rad, Hercules, CA) on a CFX96-TOUCH system (Life Science Research, Bio-Rad), following a two-step protocol recommended by the manufacturer. Each 20 μL reaction contained primers at a concentration of 300 nM and probe at 200 nM (Applied Biosystems, Life Technologies, Carlsbad, CA). To specifically identify *A. phagocytophilum* genotype, we sequenced the 23S-5S rRNA intergenic spacer (*rri-rrs*) of each qPCR-positive animal sample using a nested protocol and high-fidelity Phusion taq polymerase (Thermo Fisher Scientific, Waltham, MA).²¹ PCR product was further purified using the QIAquick kit (Qiagen) and then sequenced using capillary Sanger sequencing on an ABI 3730 sequencer (EnGGen; Northern Arizona University, Flagstaff, AZ). To initially identify pathogen homology, each sequence was compared with other pathogen sequences available from GenBank using basic local alignment tool (BLAST) (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD).

DNA sequences were then aligned using MUSCLE²² as implemented in MEGA 5.0 (<http://megasoftware.net/>)²³ and then analyzed for Bayesian phylogenetic inference using MrBayes version 3.1.2 (<http://mrbayes.csit.fsu.edu/>).²⁴ For Bayesian analysis, tree sampling was conducted on 10^7 generations with the initial 1,000 trees being discarded (burn in = 1,000). Consensus trees were midpoint rooted and visualized using the software package FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS

We collected a total of 2,033 *I. pacificus* ticks—568 nymphs and 1,465 adults—from 20 sites in the San Francisco Bay Area region in 2012 and 2013 (Table 1). In total, *A. phagocytophilum* infection prevalence was 0.8% (11/1,465) in adult and 4.2% (24/568) in nymphal western black-legged ticks, though prevalence varied depending on the site and year. We identified *A. phagocytophilum* in 11/20 sites, and when observed at a location, infection prevalence varied from 0.7% to 33%. Where sample sizes exceeded 30, infection prevalence of *A. phagocytophilum* tended to range from 0.7% to 3.9%. An exception was found at Foothills Park where 29.7% (11/37) nymphal ticks were infected in 2012, though combining nymphal ticks from both 2012 and 2013 reduced nymphal infection prevalence of *A. phagocytophilum* to 17.2% (11/64).

FIGURE 2. Midpoint rooted Bayesian phylograms of *Anaplasma* sp. 23S-5S rRNA sequences from ticks, vertebrate hosts, and clinical patients. The tree was constructed using MrBayes (<http://mrbayes.csit.fsu.edu/>). Numbers at the nodes correspond to the posterior probabilities following 1,000,000 iterations. The 23S-5S rRNA sequences from this study are labeled with the study site name and Ixpac (*Ixodes pacificus*). The GenBank accession numbers for representative *Anaplasma* sp. sequences are as follows: Lizard 1 (JF487930), Lizard 2 (JF487929), gray squirrel (JF451142), and dusky-footed woodrat (JF451141) were described previously from northern California.²¹ MRK, horse origin (JF451139); HZ, human isolate (JF451140); *Anaplasma marginale* (AY048815); *Anaplasma centrale* (NR_076686); and *Ehrlichia chaffeensis* (NR_076400) are all clinical isolates from North America.

We were able to obtain sequences of the intergenic spacer region of *A. phagocytophilum* from nine of 35 qPCR-positive samples observed in western black-legged ticks (GenBank accession nos. KU588996–KU589004). Following an initial BLAST analysis, all nine sequences matched closely to the western and eastern U.S. clinical isolates of *A. phagocytophilum*—equine (MRK) and human (HZ), respectively (accession nos. JF451139, JF451140). However, following Bayesian phylogenetic analysis, our sequences grouped into two distinct clades. One clade corresponded to clinical cases of HZ, MRK, and reservoir host (*Sciurus griseus* and *N. fuscipes*) *A. phagocytophilum* sequences. The second clade included sequences obtained from ticks from this study and from a lizard (*Sceloporus occidentalis*) from Hoopa Valley, Humboldt County, CA (accession no. JF487930). Both clades maintained maximum posterior probability following 1,000,000 iterations (Figure 2).

DISCUSSION

We found *A. phagocytophilum* in tick populations across the San Francisco Bay Area, though prevalence varied between sites, ranging from 0.7% to 25% infection prevalence in adult western black-legged ticks and from 2.3% to 33.3% infection prevalence in nymphs. It is important to note that the higher prevalence values were often garnered from small sample sizes of ticks, suggesting that the prevalence value was highly skewed by one or two positive samples with a small denominator, for example, 2/6 nymphal ticks from Edgewood Park or 1/4 adult ticks in Annadel State Park (Table 1). Our sites were often in areas without prior studies of *A. phagocytophilum* prevalence, making direct comparisons difficult. Furthermore, prevalence of tick-borne diseases and abundance of ticks can fluctuate at sites.^{14,25} Thus, our lower *A. phagocytophilum* prevalence compared with some other studies^{15,26,27} may reflect inter-annual variation. Differences may also arise because we sampled habitats not commonly associated with HGA risk, for example, chaparral and grasslands, whereas other studies deliberately focused on habitats exhibiting high abundance of ticks and/or known *A. phagocytophilum* presence.^{15,26} Certainly, the variation in reported infection prevalence illustrates the patchy and sporadic nature of tick-borne disease risk in the varied landscape of California and demonstrates that *A. phagocytophilum* is present across the San Francisco Bay Area.

A wide diversity of vertebrate hosts and tick vectors are a hallmark of *A. phagocytophilum* eco-epidemiology.^{9,12,28,29} This has led to the identification of regionally diverse strains, some of which may be associated with human infection and others that may be completely restricted to suites of wildlife hosts and cause limited or no clinical disease in spite of significant serological cross-reactivity.³⁰ Reservoir hosts for clinical *A. phagocytophilum* (MRK and HZ strains) are presumed to be members of the Scuridae (tree squirrels and chipmunks) in the western United States and the white-footed mouse (*Peromyscus leucopus*) in the eastern United States.^{31–33} However, in both regions there are also host species infected with *A. phagocytophilum* strains that are not associated with human disease, for example, maintained within populations of dusky-footed woodrats (*N. fuscipes*) and reptiles in the west^{11,13} and white-tailed deer (*Odocoileus virginianus*) in the east.³⁴ Here, we were able to obtain valid sequences from nine of our qPCR-positive samples and these

include sequences that were closely related to both clinical *A. phagocytophilum* strains and those that have not been demonstrated as pathogenic in humans (Figure 2). The diversity of *A. phagocytophilum* strains present in the San Francisco Bay Area is as high as in other parts of the state, but measuring actual human disease risk is complicated by the observations that multiple strains are circulating in ticks.

Perhaps reflective of the low prevalence of infection in ticks and the diversity of *A. phagocytophilum* observed, HGA is not commonly reported in California. However, cases do occur in most years, and across a broad swathe of the state, implying that physicians across California, and particularly in the San Francisco Bay Area, should be cognizant of the potential for HGA after tick bites. An additional complication is the misdiagnosis of HGA if the diagnosis is based only on clinical examinations and not confirmed by specific laboratory assays.³⁵ In the northeastern United States, *Borrelia miyamotoi* infections have been mistakenly diagnosed as anaplasmosis.³⁵ Given the relatively high prevalence of *B. miyamotoi* in western black-legged ticks in the same sites reported here,^{3,36} physicians suspecting tick-borne diseases with symptoms similar to HGA may wish to confirm the identity of the disease agent with appropriate laboratory tests.³⁵

Received September 28, 2015. Accepted for publication February 17, 2016.

Published online May 2, 2016.

Acknowledgments: We thank the Bay Area Lyme Foundation for generously supporting this research; Stephanie Cinkovich and Carter Hranac for their assistance in the laboratory; Chrissy Esposito for creating maps; and California Department of Public Health's Vector-Borne Disease Section—especially Anne Kjemtrup—for collaboration and advice.

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