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A putative marker for human pathogenic strains of *Anaplasma phagocytophilum* correlates with geography and host, but not human tropism

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ABSTRACT

Anaplasma phagocytophilum is an *Ixodes* species tick-transmitted bacterium that is capable of infecting a variety of host species, although there is a diversity of bacterial strains with differing host tropism. Recent analysis of *A. phagocytophilum* strains suggested that "*drhm*", a gene locus designated "distantly related to human marker" (*drhm*), which was predicted to be an integral membrane protein with possible transporter functions was not present in available canine and human isolates. By assessing 117 strains from 14 host species from across the US, we extended this analysis. Phylogenetic clades were associated with geography, but not host species. Additionally, a virulent clade that lacks *drhm* and infects dogs, horses, and humans in northeastern US was identified.

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1. Introduction

Anaplasma phagocytophilum is an *Ixodes* species tick-transmitted bacterium, with a Holarctic geographical distribution and wide host range (Foley et al., 2008a, 2004; Stuen et al., 2013). *A. phagocytophilum* was initially described in small hoofstock in Scotland, designated *Ehrlichia phagocytophila*, and subsequently in horses (*Equus caballus*) in California, designated *Ehrlichia equi* (Dumler et al., 2001; Foggie, 1951; Madigan and Gribble, 1987). Illness in cats (*Felis catus*), dogs (*Canis lupus domesticus*), horses, humans (*Homo sapiens*) and sheep (*Ovis ovis*) can be subclinical to fatal, most commonly presenting as an undifferentiated febrile illness (Carlyon and Fikrig, 2006; Dumler et al., 2005; Rikihisa, 2011). Although *A. phagocytophilum* represents a single species (Dumler et al., 2005), recent research documents host-adaptation among strains. For example, a strain found in deer (*Odocoileus* spp.), designated Ap-variant 1, is genetically distant from human-associated strains described to date. Current evidence, although not comprehensive of all possible species, indicates that the Ap-variant 1 infects only deer, goats (*Capra aegagrus hircus*), and tick-origin cell lines, while experimental infection of mice was not successful (Massung et al., 2007). Strain DU1 (isolate obtained from the "day-use" area of Hendy Woods State Park, Mendocino County, CA), has only been found in bears (*Ursus americanus*) and woodrats (*Neotoma fuscipes*) throughout the state (Rejmanek et al., 2012; Stephenson et al., 2015a). This strain is non-infectious for horses and is genetically distinct from strains found in dogs, horses, people, and other small mammals (Foley et al., 2008b; Rejmanek et al., 2012).

Host specificity can result from ancestral or evolutionarily derived conditions. Greater understanding of the origin(s) of *A. phagocytophilum*, the Holarctic geographical spread of these bacteria, and coevolution with mammalian hosts and tick vectors could enhance surveillance and facilitate medical management for sick animals and people.

Recently, analysis of full genomes from various *A. phagocytophilum* strains identified a gene locus designated "distantly related to human marker" (*drhm*), which was predicted to be an integral membrane protein, possibly with transporter functions (Al-Khedery and Barbet, 2014). *dhrm* was not found in

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available canine and human isolates (Al-Khedery and Barbet, 2014). In contrast, this locus was found in equine and ovine strains and Ap-variant 1. Because canine anaplasmosis can be severe and resemble the human disease (Carrade et al., 2009), the *drhm* locus was proposed as a virulence marker. However, because of a lack of geographical and host range overlap (Al-Khedery and Barbet, 2014), the inference of a marker for virulence was potentially confounded. Therefore, we aimed to broaden the geographical and host ranges over which *drhm* could be assessed. We aimed to test two hypotheses: that *drhm* would vary in its presence or absence among *A. phagocytophilum* strains from different host species within a geographical region with high *A. phagocytophilum* diversity (California), and that *drhm* from clades from California would be distinct from strains found in other geographical regions.

2. Methods

Data on *drhm* were compiled from analyzed, previously published *A. phagocytophilum* genomes (Al-Khedery and Barbet, 2014) and from PCR and DNA sequencing results from animals in a variety of locations. For display and analysis, states were grouped by region: with Midwest comprising Iowa, Ohio, Wisconsin and Minnesota; northeast including Vermont, Massachusetts, Maryland, New York, New Hampshire, Pennsylvania, and Connecticut; southeast including North Carolina and Virginia; and all western samples originating from California. Animal blood samples were obtained from trapping for various studies of tick-borne disease in California and from diagnostic laboratories testing domestic animals for anaplasmosis. DNA was extracted using standard protocols for each laboratory and then all DNA samples included in this study were confirmed PCR-positive for the *A. phagocytophilum msp2* gene (Drazenovich et al., 2006). Samples for *drhm* analysis included black bears, cats, chipmunks (*Tamias* spp.), dogs, Douglas squirrels (*Tamiascirus douglasii*), dusky-footed woodrats, grey foxes (*Urocyon cinereoargenteus*), horses, humans, western and eastern grey squirrels (*Sciurus griseus* and *S. carolinensis*), and white-tailed deer (*Odocoileus virginianus*) from across the continental United States. Samples typed as strain Ap-variant 1 were verified by DNA sequencing of the 16S rRNA gene, and DU1 strains were verified based on *ankA* gene sequencing PCR (Massung et al., 1998; Stephenson et al., 2015b).*drhm* gene PCR was performed as previously described (Al-Khedery and Barbet, 2014). Because samples from humans and dogs were reported previously not to contain this gene, we ensured that our negative *drhm* PCR results were truly negative by first confirming each sample contained adequate DNA using an *A. phagocytophilum*-specific *msp2* quantitative real-time PCR with a cycle threshold <30, a value in our laboratory that routinely allows for successful DNA sequencing for a diversity of genes (Unpub. data). A stratified selection by host species of positive *drhm* amplicons (those with lowest cycle threshold) was subjected to DNA sequencing. Bands of the expected size were excised from the agarose gel and cleaned with a QIAquick gel extraction kit (Qiagen, Valencia, CA) per manufacturer instructions. Products were sequenced in the forward direction using the PCR primer on an ABI Prism 3730 Genetic Analyzer (UC DNA Sequencing Facility, Davis, CA, USA). Electropherograms were trimmed for end-read errors and then aligned using the CLC Main Workbench 6 (CLC Bio, Boston, MA). Single nucleotide polymorphisms apparent in the alignment were also manually corrected, after which a phylogenetic tree was constructed in CLC using the UPGMA algorithm.

3. Results

drhm PCR results were compiled from three sources: (1) samples tested in our laboratory, (2) sequences reported in a previous publication (Al-Khedery and Barbet, 2014) and (3) GenBank (Table 1). The *drhm* locus was not amplified from dog, horse or human samples from the Midwest and Northeast, whereas one Ap-variant 1 white-tailed deer sample from Minnesota was *drhm* PCR-positive. In contrast, both *drhm* positive and negative bear, dog, fox, horse and woodrat *A. phagocytophilum* strains were found in the southeastern and western US. One cat *A. phagocytophilum* strain from the western US lacked the *drhm* locus, as did the 32 out of 33 available rodent samples. However, because of the presence of multiple DU1 genotypes in the western US, we looked for patterns in *drhm* PCR results between the two most common strains, DU1 and one with high homology to strains associated with human, equine, and canine disease (Table 2) (Rejmanek et al., 2012). Among DU1 strains, only two (woodrat and black bear) contained the *drhm* locus, while the remaining 13 woodrat and seven bear DU1 strains

Table 1
Results of PCR testing for the *drhm* locus from samples from people and other animals, grouped by region. All samples included here were strongly positive for *A. phagocytophilum*, as indicated by a real-time PCR cycle threshold of less than 30.

	Negative	Positive	Total	Prevalence
Midwest	7	1	8	0.1250
Dogs	5	0	5	0.0000
White-tailed deer	1	1	2	0.5000
Humans[a]	1	0	1	0.0000
Northeast	17	0	17	0.0000
Dogs	7	0	7	0.0000
Horses	5	0	5	0.0000
Humans[a]	5	0	5	0.0000
Southeast	4	5	9	0.5556
Dogs	0	2	2	1.0000
Horses	4	3	7	0.4286
West	53	30	83	0.3614
Dogs	4	2	6	0.3333
Horses	1	8	9	0.8889
Domestic cats	1	0	1	0.0000
Dusky-footed woodrats	14	1	15	0.0667
Eastern grey squirrels	3	0	3	0.0000
Western grey squirrels	6	0	6	0.0000
Redwood chipmunks	3	0	3	0.0000
Allen's chipmunks	1	0	1	0.0000
Siskiyou chipmunks	1	0	1	0.0000
Sonoma chipmunks	2	0	2	0.0000
Douglas squirrels	2	0	2	0.0000
Grey foxes	3	2	5	0.4000
Black bears	12	17	29	0.5862
Grand total	81	36	117	0.3077

[a] Data included in a previous publication (Al-Khedery and Barbet, 2014) or from GenBank.

Table 2
Numbers of *Anaplasma phagocytophilum drhm* PCR-positive or negative strains in animals from the western US, based on whether the strains were typed by the *ankA* gene as *sensu stricto* (APSS) or DU1.

	Negative	Positive	Total
APSS			
Dogs	2	2	4
Horses	1	8	9
Dusky-footed woodrats	1		1
Eastern grey squirrels	2		2
Western grey squirrels	4		4
Redwood chipmunks	1		1
Sonoma chipmunks	2		2
Douglas squirrels	2		2
Grey foxes	2	1	3
Black bears	2	16	18
DU1			
Dusky-footed woodrats	13	1	14
Western grey squirrels	1		1
Redwood chipmunks	2		2
Black bears	7	1	8
Total	42	29	71

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