Ticks and Tick-borne Diseases 4 (2013) 482–487

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Unique strains of *Anaplasma phagocytophilum* segregate among diverse questing and non-questing *Ixodes* tick species in the western United States

Daniel Rejmanek[a,*], Pauline Freycon[b], Gideon Bradburd[c], Jenna Dinstell[a], Janet Foley[a]

[a] Davis School of Veterinary Medicine, University of California, Department of Medicine and Epidemiology, Davis, CA 95616, USA
[b] Vetagro Sup – Campus Vétérinaire de Lyon, 69280 Marcy l'Etoile, France
[c] Center for Population Biology, University of California, Department of Evolution and Ecology, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 18 February 2013
Received in revised form 24 June 2013
Accepted 25 June 2013
Available online 30 August 2013

Keywords:
Anaplasma phagocytophilum
Ixodes spp.
Nidicolous ticks
Ixodes pacificus
ank gene
Phylogeny

ABSTRACT

The emerging tick-borne pathogen *Anaplasma phagocytophilum* infects humans, domestic animals, and wildlife throughout the Holarctic. In the western US, the ecology of *A. phagocytophilum* is particularly complex, with multiple pathogen strains, tick vectors, and reservoir hosts. A recent phylogenetic analysis of *A. phagocytophilum* strains isolated from various small mammal hosts in California documented distinct clustering of woodrat strains separate from sciurid (chipmunk and squirrel) strains. Here, we identified strains of *A. phagocytophilum* in various *Ixodes* tick species in California and related these genotypes to those found among reservoir and clinical hosts from the same areas. The sequences from all of the nidicolous (nest-dwelling) *Ixodes* ticks grouped within a clade that also contained all of the woodrat-origin *A. phagocytophilum* strains. Two of the *I. pacificus* sequences were also grouped within this woodrat clade, while the remaining five belonged to a less genetically diverse clade that included several sciurid-origin strains as well as a dog, a horse, and a human strain. By comparing *A. phagocytophilum* strains from multiple sources concurrently, we were able to gain a clearer picture of how *A. phagocytophilum* strains in the western US are partitioned, which hosts and vectors are most likely to be infected with a particular strain, and which tick species and reservoir hosts pose the greatest health risk to humans and domestic animals.

Introduction

Anaplasma phagocytophilum, the causative agent of granulocytic anaplasmosis (GA) is a tick-transmitted, intra-leukocytic rickettsial parasite of humans and other animals. Reservoir hosts include small mammals, such as white-footed mice (*Peromyscus leucopus*) in the eastern US (Telford et al., 1996) and woodrats (*Neotoma fuscipes*), squirrels (*Sciurus* spp.), and chipmunks (*Tamias* spp.) in the western US (Nicholson et al., 1999; Nieto and Foley, 2008, 2009). The tick vectors for *A. phagocytophilum* are most frequently reported to be the questing ticks of the *Ixodes ricinus* group, including *I. pacificus* in the western US and *I. scapularis* in the eastern US (Foley et al., 2004). While these tick species likely serve as the primary bridge vectors transmitting GA to humans and domestic animals, other nidicolous (i.e. primarily nest dwelling) *Ixodes* ticks including *I. spinipalpis*, *I. ochotonae*, and *I. trianguliceps* harbor *A. phagocytophilum* and likely help maintain enzootic cycles of GA (Zeidner et al., 2000; Bown et al., 2003; Foley et al., 2011).

Although *A. phagocytophilum* was originally classified as 3 distinct organisms – *Ehrlichia equi*, *Ehrlichia phagocytophila*, and the agent of human granulocytic ehrlichiosis – morphological, phenotypic, and genetic evidence led to the reclassification of these 3 organisms as the modern *A. phagocytophilum* in 2001 (Dumler et al., 2001). Despite this reorganization, phenotypic and preliminary genetic data strongly support the presence of multiple distinct strains. For example, strains in Europe commonly cause clinical disease in small hoofstock, but North American strains are neither particularly infectious nor virulent based on data from experimental infections of sheep or cattle (Pusterla et al., 1997; Stuen, 2007; Gorman et al., 2012). Similarly, in England, 2 genetically distinct subpopulations of *A. phagocytophilum* coexist in separate enzootic cycles, one involving deer and *I. ricinus* ticks and the other involving field voles and *I. trianguliceps* (Bown et al., 2009). The North American strain designated Ap-Variant 1 occurs in ticks and deer and is infectious to goats, but not rodents (Massung et al., 2003, 2007). The California strain DU1, originating from a woodrat, can infect rodent species, but not horses (Nieto et al., 2010). Strain MRK, which was

* Corresponding author. Tel.: +1 530 754 9740; fax: +1 530 752 0414.
E-mail address: drejmanek@ucdavis.edu (D. Rejmanek).

1877-959X/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.ttbdis.2013.06.003

isolated from a horse, reproducibly induces severe clinical disease in horses indistinguishable from that induced when human-origin *A. phagocytophilum* is inoculated into horses and is also infectious to small mammals (Pusterla et al., 1999; Foley et al., 2009b). These clinical and epidemiological distinctions are associated with genotypic segregation into specific clades based on analyses of the 16S rRNA, *msp4*, *msp2*, and *ank* genes (Massung et al., 2003; de la Fuente et al., 2005; Bown et al., 2009; Rejmanek et al., 2011a,b).

In the western US, the ecology of *A. phagocytophilum* is particularly complex, with multiple pathogen strains, tick vectors, and reservoir hosts (Foley et al., 2004). A recent phylogenetic analysis of *ank*, *groESL*, and the 23S-5S rRNA genes from 28 Californian rodent strains, the MRK horse strain, and a California dog strain documented distinct clustering of woodrat strains separate from a genetically uniform group consisting of sciurid (chipmunk and squirrel), horse, and dog strains (Rejmanek et al., 2011a,b).

One question that has not been addressed in previous studies of *A. phagocytophilum* is whether particular *A. phagocytophilum* genotypes are associated with certain host-specialist ticks. California is home to numerous nidicolous *Ixodes* spp., many of which infest some of the same small mammal species as *I. pacificus* (Furman and Loomis, 1984). A recent survey of ticks on small mammals from numerous sites across California revealed that 66% of tick-infested small mammals hosted nidicolous *Ixodes* species, while the remaining 34% were infested with *I. pacificus* (Foley et al., 2011). In the current study, our goal was to identify strains of *A. phagocytophilum* in various *Ixodes* tick species in California and relate these genotypes to those found among reservoir and clinical hosts from the same areas. For our analysis, we focused on a single gene (*ank*), which was chosen because it exhibited a high level of polymorphism even among closely related *A. phagocytophilum* strains and was largely concordant with phylogenetic results from analysis of other gene regions (Rejmanek et al., 2011a,b).

Materials and methods

Sample collection

DNA samples for molecular characterization were obtained from *I. pacificus*, *I. spinipalpis*, *I. angustus*, *I. ochotonae*, and *I. woodi* from April 2006 to September 2011. Ticks were collected in central and northern California at the following sites: Soquel Demonstration Forest (SD, Santa Cruz County; 37° 03.25′, 121° 50.68′), Henry Cowell State Park (HC, Santa Cruz County; 37° 08.705′, 122° 11.12′), Samuel P. Taylor State Park (SPT, Marin County; 38° 01.232′, 122° 40.774′), Hendy Woods State Park (HW, Mendocino County; 39° 04.25′, 123° 28.238′), Humboldt Redwoods State Park (HR, Humboldt County; 40° 17.770′, 123° 59.178′), and Archer Taylor Preserve (ATP, Napa County; 38° 20.54′, 122° 25.34′). Two ticks were obtained from the Green Diamond Resource Company (GD), found on a dusky-footed woodrat in an unspecified site of Humboldt County.

Ticks were collected from small mammals trapped and sampled as described previously (Foley et al., 2011). Ticks were found on dusky-footed woodrats, deer mice, a chipmunk (*Tamias ochrogenys*), an eastern gray squirrel (*Sciurus carolinensis*), and a human. Trapping and sample collection were carried out as previously described (Foley et al., 2011). To obtain questing ticks, flagging was performed over herbaceous and shrubby vegetation as well as duff and leaf litter using a 1-m^2 white cotton flag.

Nucleic acid extraction, PCR, and sequencing

DNA was extracted from ticks following a modified protocol described previously (Humair et al., 2007). Briefly, individual ticks were placed in microcentrifuge tubes, cooled in liquid nitrogen for 3 min, and crushed using a microcentrifuge pestle. Next, 100 μl of 0.7 M NH_4OH was added, and the tubes were placed on a 100 °C heat block for 15 min. Tubes were then cooled on ice for 30 s followed by an additional 15 min of heating at 100 °C with open lids in order to evaporate the ammonia.

All DNA samples were initially screened for the presence of *A. phagocytophilum* DNA using a highly sensitive real-time TaqMan PCR assay targeting the msp2/p44 gene (Drazenovich et al., 2006). Results of real-time PCR were considered positive if they had a cycle threshold (CT) value <40 and a characteristic amplification curve. Nested conventional PCR assays targeting a section of the *ank* gene were then performed on all positive samples. Amplification of the *ank* gene was performed as described by Massung et al. (2007) using external primers ANK-F1 (5′-GAAGAAATTACAACTCCTGAAG-3′) and ANK-R1 (5′-CAGCCAGATGCAGTAACGTG-3′), followed by internal primers ANK-F2 (5′-TTGACCGCTGAAGCACTAAC-3′) and ANK-R2 (5′-ACCATTTGCTTCTTGAGGAG-3′), yielding an approximately 600-bp amplicon.

Amplified DNA was visualized on a 1% agarose gel stained with GelStar nucleic acid stain (Lonza, Rockland, ME). Bands of the expected size were excised and cleaned with a Qiagen (Valencia, CA) gel extraction kit. Gel-extracted amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI), transformed into *Escherichia coli* DH5α cells, and plated onto LB agar containing 100 μg/ml ampicillin. Individual colonies were grown overnight in LB broth containing 100 μg/ml ampicillin, and plasmids were purified using a Quantum Prep plasmid miniprep kit (BioRad, Hercules, CA). Following *Eco*RI digestion, plasmids were assessed for appropriate insert size. Gel-extracted or cloned PCR products were sequenced in both forward and reverse directions on an ABI 3730 sequencer (Davis Sequencing). Consensus sequences were initially aligned using the CLUSTAL_X sequence alignment program (Larkin et al., 2007) and trimmed to a final length of 567 bp. All unique sequences were deposited in GenBank and issued the following accession numbers: KC249918–KC249925.

Phylogenetic analysis

In addition to the *ank* sequences acquired in the current study, 31 previously reported *A. phagocytophilum ank* sequences were used in the phylogenetic analysis. These included sequences from 22 *A. phagocytophilum* strains isolated from small mammal hosts (woodrats, chipmunks, and gray squirrels) sampled in the same areas and during the same time frame as most of the ticks (GenBank accession numbers JF303732–JF303741 and JF776828) (Rejmanek et al., 2011a,b). Additional strains included HZ_NY (the fully sequenced human-origin strain from New York State) (Dunning Hotopp et al., 2006), 2 other human isolates from New York (NY_2 and NY_3), Webster_WI and WI_2 (human isolates from Wisconsin), Mn_Dog (a dog isolate from Minnesota), RI_1 (isolated from an *I. scapularis* tick in Rhode Island), MRK_CA (originally isolated from an infected horse in Shasta County, CA), Dog_CA, (a strain isolated from an infected dog from Tuolumne County, CA), and CAHU_HGE2, a strain isolated from an infected human from southern Humboldt County, CA (GenBank accession numbers: CP000235 and AF172153, AF100884, AF100885, GU236811, AF100890, AF100894, DQ320648, AF153716, JF303732, and AF172153, respectively).

All DNA sequences were aligned using MUSCLE v3.8 (Edgar, 2004), then translated into amino acid codons, and adjusted by eye to ensure that the sequences were in frame and did not contain stop codons. Identical sequences were excluded from the analysis, then reincluded as polytomies for the phylogeny figures. To examine the effect of missing data, 2 separate data matrices were used: one in which all sequence data in hand were included for each accession, and one in which all sequences were trimmed to