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Original article

Detection and characterization of a novel spotted fever group *Rickettsia* genotype in *Haemaphysalis leporispalustris* from California, USA

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ABSTRACT

The rabbit tick, *Haemaphysalis leporispalustris* Packard, is known for its association with *Rickettsia rickettsii* as it harbors both virulent and avirulent strains of this pathogen. In this manuscript we report findings and preliminary characterization of a novel spotted fever group rickettsia (SFGR) in rabbit ticks from California, USA. *Rickettsia* sp. CA6269 (proposed "*Candidatus* Rickettsia lanei") is most related to known *R. rickettsii* isolates but belongs to its own well-supported branch different from those of all *R. rickettsii* including strain Hlp2 and from *Rickettsia* sp. 364D (also known as *R. philipii*) and *R. peacockii.* This SFGR probably exhibits both transovarial and transstadial survival since it was found in both questing larvae and nymphs. Although this rabbit tick does not frequently bite humans, its role in maintenance of other rickettsial agents and this novel SFGR warrant further investigation.

1. Introduction

Most of the ecological and epidemiological arthropod surveillance studies conducted on tick-borne rickettsioses are focused on *Amblyomma*, *Dermacentor, Ixodes* and *Rhipicephalus* genera which frequently bite humans. Other tick genera receive less attention presumably due to their infrequent attacks on humans; however, their associations with human rickettsial pathogens are well known and they likely serve as part of the larger maintenance mechanisms for these rickettsiae in nature.

The rabbit tick, *Haemaphysalis leporispalustris* Packard is a three-host tick that is widely distributed from Alaska to Mexico and Central America and to Argentina (Burgdorfer, 1969). The adult ticks primarily parasitize wild rabbits and hares, while larvae and nymphs may infest other small animals and several species of birds (Green et al., 1943; Campbell and Glines, 1979; Kinsey et al., 2000; Kollars and Oliver, 2003); however, an attachment to humans is extremely rare (Lane, 1996; Harrison et al., 1997). Two generations of ticks may occur annually in the southern United States, while a single life cycle may take two years in colder summers like those in Nova Scotia (Green et al., 1943; Campbell and Glines, 1979; Keith and Cary, 1990). The rabbit tick is well known for its contribution to the epizootiology *Francisella tularensis*, the etiological agent of tularemia (Brown, 1945; Foley and Nieto, 2010). It can also be infected with *Borrelia burgdorferi*, *Anaplasma* sp. or *Babesia* sp. while feeding on infected animals; however, it is unlikely to be an efficient vector for these pathogens (Goethert and Telford, 2003; Yabsley et al., 2006; Lane and Burgdorfer, 1988; Levine et al., 1991; Gurfield et al., 2011). A recent report also indicated a possible link between *H. leporispalustris* collected in northern California and *R. felis* (Roth et al., 2016); this rickettsia is almost ubiquitously detected in association with various ectoparasites, particularly fleas, and environmental conditions (Blanton and Walker, 2017; Angelakis et al., 2016).

It is well established, that *H. leporispalustris* contributes to the enzootic maintenance of *Rickettsia rickettsii* by harboring both virulent and avirulent strains of this pathogen, respectively, in Central and Northern America (Parker et al., 1951; Bozeman et al., 1967; Philip et al., 1978; Fuentes et al., 1985). Experimental evidence also suggests that *H. leporispalustris* contributes to enzootic maintenance of *R. rickettsii* throughout Latin America (Freitas et al., 2009). Rabbit ticks infected with *Rickettsia canadensis* and *R. bellii* have been also detected infrequently although the geographic areas where these rickettsial agents

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Table 1
Haemaphysalis leporispalustris tick collections and PCR assay results by site, county, sex, and life stage.

County (number of sites)	Number of ticks collected	Life stage	Sex	Positive by SYBR Green PCR (%, minimal infection prevalence; 95% CI)[1]	Positive by semi-nested and conventional PCR[2]	Identification
Sonoma (20)	224	Adults (2)	Female (2)	0/2 (0%)	0/2	N/A[3]
		Nymphs (66)		2/66 (3%; 0.8–10.4%)	1/2	New genotype
		Larvae (156)		6/156 (3.8%; 1.8–8.1%)	1/6	New genotype
Orange (1)	179	Adults (4)	Female (3)	0/2	N/A	N/A
			Male (1)	0/1	N/A	N/A
		Nymphs (69)		14/69 (20%; 12.5–31.2%)	1/14	Unknown
		Larvae (106)		8/106 (7.5%; 3.9–14.2%)	0/8	N/A
Total (21)	403			30/403 (7.4%; 5.3–10.4%)	3/32	

[1] Denominator corresponds to total numbers of ticks tested for each life stage, numerator corresponds to a number of positive adults and nymphs tested individual or number of positive pools of larvae tested.

[2] Denominator corresponds to the number of SYBR Green or EvaGreen PCR positive DNA used for MLST analysis, numerator corresponds to a number of DNA tested positive for at least one MLST gene.

[3] N/A, not applicable.

were detected do not form a continuum (McKiel et al., 1967; Lane et al., 1981a; Lane et al., 1981b; Philip et al., 1982).

The purpose of this study was to evaluate the contemporary presence of rickettsiae in *H. leporispalustris* from California using molecular and culture methods.

2. Material and methods

2.1. Collection of ticks and preparation of DNA

Questing *H. leporispalustris,* including larvae, nymphs and adults were collected from rocks and leaf litter using 1-m^2 white flannel tick flags from 2 sites in Northern and Southern California during 2009–2011 (Table 1). Ticks were identified to species using standard taxonomic keys (Furman and Loomis, 1984), preserved in 70% ethanol or kept alive and frozen prior to further processing.

2.2. Processing and testing of ticks

Live adult ticks were surface disinfected by a series of washes in 10% bleach, 70% ethanol and distilled water, and hemolymph was obtained and stained for microscopic observations according to classic procedure (Burgdorfer, 1970). Ticks were bisected; one half was triturated and used for inoculation of VERO E-76, and incubated at 34 °C and 28 °C for 2 blind passages as described previously (Eremeeva et al., 2006). Cultures were observed for the signs of cytopathic effects and smears were prepared and stained by acridine orange to detect the presence of rickettsiae. DNA was prepared from the aliquots of infected cells and tested for rickettsial DNA using *omp*A SYBR Green PCR (Eremeeva et al., 2003).

DNA was extracted from the other half of the bisected ticks, and individual preserved ticks and nymphs, and pools of larvae using QIAamp DNA Mini kit (Qiagen, Valencia, CA) or (Promega, Madison, WI) extraction and Robotic station set up as reported previously (Wikswo et al., 2007, 2008). DNA, stored at 4 °C, was tested for rickettsiae using *omp*A SYBR Green PCR, and 17 kDa antigen gene TaqMan assays as described previously (Eremeeva et al., 2003; Loftis et al., 2006; Wikswo et al., 2008). EVAGreen PCR assay was also done using Rr190.547 and Rr190.701 primers and the same cycling conditions as for SYBR Green PCR assay according to the manufacturer's instruction (Bio-Rad, Hercules, USA); the assay performance was comparable with two different dyes. Primers and probes used in individual assays are listed in Table 2. DNAs of *Rickettsia montanensis* or *R. sibirica* were used as positive controls, and two negative controls containing no DNA were included in each testing run.

2.3. Identification of Rickettsia species

Samples tested positive by SYBR Green PCR were further characterized using multiple locus sequence typing (MLST) as described elsewhere (Eremeeva et al., 2006; Fournier et al., 2003). *Rickettsia* DNA gene fragments including 381-bp of *glt*A, 532-bp of *omp*A, 749-bp of *omp*B and 928-bp of *sca*4 were amplified as described previously. The amplicons were purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (ABI) according to the manufacturer's recommendations on an ABI 3130*xl* genetic analyzer. Sequencing reads were assembled using Sequencher 5.1 (Gene Codes, Ann Arbor, MI). Primer sequences were removed and homologous sequences were detected using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Sequence Tool (BLAST) search engine. Homologous sequences of formally named SFGR species including *R. rickettsii* isolates and *Candidatus* species were downloaded from NCBI GenBank, *glt*A-*omp*A-*omp*B-*sca*4 sequences were concatenated, aligned using ClustalW, and a phylogenetic tree was drawn using MEGA5.0 software (Tamura et al., 2011). New sequences generated during this study were submitted to the NCBI GenBank under the following accession numbers: *glt*A- JN990594, *omp*A- JN990595, *omp*B- JN990596, *sca*4- JN990597.

2.4. Data analysis

When applicable prevalence or minimal infection prevalence (MIP) were calculated; MIP was defined as percentage of a ratio between the total number of tick pools positive for *Rickettsia* and the total number of ticks tested. Confidence intervals (CI) for prevalence rates were calculated using the Wilson score method without continuity correction (Newcombe, 1998).

3. Results

In total, 403 *H. leporispalustris* tick specimens collected by flagging from two counties in California were tested (Table 1).

Fifty live ticks, including 4 adults and 46 nymphs were triturated and each inoculated into cell cultures; seven other cultures were inoculated with triturates made from 71 live larvae (8–12 larvae per pool). Four inoculated cultures were discarded due to severe bacterial (3) or yeast (1) contamination. Other cultures remained without contamination and underwent 2 more passages. DNA prepared from supernatants and/or pellets of infected Vero cells tested Eva-Green PCR positive for 547-701 nt *omp*A fragment with 10 of 46 nymph triturate cultures and 2 of 4 larva triturate cultures. Six of 13 dead nymphs also tested positive with an average Ct cycle was 37.8 (median 37.8) by the same PCR assay. Further attempts to amplify larger fragments of rickettsial DNA were not successful using end-point or nested PCR so they